University of Cincinnati

Date: 4/26/2023

I, Phillip C Witcher, hereby submit this original work as part of the requirements for the degree of Doctor of Philosophy in Cancer and Cell Biology.

It is entitled:

Assessing the impact of Myomaker and Myomerger in mature muscle fibers

Student's name: **Phillip C Witcher**

This work and its defense approved by:

Committee chair: Douglas Millay, Ph.D.

Committee member: Jiajie Diao, Ph.D.

Committee member: Daniel Lucas

Committee member: Xiaoyang Qi, Ph.D.

Committee member: Kathryn Wikenheiser-Brokamp, M.D.

44418

Assessing the impact of Myomaker and Myomerger in mature muscle fibers

A dissertation submitted to the
Graduate School of the University of Cincinnati
in partial fulfillment of the
requirements for the degree of

Doctor of Philosophy

in the Department of Cancer and Cell Biology
of the College of Medicine
April 26, 2023
by

Phillip C Witcher
B.S. Indiana University Purdue University Indianapolis (2017)

Committee Chair: Douglas Millay, Ph.D.

Abstract

Skeletal muscle plays a pivotal role in everyday life. Not only does it allow locomotion but it also helps with temperature regulation and a myriad of metabolic pathways. The organized structure and niche of skeletal muscle, in part, allows it to achieve these tasks. Skeletal muscle is comprised of bundles of myofibers packaged within fascicles. Hundreds of myonuclei reside in each myofiber. Each nucleus specializes within the myofiber based on its location, with myonuclei near the neuromuscular junction assisting with signal transmission at the synaptic cleft and myonuclei near the myotendinous junctions helping maintain integrity. The multinucleated skeletal muscle syncytium arises from myogenic progenitors which fused to each other to form mature muscle fibers. In disease, such as Duchenne muscular dystrophy (DMD), these progenitors, also known as muscle satellite cells (MuSCs), are activated to repair damaged muscle fibers and form new muscle fibers. The regenerative program employed by MuSCs utilizes identical myogenic regulatory factors, such as MyoD and Myogenin, that govern myogenic progression during development. Previous studies have implicated an overactive regenerative program in advancing the pathogenesis of DMD. Given the broad array of genetic programs regulated by MyoD and its important role in priming myoblasts for fusion, we sought to determine if a distinct program activated by MyoD, namely the fusogenic program, had an effect on myofibers. To test the hypothesis that reactivation of the fusogenic program in myofibers is deleterious, we employed a doxycycline-inducible system to activate expression of the skeletal muscle fusogens Myomaker and Myomerger in developed myofibers. We found that both Myomaker and Myomerger destabilized the myofiber membrane in a dose-dependent manner, leading to muscle damage and pathology. Dual expression of both Myomaker and Myomerger exacerbated these effects on skeletal muscle myofibers. In summary, reactivation of the fusogens within mature muscle fibers leads to membrane damage, muscle atrophy, and pathology. These data explain why Myomaker and Myomerger expression is so tightly regulated in myoblasts during development and

regeneration. They also reveal that myofiber-expression of the fusogens are a potential therapeutic target to reduce disease progression in muscular dystrophy.

Acknowledgements

I am grateful for my mentor, Doug Millay, for fostering a culture of curiosity and excellence. His tenacious approach to lab meetings and informal discussions was infectious, helping elevate projects from good to great. Combined with the valuable perspectives and skills contributed by my thesis committee, whom I am also grateful for, I learned about the importance of asking the right scientific question and designing experiments to answer these inquiries.

I would also like to thank all of the lab members who made the everyday life in the lab welcoming and productive. Dilani Gamage taught me core molecular biology principles to establish my footing in the lab. I would also like to thank John Olthoff, Qing Goh, Sajedah Hindi, Chengyi Sun, Thiru Govindarajan, Fabián Montecino, Alyssa Cramer, Leah Focke, Cris Calvo, Tanner Wherley, and Michael Petrany for their technical assistance and guidance during my graduate years. Stephanie Kidder was instrumental for teaching me how to use the atomic force microscopy instrument. Finally, I will forever be indebted to Vikram Prasad, whose expert immunoblotting skills and inquisitive nature helped propel my project to completion.

I am thankful for fellow graduate students Johnny Donovan, Oliver Ströbel and Wesley Stansberry who were readily available to commiserate the trials and tribulations of graduate school. For the times when I just needed someone to talk to, I am grateful for Kumar Bogireddi freely lending his time and support. I would not be where I am without the unselfish support of my previous mentors, Richard Gregory, Robert Wolter, Shoji Ichikawa, Alex Robling, and Justin Grobe. My family has also been instrumental in my journey, especially my dad, encouraging me to push forward and persist through the trials of graduate school. Last, but not least, I would like to thank my wife for her patience and kind understanding as I persisted through the ups and downs of graduate school.

Table of Contents

List of Figures

Chapter 1: Introduction

I. Embryological Origins of Skeletal Muscle

Embryological development in vertebrates is a highly regulated process governed by stem cell proliferation and differentiation. Morphogenesis commences with gastrulation, the formative process by which the three germ layers – ectoderm, mesoderm, and endoderm – arise[1]. Coordinated expression of BMP, Shh, and Wnt signaling pathways orchestrates gastrulation, prodding epiblast thickening and the formation of the primitive streak in the caudal region of the median plane[2–6]. Caudal elongation of the primitive streak occurs alongside proliferation of its cranial end, leading for the formation of a primitive node[7]. A portion of the cells which sit atop the yolk sack, termed epiblasts, invaginate and form a monolayer from which endoderm is derived[8,9]. Epiblasts which undergo epithelial to mesenchymal transition but do not fully migrate form the mesoderm while those that do not migrate form the ectoderm[10,11].

A portion of the invaginating epiblasts migrate cranially from the primitive node to form the notochordal process[12]. The notochordal process expands cranially until reaching the prechordal plate, the site where ectoderm and endoderm are fused. In addition to forming the notochord, these epiblasts pattern the mesoderm of the median plane, forming (medially to laterally) paraxial mesoderm, intermediate mesoderm, and lateral mesoderm (Fig. 1)[3,13–16].

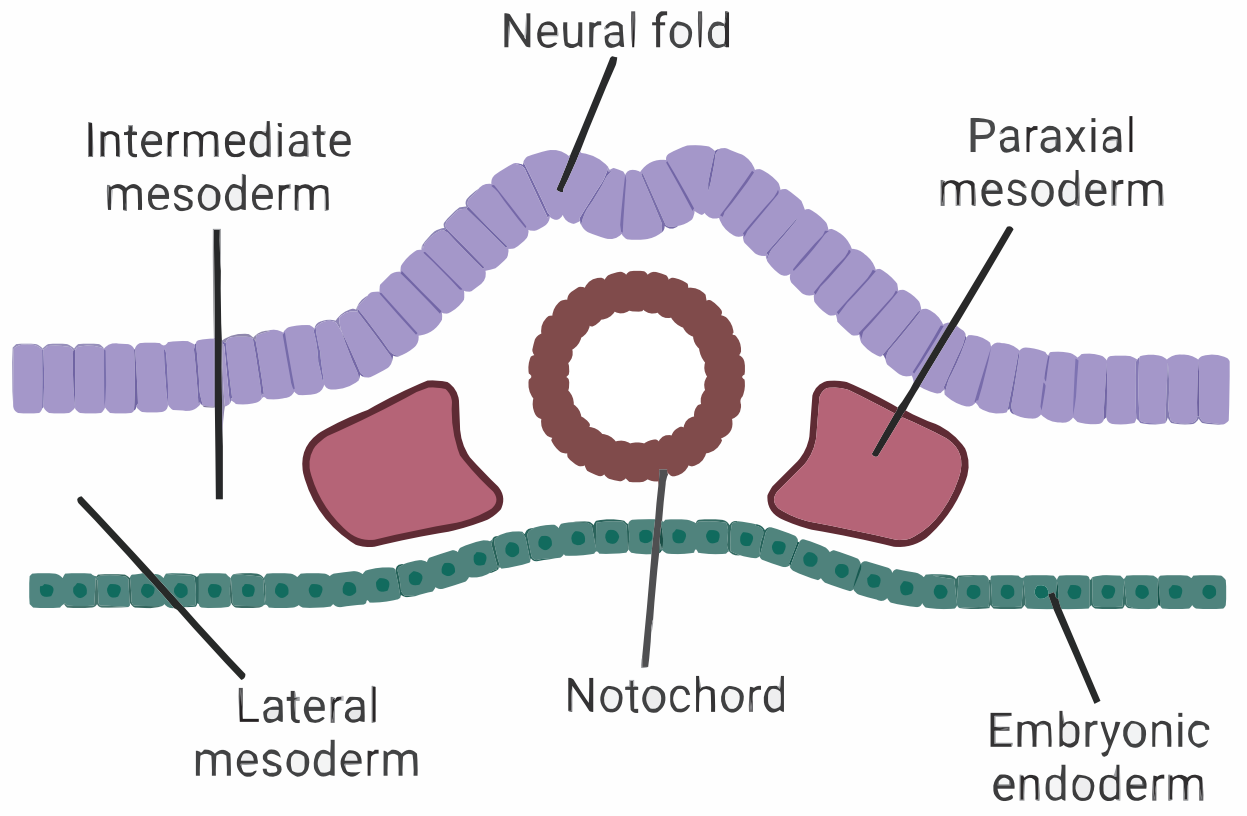

Figure 1: Norchord development. Epiblast invagination leads to the formation of the notochord. Other populations of epiblasts partially migrate through the neural fold to form separate mesodermal structures: paraxial mesoderm, intermediate mesoderm, and lateral mesoderm.

The paraxial mesoderm differentiates and forms cuboidal bodies called somites on both sides of the notochord. Shh, Wnt, and BMP signaling from the notochord, neural tube, surface ectoderm, and lateral plate mesoderm partition the somites into ventromedial and dorsolateral regions[17]. Epithelial-to-mesenchymal transitioning within the ventromedial region and migration towards the ventral region of the neural tube leads to sclerotome formation from which the vertebrae and ribs form[18]. Cells within the dorsolateral region remain epithelial and comprise the dermomyotome (Fig. 2)[19]. The dermomyotome further develops, expanding laterally. Cells in the lateral lips of the dermomyotome expressing *Myf5* and *Mrf4* migrate ventrally to form a distinct

myotome beneath the dermatome from which skeletal muscles of the body and limbs are derived[20,21].

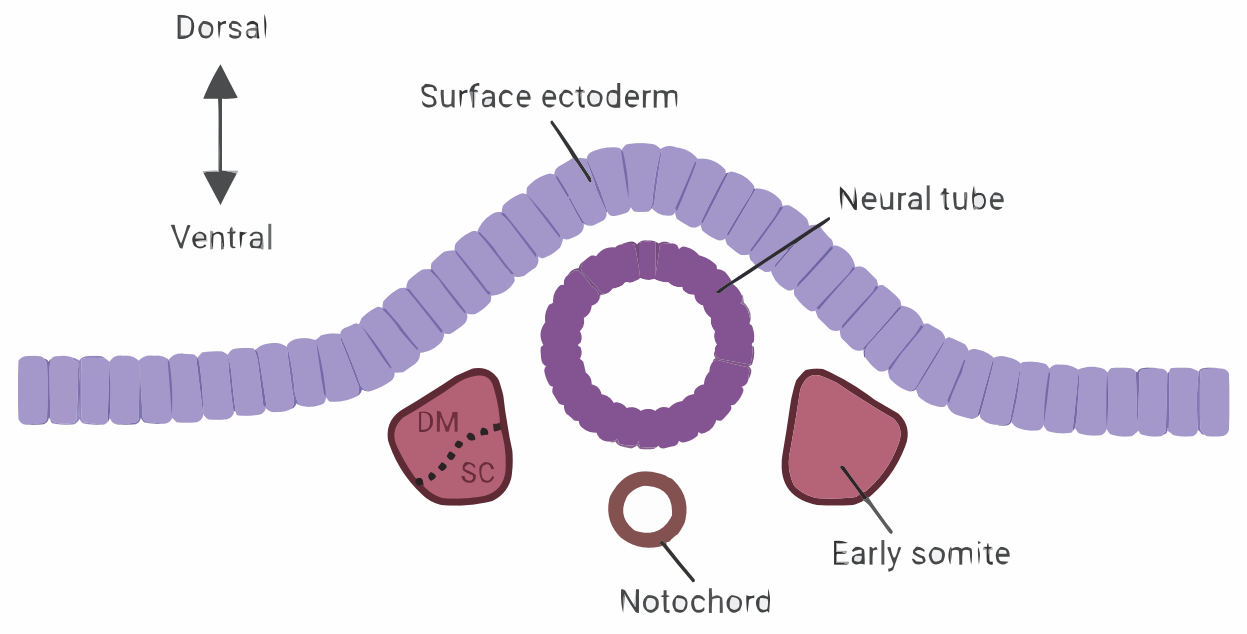

Figure 2: Neurulation and somite development. Invagination of the neural plate at the neural folds leads to the development of a neural tube and dorsal surface ectoderm[22]. Differentiation of the paraxial mesoderm leads to somite development. The ventral half of the somite undergoes epithelial to mesenchymal transition to form the sclerotome (SC), which later gives rise to the vertebral column, meninges, and ribs[23]. The remaining dorsal portion of the somite subsequently differentiates to form the dermomyotome (DM), the origin of muscle, connective tissue, endothelium, and cartilage[24].

Oddly enough, craniofacial musculature has a different origin than trunk and limb musculature. While commonly mistaken as having a neural crest origin, head muscles originate from cranial paraxial mesoderm, more specifically the prechordal, paraxial, and splanchnic mesoderm components[25,26]. Muscles which comprise the head are broadly divided into three groups: 1) tongue and posterior neck muscles, 2) pharyngeal muscles (those of the jaw, anterior

neck, and face), and 3) extraocular muscles (EOMs)[26]. Although muscles of the tongue and posterior neck muscles were originally thought to both be derived from the occipital somites, it was more recently discovered that the posterior neck muscles actually originate from the lateral plate mesoderm adjacent to the most anterior somites[27]. The latter two groups arise from cranial paraxial mesoderm[26,28].

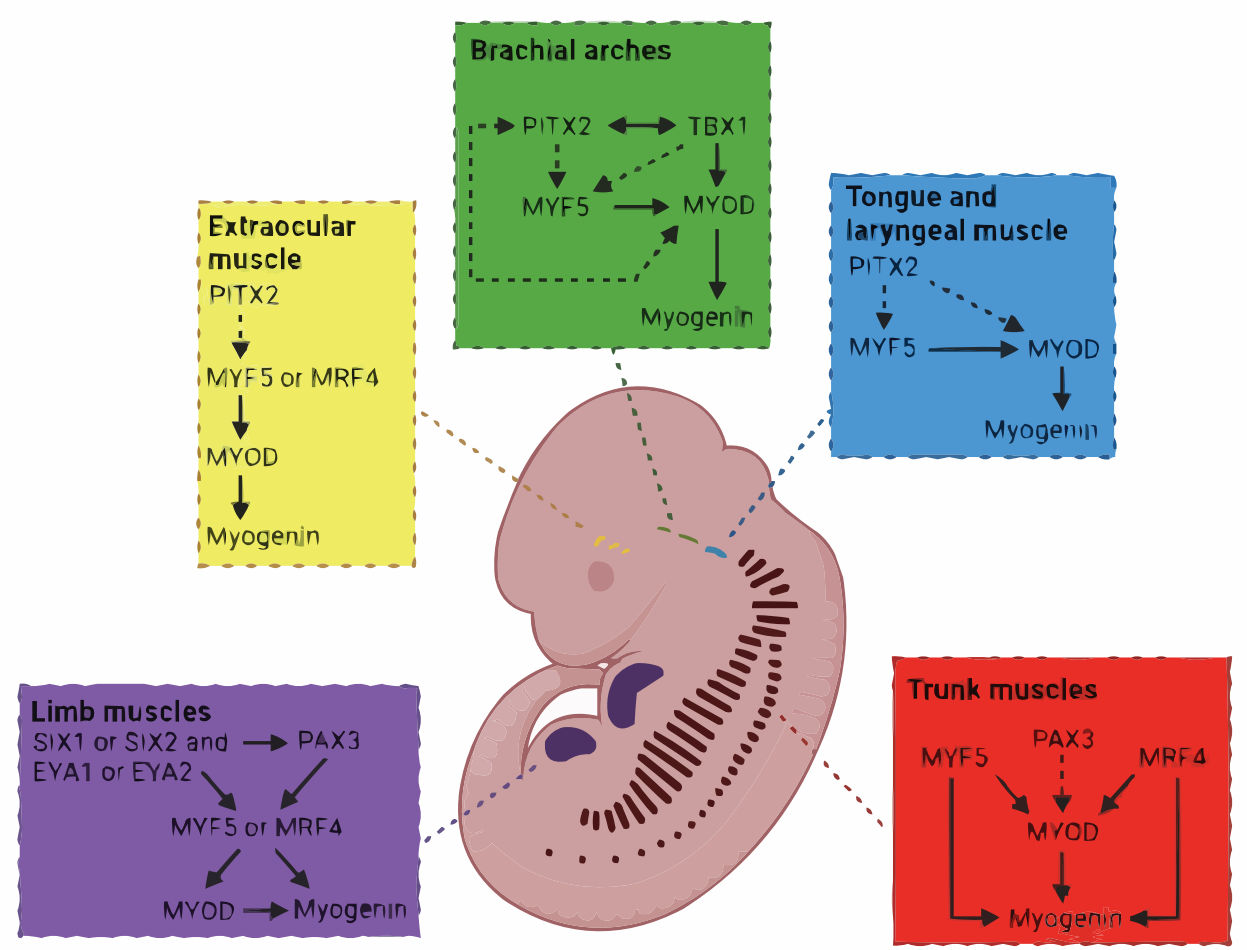

Figure 3: Myogenic genetic networks (adapted from Braun and Gautel. *Nat Rev Mol Cell Bio*, 2011). Different genetic networks of transcription factors regulate myogenesis based on developmental origin. A core set of transcription factors are expressed in all groups of muscles: Myf5, Mrf4, MyoD, and Myogenin. Craniofacial muscle development is initiated by Pitx2 whereas limb and trunk muscle development is initiated by Pax3.

Highly coordinated genetic networks determine cell fate of skeletal muscle stem cells (Fig. 3). These genetic programs have largely been unraveled through loss-of-function experiments in mice and chick embryos. Limb and facial muscles fail to develop in embryos lacking *Myf5* and *MyoD*, but some trunk muscles are still present[20]. Conversely, mice deficient in *Pax3*, *Myf5*, and *Mrf4* fail to develop trunk muscles but have normal head muscles[29]. Thus, somitic myogenesis requires *Pax3*-complementation of the canonical myogenic developmental pathway[20,29]. In the absence of *Myf5* and *Mrf4* or the homeobox transcription factor *Pitx2*, however, EOMs fail to development, indicating a lack of alternative signaling pathways to activate *Myogenin* and downstream myogenic stem cell differentiation[30,31]. A small number of EOMs and pharyngeal arch muscles are observed in *Myf5*;*Mrf4* double mutants, hinting at a parallel pathway for *Pitx2* to activate MyoD[32]. Despite these differences in origin, *MyoD* or *Myf5* is required for activation of *Myogenin* and subsequent differentiation of skeletal muscle stem cells[33].

The stem cells which form the bulk of prenatal skeletal muscle can be binned into three groups termed founder stem cells (FSC). FSC1 arises from the dorsal and ventral lips of the dermomyotome, expressing *Pax3* (and eventually *Myf5/Mrf4/Myod*)[26]. However, not all cells from FSC1 require *Pax3*, as Pax3-null embryos still develop a myotome[29,34,35]. FSC2 migrates from the central dermomyotome to the underlying myotome, expressing *Pax3/Pax7* (and eventually *Myod/Myf5*)[26]. Finally, the FSC3 population originates from the ventral dermomyotome of select somites, developing into limb, tongue, and diaphragm skeletal muscle, expressing *Pax3*, *Met*, *Lbx1*, and *Meox1* (and eventually *Myf5/Myod/Mrf4*)[20,31,34]. The majority of adult skeletal muscle stem cells is understood to arise primarily from FSC2 and FSC3, as FSC1 is exhausted early in embryogenesis[31]. However, additional work is needed to validate this premise. Overall, all skeletal muscles, sparing those in the head, are derived from the dermomyotome. The epaxial portion of the dermomyotome, that which is adjacent to the neural tube, gives rise to dorsal muscle groups

while the hypaxial portion gives rise to limb muscles[36]. With regards to specific origin, trunk and tongue muscle stem cells originate from the $Pax3^{+}$ lineage, head and EOM muscle stem cells are derived from the $MesP1^{+}$ lineage, and the Isl1 lineage comprises the stem cells of pharyngeal muscles[37].

II. Myogenesis

Upon formation of the dermomyotome, key transcription factors, termed myogenic regulatory factors (MRFs), coordinate the next steps of myogenesis. During murine development, myogenesis can be separated into two phase: an early (primary) phase around E10.5-E12.5 and a later (secondary) phase around E14.5-17.5[38,39]. In primary myogenesis, *Myf5*- and *Mrf4*-expressing cells in the lateral regions of the dermomyotome delaminate, migrating ventrally to form the myotome[20,21]. As myocytes develop in the myotome, they begin to differentiate, expressing slow (*Myh7*) and embryonic (*Myh3*) myosin heavy chains (MyHCs), α-actins (cardiac (*Actc1*) and skeletal (*Acta1*)) and desmin, and metabolic enzymes such as β-enolase and carbonic anhydrase III[40–47]. Wnt11 signaling directs their elongation along the anterior-posterior axis[48–50]. Slow myosin heavy chain fibers begin to develop as more cells continually migrate from the dermomyotome[51,52]. Shortly after depletion of the *Myf5*- and *Mrf4*-expressing cells of the dermomyotome, the central dermomyotome loses its epithelial character and *Pax3*-expressing cells from this region delaminate and begin to seed the myotome[35,53–55].

As the embryo elongates, myogenesis proceeds in a rostral to caudal fashion, sequentially adding somites[56,57]. Limb muscles develop as cells from the lateral dermomyotome migrate to the developing limb buds[58,59]. While the myogenic program of the trunk and limbs is slightly divergent from that of the EOM and muscles derived from the pharyngeal arches, a key set of transcription factors drive myogenesis: Pax3 and the MRFs Myf5, MyoD, Mrf4, and myogenin[33,60–62].

Regardless of the location (somite, extraocular, pharyngeal arch), however, myogenin is globally responsible for the terminal differentiation of myoblasts to myocytes[63–65].

During secondary myogenesis, a portion of the $Pax3^+$ myogenic progenitors begin to express Pax7 and downregulate Pax3[66]. Secondary (fetal) myofibers, characterized by β-enolase, Nfix, or MyLC3 expression, arise from the fusion of $Pax7^+$ myogenic progenitors to themselves or to the primary myofibers[67–70]. Muscle growth during secondary myogenesis is sustained by the fusion and proliferation of $Pax7^+$ progenitors[71]. Postnatally, however, muscle growth primarily occurs through fiber hypertrophy and myofibril addition[72,73]. As muscle fibers develop, they become more specialized through the expression of specific MyHC isoforms[74]. Oxidative slow twitch muscle fibers are characterized by slow MyHC (*Myh7*) expression. More glycolytic, or fast twitch, muscle fibers express fast MyHC (*Myh2*, *Myh1*, *Myh4*, in order of decreasing oxidative capacity)[75]. Developmentally, the MyHC isoforms are expressed in a set sequence, with embryonic and slow MyHC expressed first, followed by perinatal MyHC in the fetal and neonatal stages and fast MyHC isoforms during the later fetal stages[66]. Fast-type fiber diversity is regulated, in part, by the Six and Eya transcription factors[76,77]. In concert with physiological demands and innervation, these transcription factors help pattern the fiber type in each muscle fiber, leading to unique metabolic traits and electrophysiological properties[74].

Muscle fibers hypertrophy not only through fusion of myogenic progenitors but also through myofibrillogenesis. The sarcoplasm of muscle fibers is comprised primarily of myofibrils which themselves are composites of sarcomeres, the contractile unit of muscle[66]. The myofibrils span the entire length of the muscle fiber, connecting at myotendinous junctions at each end[73,78]. A specialized plasma membrane, the sarcolemma, envelops each myofiber and anchors to a specialized basal lamina, the endomysium[79,80]. The dystrophin-glycoprotein complex anchors the sarcolemma to the extracellular matrix (ECM)[81]. Invaginations of the sarcolemma called T-tubules abut cisternae, specialized sarcoplasmic reticulae which store calcium, which altogether comprise

triads (a T-tubule surrounded by terminal cisternae on both sides laterally). These triads, which develop perinatally, are closely associated with the myofibrils, allowing for transduction of sarcolemmal depolarization through the fiber during neuronal excitation[82–84].

The Pax7$^+$ myogenic progenitors not only participate in secondary myogenesis but they also form the pool of adult muscle stem cells[35,53,54,85,86]. In some muscles, such as the diaphragm, Pax3 expression is maintained in the stem cell pool[35,87]. Maintenance of these satellite cells requires Notch signaling; these myogenic progenitors cannot properly develop in its absence[88]. While nearly all satellite cells transiently express MyoD prenatally, a subset of Pax3/7+ cells proliferate without upregulating other MRFs, such as Myf5[35,53,89]. During the fetal and perinatal stages of muscle growth, when the rate of muscle mass accrual is highest, satellite cell progenitors comprise approximately 30% of the mononuclear cell population[66,90]. Shortly after birth, however, these progenitors reduce in number to a smaller pool of quiescent Pax7$^+$ satellite cells, accounting for a small percentage of the total mononuclear cell population[90–92].

III. Disease in Skeletal Muscle

Disruptions in any component of the skeletal muscle motor unit or its surrounding extracellular environment may lead to disease. Skeletal muscle diseases can broadly be categorized into four categories: dystrophinopathies, inflammatory myopathies, neuromuscular, and metabolic diseases[93]. Duchenne muscular dystrophy (DMD), classified as a dystrophinopathy, is the most prevalent of these, affecting approximately 1 in 5,000 live male births[94]. Although it primarily affects males, given its X-linked inheritance, females can also be affected, but they usually exhibit a milder phenotype. DMD typically presents around 2-3 years of age, manifesting with difficulties walking and climbing stairs. To compensate for weakness in the pelvic and lower limb muscles, affected children will utilize a technique termed Gower's maneuver to stand up[95]. This method involves using their hands to push up on their knees from a prone

position to stand up, leading to hypertrophied calf muscles[96]. Patients usually become wheelchair-bound by the time they reach their teenage years[97].

The most prevalent cause of mortality in patients with DMD used to be respiratory failure[98]. However, improved treatment strategies and management of DMD patients has extended the median lifespan from approximately 20 years of age to over 30 years of age[98,99]. As a result, cardiac disease has emerged as a leading cause of death in these patients[98,100–102]. Not only have cardiac complications been underappreciated in DMD patients but also intellectual disabilities[103,104]. Delayed speech acquisition, intellectual disability, autism spectrum disorder, and attention deficit disorder have been implicated in DMD. The variability of neurological symptoms, which are not progressive like the skeletal and cardiac symptoms[97], may be due to the isoform of dystrophin affected. In muscle, only one isoform is expressed while multiple isoforms are expressed in the brain. The location of the dystrophin mutation, therefore, may impact the manifestation of neurological symptoms in each DMD patient[105]. Although there are numerous neurological symptoms reported to be associated with DMD, they are not progressive like the skeletal and cardiac muscle symptoms[97].

Elucidating the genetic cause of DMD was difficult at first. Clinical observations had determined that it had an X-linked inheritance pattern[106]. It wasn't until the late 1970s and early 1980s that the DMD locus had been narrowed down to the Xp21 region of the X chromosome[107]. Following a heated competition between several labs to discover the causative gene and a monumental cloning endeavor, the DNA sequence was successfully cloned and a protein sequence was predicted, indicating that the DMD protein might serve a structural role in muscle[108]. The complete cDNA sequences of the mouse and human homologues were generated soon thereafter, distinguishing the gene as the largest ever known[109,110]. Successful purification of the protein product, dystrophin, confirmed previous predictions of size and absence of expression in muscle biopsies from DMD patients[111].

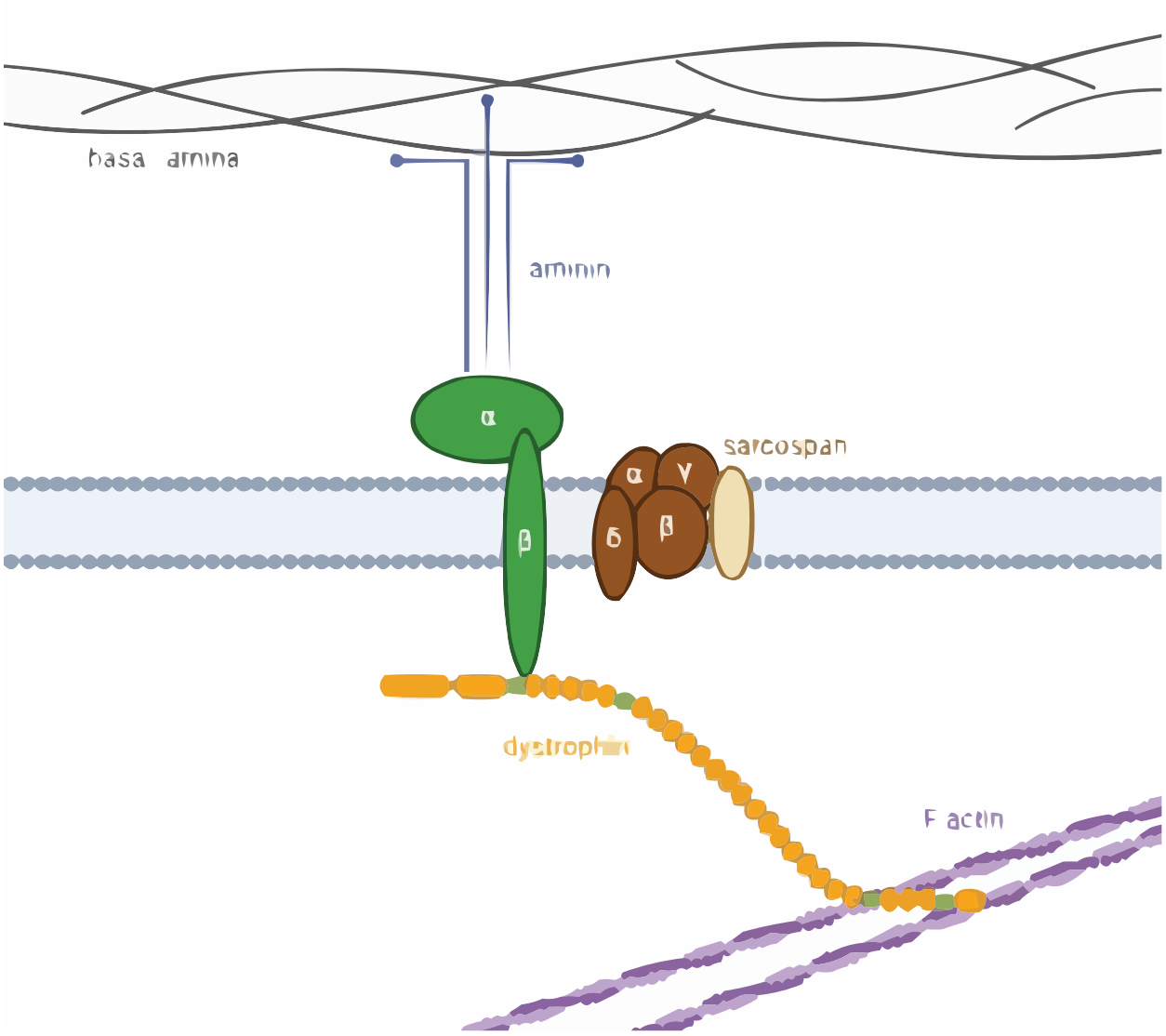

Figure 4: The Dystrophin-Glycoprotein Complex. Dystrophin is a critical structural protein which links the intracellular F-actin elements of the cytoskeleton to the extracellular matrix. It both binds to and serves as a membrane scaffold to other proteins in the sarcolemma, including dystroglycans and sarcoglycans.

The discovery of dystrophin catalyzed a myriad of studies seeking to reveal its precise function. These studies have uncovered dystrophin as a critical structural protein linking intracellular cytoskeletal components to a plasma membrane complex termed the dystrophin-

glycoprotein complex (DGC)[112]. Not only is dystrophin necessary for membrane stabilization but also as a scaffold for many dystrophin-associated proteins (Fig. 4)[106]. More recently, post-translational modifications, especially the addition of glycosylation moieties to the extracellular components of the DGC, have been shown to play an important role maintaining the stability of this complex[113].

DMD has been historically characterized as a skeletal muscle disease ultimately resulting in necrosis[114]. However, the mechanism of cell death within skeletal muscle has been disputed. Harmful factors, such as calpain activity[115], reactive oxygen species[116], and nuclear factor kappa beta activation[117] have been reported in DMD. Given the important role of dystrophin in mediating muscle cell stability, there is a growing consensus that the plasma membrane stability may be culpable in initiating disease[118]. When dystrophin is absent, the plasma membrane becomes more susceptible to injury, leading to excess calcium ion influx into the myofiber and muscle damage[119–124].

Unique susceptibilities to myopathies arise from the divergent origins of skeletal muscle. DMD, for example, affects the majority of limb skeletal muscles while oddly sparing EOM and laryngeal muscles even though every cell in DMD patients lack dystrophin[125,126]. Because EOM and laryngeal muscles are both embryologically derived from different cell origins compared to limb muscles, this has been proposed as a reason for their enhanced myogenic progenitor cell activity[127–130] and altered calcium homeostasis[131,132]. These observations provide support for the notion that dystrophin deficiency itself is not sufficient for disease but rather events secondary to its absence[133].

In addition to diseases, birth defects have also been linked to the different cell origins of skeletal muscle. Certain birth defects manifest in both skeletal and cardiac muscle, such as DiGeorge Syndrome. There exists a substantial overlap in the expression of head muscle markers and cardiac markers in the pharyngeal mesoderm, suggestive of a dual contribution to

myogenesis and cardiogenesis[134–136]. Furthermore, lineage-tracing studies have demonstrated an overlap in the progenitor populations which contribute to pharyngeal muscles and the second heart field derivatives[37,135,137,138]. The overlap in progenitor contributions and physical proximity during early embryogenesis leads to linked cardiac and craniofacial birth defects[139–141]. Better understanding the embryologic origins of skeletal muscles could be leveraged in the future to develop more targeted treatments for myopathies and birth defects.

DMD has historically been treated with corticosteroids to curtail disease progression[142]. While the precise mechanism by which this therapeutic acts is not fully understood, steroids are believed to have a dampening effect on the immune system, reducing cytokine production and lymphocyte reaction, as well as stimulating muscle growth through insulin-like growth factors and enhanced myoblast proliferation[143]. Despite the benefits of improved motor function, reduced cardiomyopathy, and blunted progression of scoliosis, steroids have several undesirable side effects, not limited to immunosuppression, muscle weakness, osteopenia, and blunted growth[144]. In order to reduce these side effects, a novel steroid, vamorolone, has been developed that has distinct anti-inflammatory activity without the promiscuous gene transcriptional activities which contribute to the negative effects of gold standard corticosteroids, such as prednisone[145,146].

Cell-based therapies, which first showed promise after donor myoblasts were observed to fuse with existing myofibers following skeletal muscle graft, arose as an alternative to symptomatic treatment[147]. A decade later, successful fusion of dystrophin-positive myoblasts into dystrophic muscle was demonstrated, providing support for cell therapy as a potential therapy for DMD[148]. Despite these promising studies, satellite cell-based therapies have not yielded promising results, potentially due to the host immune response[149,150]. More successful cell-based therapies have involved nondonor-derived mesoangioblasts in animal models, but preliminary results expose unanticipated challenges in developing such treatments for DMD[151–153].

To circumvent the challenges of cell-based therapies, other groups have attempted to restore functional dystrophin in dystrophic muscle via alternative methods. Because the large size of dystrophin makes it unfavorable for viral transfection of dystrophic tissue, groups have attempted adding an analogous structural component to dystrophic muscle, such as minidystrophins[154–159] or microdystrophins[160–165]. In addition to dystrophin substitutes, antisense oligonucleotide-mediated exon skipping has emerged as a potential alternative to traditional treatments. These agents utilize synthetic nucleotide analogues to skip the mutation-containing exons responsible for the patient's DMD[166]. Although they are only effective for certain mutations and do not restore full-length dystrophin, the resulting protein product is more functional than the lack thereof[167]. To date, four exon skipping agents have been approved by the FDA, covering nearly one third of all DMD mutations[167,168].

Although dystrophin restoration is a promising treatment for DMD patients, therapeutic success still hinges on the immune system. Not only have cellular and humoral responses been documented against the delivery vehicle (i.e. viral components) but also against dystrophin-positive myofibers[169,170]. These immune responses have the potential to reduce the therapeutic efficacy[171]. Immunomodulatory agents, such as steroids[172], rapamycin[173], and immunoproteasome inhibitors[174], have variable effectiveness reducing this counterproductive immune response but may augment dystrophin restoration treatments[175]. Altogether, these studies indicate that fixing the causative genetic contributor to DMD, by itself, may not be sufficient to prevent disease progression, highlighting a need to develop parallel treatments which cooperate with gene therapies to provide meaningful clinical benefit to the patient.

IV. Regeneration of Skeletal Muscle

Satellite cells not only play a critical role in the development of skeletal muscle but also its regeneration. Depletion of the satellite cell pool in muscle leads to impaired regeneration[176,177].

Progenitor-null muscle has fewer myofibers, increased fibrosis, and elevated intracellular fat accumulation following injury[178–180]. This pathology is rescued with satellite cell transplantation into the depleted muscle[180].

Broadly, skeletal muscle regeneration can be divided into three overlapping stages: 1) inflammatory response, 2) activation, differentiation, and fusion of the satellite cell pool, and 3) remodeling of the myofiber milieu[181]. The second stage can be subdivided into distinct phases, broadly characterized by their expression of different MRFs, of satellite cell quiescence, stimulation, migration, proliferation, differentiation, and fusion (Fig. 5)[36]. Relative MRF expression is a key determinant of myogenic progenitor activation and progression through these stages of regeneration. Indeed, a high ratio of Pax7 to MyoD is suggested to promote quiescence while a low ratio may favor differentiation[182]. Each stage of myogenic regeneration will be reviewed.

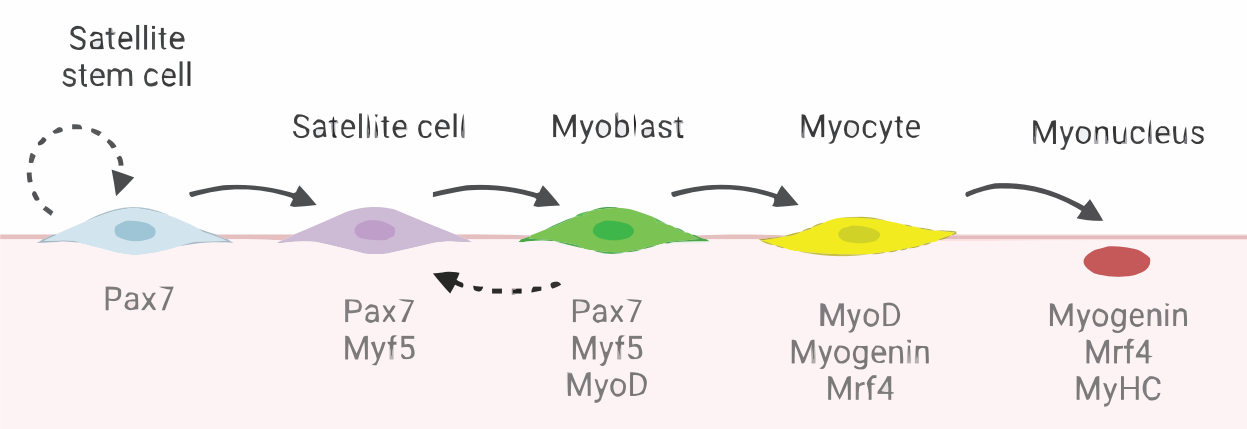

Figure 5: Skeletal Muscle Regeneration (adapted from Dumont et al. *Comprehensive Physiology*, 2015). Upon muscle injury, quiescent satellite cells (Pax7+ and Myf5 +/–) are activated and become myoblasts, gaining expression of MyoD. After proliferating, they may fuse to each other or to damaged myofibers.

A. Quiescence

The majority of satellite cells within unperturbed skeletal muscle are quiescent[183]. This stage is characterized by a lack of cell cycle progression, low rate of metabolism, and low RNA content[184]. Although quiescent satellite cells are intuitively understood to be dormant, maintenance of this cell state is an active process. Compared to activated satellite cells, over five-hundred genes are upregulated in quiescent satellite cells, most of which are involved in negative regulators of the cell cycle and myogenic inhibitors[185]. In addition to cell cycle regulation, the satellite cell niche plays a critical role in stem cell quiescence. M-cadherin expression on the satellite cell and its neighboring myofiber membrane interact and molecularly immobilize it[186]. Additionally, quiescent satellite cells express elevated levels of tissue inhibitor of metalloproteinases to counteract ECM degradation by metalloproteinases[187].

Notch signaling plays a pivotal role in satellite cell quiescence[188]. Deficient Notch signaling triggers a loss of satellite cell quiescence[189]. Interestingly, satellite cells with reduced Notch signaling do not undergo apoptosis but rather prematurely differentiate without undergoing cell division, fusing with adjacent myofibers[190,191]. Notch signaling has been shown to promote quiescence in several different manners. First, Notch signaling may directly activate Pax7 expression, leading to self-renewal of the myogenic progenitor pool[192]. Second, upregulation of the Hes and Hey family genes, canonical Notch targets, inhibits MRF expression, indirectly blunting differentiation[192,193]. Finally, crosstalk with other pathways regulates the quiescence of satellite cells. Coordination of Notch signaling and BMP4/SMAD1 inhibits myogenic differentiation[194]. Also, hypoxic conditions of the stem cell niche permit HIF-1α to activate expression of Notch-responsive promoters, helping maintain quiescence[195]. Finally, crosstalk between the Wnt and Notch signaling pathways via GSK3β determines satellite cell progression to proliferative expansion[196].

B. Activation

Satellite cells respond to changes in their niche, some of which coerce activation. Upon injury, growth factors such as FGF2 are released from the ECM, triggering satellite cell activation[197]. Indeed, FGF receptors are upregulated in activated stem cells compared to quiescent stem cells[198]. Syndecan-4 mediates FGF2 signaling, triggering an increase in intracellular calcium which leads to NFATc translocation to the nucleus and satellite cell activation[199,200]. Satellite cell activation is also stimulated by FGF2-mediated activation of the p38 MAPK pathway and inhibiting this pathway prevents activation[201,202]. In parallel to FGF2, HGF is another critical factor for satellite cell activation. It, too, is released from the ECM upon injury[203]. Syndecan-4 is also necessary to mediate HGF signaling[199], which involves HGF binding to c-Met, a receptor on quiescent satellite cells, to promote entry into the cell cycle[204].

Another molecule known to be released from tissue upon injury is nitric oxide (NO). Inhibition of the enzyme responsible for NO production, NO synthase, blunts satellite cell response to injury[205]. NO has been shown to stimulate matrix metalloproteinase (MMP) expression and increase the release of growth factors from the ECM[206].

The canonical growth factor IGF-1 may also activate satellite cells[207]. Several different cell types serve as sources of IGF-1, such as fibroblasts and myofibers[208]. IGF-1-mediated stimulation of the Akt-mTOR pathway and subsequent downregulation of the FOXO transcription factor has been well studied in skeletal muscle[209]. FOXO downregulation by IGF-1 inactivates $p27^{kip}$, a cell cycle repressor, and triggers satellite cell cycling[210].

TNF-α is a cytokine is that is produced and released upon muscle injury[36]. It has been shown to activate satellite cells, leading them to enter the cell cycle[211]. TNF-α-mediated activation of the NF-κB pathway silences Notch1 expression, obstructing quiescent cell signaling and promoting satellite cell activation[212].

The plasma membrane composition of satellite cells also plays a role in their quiescence. Quiescent, but not proliferating, satellite cells have abundant sphingomyelin stores in their plasma membrane[213]. A series of enzymatic reactions may catalyze the conversion of sphingomyelin to sphingosine-1-phosphate (S1P), which can prompt satellite cell entry into the cell cycle and induce its differentiation[214–216]. Interestingly, S1P signaling has differential effects on cell migration depending on which receptors it activates[217]. Altogether, stimulation of S1P preferentially nudges satellite cells from quiescence and into a more differentiated state.

C. Migration

Quiescent satellite cells reside more or less randomly dispersed along the length of myofibers[218]. Upon muscle injury and subsequent activation, they migrate to the lesion locus[219]. A multitude of studies have unveiled the extraordinary migratory capacity of activated satellite cells, even venturing to neighboring fibers to repair damage[219–221], which is higher before they begin proliferating[220,222]. Both adhesive and soluble molecules play a critical role in directing satellite cells to the appropriate region.

Although fibronectin and collagen have been shown to enhance satellite cell migration *in vitro*, laminin is generally considered the best inducer *in vivo*[220]. In addition to the membrane proteins integrin-α7 and integrin-β1, which play a role in laminin binding, CD34 is another membrane protein expressed in satellite cells critical for migration, as CD34-deficient satellite cells exhibit impaired migratory capacity[36,223]. CD44, a multidomain signaling platform with the capability of interacting with collagen, fibronectin, and laminin[224], has also been shown to regulate satellite cell motility[225].

While the ECM may serve as a scaffold for satellite cell migration, it may also act as an obstacle to overcome. Satellite cells exploit MMPs to surmount this impediment. MMPs consist of a broad range of enzymes capable of remodeling the ECM[226]. MMP inhibition has been shown to

blunt satellite cell migration[227,228]. More specifically, MMP-1 is capable of degrading collagen types I, II, and III to facilitate myoblast migration[229]. MMP-2 and MMP-9 are upregulated in regenerating skeletal muscle[230] and overexpression of MMP-9 enhances myogenic cell migration and engraftment[231]. More recently, satellite cell-derived MMP-13 has been found to be required for efficient muscle growth and regeneration[232]. Regulation of MMP activity is thought to occur primarily through tissue inhibitors of MMP (TIMPs). While TIMPs were originally thought to function as MMP inhibitors, as their name suggests, there is accumulating evidence that interactions between TIMPs, MMPs, and the ECM is more nuanced than originally predicted[233,234].

Not only is the ECM a critical element of satellite cell migration but also soluble molecules. Among the components of crushed skeletal muscle, which itself has been found to modulate satellite cell migration, TGF-β and HGF were found to individually exhibit chemotactic activity[235]. The chemotactic activity of the growth factors FGF-2 and FGF-6 have also been reported[220,236]. Wnt7a has been shown to mediate myogenic progenitor dispersion through the Fzd7 receptor via the planar cell polarity pathway[237]. Integrin expression, such as β1-integrin, which is critical for laminin binding[220], is stimulated by interleukin-4[238]. In addition to chemoattractant molecules, repulsive molecules direct satellite cell migration. Eph receptor and ephrin ligand interactions nudge satellite cells away from uninjured myofibers, supplementing the positive chemoattractants in directing them to repair damaged myofibers[239].

D. Proliferation

Upon reaching a damaged locus of skeletal muscle, activated satellite cells proliferate prior to fusion[240]. When proliferation is blocked *in vitro*, myoblasts undergo terminal differentiation[241]. Due to activation of redundant pathways, however, it has been challenging to fully dissect factors which activate satellite cells from those that promote their proliferation. For example, although FGF-2 may stimulate proliferation of myoblasts *in vitro* this activity is likely due

to enhanced satellite cell activation[242]. Additionally, although the proinflammatory cytokine TNF-α promotes satellite cell activation via stimulation of serum response factor[211], it, along with IL-1β, may stimulate IL-6 expression during the early regenerative phase[243,244]. IL-6, in turn, stimulates myoblast proliferation[245,246].

Despite challenges with distinguishing factors which have a binary effect on satellite cell activation or proliferation, the pathways which promote proliferation over differentiation (or vice versa) have been more clearly uncovered. The JAK1-STAT1-STAT3 pathway plays an important role in promoting the proliferation of myoblasts and preventing premature differentiation[247]. A handful of microRNAs play an important role in regulating gene expression at this axis. The microRNAs miR-221 and miR-222 thwart differentiation by delaying Myogenin and p27 expression, a crucial differentiation transcription factor and a cell cycle inhibitor, respectively[248]. Additionally, miRNA-133 represses serum response factor, a key driver of myogenic differentiation[249,250].

E. Differentiation

Satellite cells express a similar genetic program when differentiating compared to fetal myogenesis. In fact, all of the MRFs (MyoD, Myf5, Mrf4, and Myog) are re-expressed during regeneration[18]. Proliferating myoblasts, which express Pax7, Myf5, and MyoD, progress into differentiation through the downregulation of Pax7 and upregulation of Myogenin, becoming $MyoD^{+}$ $Myogenin^{+}$ myocytes[86,251,252]. MyoD lies at a critical nexus between satellite cell proliferation and differentiation. Myoblasts which lack MyoD fail to upregulate Myogenin and Mrf4, leading to impaired muscle regeneration[253–255]. MyoD helps to facilitate the transition from proliferation to differentiation by halting the cell cycle[256–258] and stimulating Myogenin expression[259].

Upon activation by MyoD, Myogenin activates a set of genes involved in muscle contractibility, such as MyHC, creatine kinase, components of voltage-dependent calcium channels, actinin α2, and troponin[260]. Interestingly, despite their relatedness, MyoD cannot compensate for the absence of Myogenin[261]. However, Mrf4 can substitute for Myogenin during early stages of myogenesis[262]. This could be attributed to the greater homology between Myogenin and Mrf4 in contrast to Myogenin and MyoD despite all four MRFs, including Myf5, having very similar topology[17].

Other transcription factors promote myogenic differentiation. In parallel to MyoD, SMAD2 also promotes Myogenin expression, independent from its canonical TGFβ receptor complex signaling[263]. A member of the myocyte enhancer factor (MEF) family, MEF2C, has been shown to physically interact with MyoD or Myogenin to activate downstream gene programs[264]. A positive regulatory feedback mechanism between MEF2C and the MRFs further potentiates the myogenic differentiation program[265,266]. Despite functional redundancy within the MEF family, the MEF transcription factors play a critical role in skeletal muscle regeneration, as myoblasts lacking these factors fail to differentiate[267].

In addition to transcription factors, signaling pathways also regulate myoblast differentiation. As previously mentioned, GSK3-mediated crosstalk between the Notch and canonical Wnt signaling pathways mediates the transition to differentiation[196]. Myoblast differentiation is also stimulated by p38α, a MAPK signaling protein[268], which antagonizes the proliferation-stimulating JNK pathway to decrease myoblast proliferation[269]. p38α also positively regulates myoblast differentiation through its involvement with MyoD/E47 heterodimerization and phosphorylation of MEF2A and MEF2C, the latter of which acts as a coactivator of MyoD[268,270].

F. Fusion and Myotube Formation

After differentiating, myocytes may fuse to damaged myofibers or to each other to form new myofibers[271,272]. This fusion process has been historically well-studied in *Drosophila* due to experimental ease for observing fusion. The first step(s) of the fusion process involves cell recognition and adhesion. These steps are modulated by Dumbfounded (Duf), (Sticks and stones) Sns, (Roughest) Rst, and (Hibris) Hbs proteins in Drosophila[273–279]. In line with its role in satellite cell quiescence, Notch signaling downregulates the expression of adhesion proteins which are important for fusion, such as Sns[280]. Similar proteins have been investigated in mice, such as Nephrin (Sns homolog), β1-integrin, focal adhesion kinase, M-cadherin, and N-cadherin[281,282]. Upon contact of myoblasts, the Duf and Sns proteins form a fusion-restricted myogenic adhesive structure (FuRMAS)[283] that contains F-actin at the sites of cell contact[284]. In response to altered distribution of phosphatidylserine, a membrane phospholipid which has been implicated in myoblast fusion[285,286], Bai3 recruits ELMO/DOCK proteins to the membrane, subsequently activating Rac1[287]. Rac1, a member of the Rho GPTase family, drives actin polymerization through activation of Actin related protein (Arp2/3)[288]. PI(4,5)P2 helps localize Arp2/3 fusion machinery to the fusion site[289]. In *Drosophila*, actin-mediated mechanical forces generate podosome-like membrane protrusions in fusion-competent myoblasts towards mononucleated founder cells[290].

Once properly positioned, myoblasts employ the fusogenic proteins Myomaker and Myomerger to complete the fusion process in vertebrates (Fig. 6). Both fusogens are activated by the MRFs MyoD and Myogenin and are necessary for muscle regeneration[291–296]. Myomaker functionally assists in achieving hemifusion between myoblasts, an intermediate stage in which the outer leaflet of the plasma membrane bilayer is united between two cells but the inner leaflet remains distinct (Fig. 6). Myomerger drives completion of the fusion process through its pore-forming ability[297]. Although the mechanism by which Myomaker participates in myoblast fusion

remains unclear, despite its homology to lipid hydrolases[298–301], more is known about the role of Myomerger in myoblast fusion. Its extracellular alpha helices interact with phosphatidylserine to drive fusion pore formation and complete the fusion process[302,303].

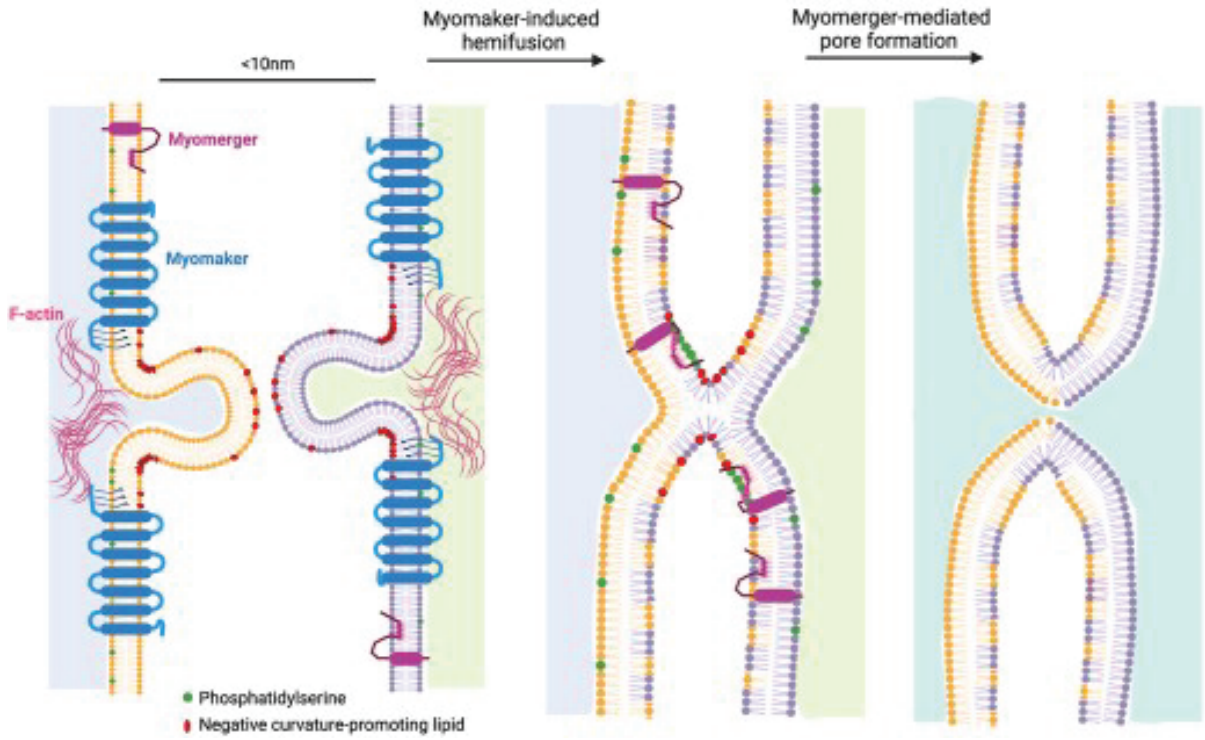

Figure 6: Membrane remodeling during myoblast fusion[299]. After close membrane apposition achieved by actin remodeling, Myomaker assists with the development of a hemifusion state whereby the outer leaflet of the plasma membrane bilayer is united between the two cells but the inner leaflet remains distinct. The pore-forming activity of Myomerger promotes complete cell fusion and cytoplasm union.

While interactions between these proteins have been observed[297,304], it remains unknown whether Myomaker and Myomerger functionally interact, especially if they have independent functions. One model suggests that Myomaker and Myomerger directly interact to promote myoblast fusion. This model is supported by findings from an elegant experiment with human

Myomaker and Myomerger double knockout myoblasts. Heterologous fusion was the highest when the mammalian homolog of Myomaker was expressed in trans to the mammalian homolog of Myomerger rather than in cis[300]. Despite this finding, Myomaker and Myomerger lack obvious domains which would facilitate such an interaction. In contrast, SNARE proteins have considerably large extracellular domains which coordinate timely vesicle fusion for neurotransmitter transmission across synapses[305].

An alternative model proposes that individual activities of Myomaker and Myomerger facilitate the fusion process. Myomerger-null myoblasts undergo hemifusion while Myomaker-null myoblasts do not, indicating the Myomaker plays a role in achieving this step of fusion. Additionally, myoblast fusion can proceed in Myomerger-null myoblasts with the addition of membrane-disrupting agents, supporting the concept that both fusogens are not necessary for myoblast fusion to occur but rather the resulting fusogen activities[297]. Although this model is more physically plausible given the current knowledge on the myoblast fusion process, the laterality of these fusogens in myoblast fusion remains a conundrum. *In vitro*, Myomaker is required on both cells while Myomerger is only required on one cell[294,304,306,307]. *In vivo*, however, data suggests Myomaker expression is only required in the myoblast and not the myofiber[308]. Resolving this enigma will elucidate the mechanism by which Myomaker and Myomerger participate in myoblast fusion.

Chapter 2: Expression of Myomaker and Myomerger in myofibers causes muscle pathology

Phillip C. Witcher[1], Chengyi Sun[1], Douglas P. Millay[1,2,*]

[1]Division of Molecular Cardiovascular Biology, Cincinnati Children's Hospital Medical Center, Cincinnati, OH, USA.

[2]Department of Pediatrics, University of Cincinnati College of Medicine, Cincinnati, OH 45229, USA.

*Corresponding author: douglas.millay@cchmc.org

Abstract

Background

Skeletal muscle development and regeneration depends on cellular fusion of myogenic progenitors to generate multinucleated myofibers. These progenitors utilize two muscle-specific fusogens, Myomaker and Myomerger, that function by remodeling cell membranes to fuse to each other or to existing myofibers. Myomaker and Myomerger expression is restricted to differentiating progenitor cells as they are not detected in adult myofibers. However, Myomaker remains expressed in myofibers from mice with muscular dystrophy. Ablation of Myomaker from dystrophic myofibers results in reduced membrane damage, leading to a model where persistent fusogen expression in myofibers, in contrast to myoblasts, is harmful.

Methods

Dox-inducible transgenic mice were developed to ectopically express Myomaker or Myomerger in the myofiber compartment of skeletal muscle. We quantified indices of myofiber membrane damage, such as serum creatine kinase and IgM^+ myofibers, and assessed general muscle histology, including central nucleation, myofiber size, and fibrosis.

Results

Myomaker or Myomerger expression in myofibers independently caused membrane damage at acute time points. This damage led to muscle pathology, manifesting with centrally nucleated myofibers and muscle atrophy. Dual expression of both Myomaker and Myomerger in the myofiber exacerbated several aspects of muscle pathology compared to expression of either fusogen by itself.

Conclusions

These data reveal that while myofibers can tolerate some level of Myomaker and Myomerger, expression of a single fusogen above a threshold or co-expression of both fusogens are damaging to myofibers. These results explain the paradigm that their expression in myofibers can have

deleterious consequences in muscle pathologies and highlight the need for their highly restricted expression during myogenesis and fusion.

Keywords

Myomaker, Myomerger/Myomixer, muscle pathology, myocyte fusion

Introduction

Skeletal muscle is comprised of multinucleated myofibers formed from the fusion of activated satellite cells, the resident stem cell of skeletal muscle. During development, satellite cells differentiate into myocytes and then fuse to each other to form the skeletal muscle syncytium[36,309,310]. Myomaker and Myomerger are two muscle-specific fusogens necessary for this fusion process during development and regeneration[291,294,304,307]. Myomaker and Myomerger are membrane active proteins that function independently at distinct points of the fusion pathway[297]. While Myomaker functions at or before the hemifusion step of the pathway, where lipids of the outer leaflet of the plasma membrane mix, Myomerger drives pore formation and fusion completion. Myomaker has seven transmembrane domains and an indispensable palmitoylated C-terminal cytoplasmic tail[306]. Although Myomaker shares structural characteristics with lipid hydrolases, its precise activity that confers hemifusion competence is not understood[298–301]. Myomerger, in contrast, is a single-pass transmembrane protein with two extracellular α-helical domains that inserts in membranes causing destabilizations needed for formation of fusion pores[302,303].

While absolutely essential for muscle regeneration, expression of these muscle fusogens is highly regulated and specific to the myoblast stage[311]. Their expression is not detected in myofibers after fusion and genetic data indicates that transcription of the *Myomaker* gene is dispensable in myofibers for their fusion with progenitor cells[308,312]. Moreover, Myomaker expression in myofibers during muscle overload and dystrophic disease is contributed from fusion of progenitor cells indicating that myonuclei within the myofiber lack the ability to transcribe Myomaker[308,313], further highlighting the degree to which the expression of these fusogens is transcriptionally restricted. Stringent control of Myomaker and Myomerger expression is likely needed due to their inherent membrane-remodeling activities. Indeed, Myomerger helices within its ectodomain insert in membranes to convert hemifusion events to full fusion[302,303]. Given

Myomaker is needed to achieve cell hemifusion, a thermodynamically unfavorable event[314], it is plausible that it, too, may have activity which remodels the plasma membrane. We propose that the consequences of these membrane-remodeling effects by the fusogens could be deleterious in certain cell types such as myofibers where they are normally not expressed. This concept is supported by evidence in which Myomaker was genetically deleted in dystrophic myofibers resulting in a reduction of membrane damage[308]. Through ectopic expression of the fusogens in otherwise normal myofibers, we sought to further test the model that consequences of their membrane-remodeling effects could be independently deleterious.

In this study, we assessed the impact of Myomaker and Myomerger activity within the myofiber compartment. To study the fusogens in an *in vivo* setting, transgenic mice were generated to ectopically express Myomaker or Myomerger within myofibers using a doxycycline-inducible system. We found that both fusogens can individually impact myofiber membrane integrity. When expressed together, muscle pathology was exacerbated compared to expression of either fusogen by itself. Altogether, these data support a paradigm whereby Myomaker and Myomerger, while necessary for myoblast fusion, can independently contribute to muscle pathology when expressed in the mature skeletal myofibers, even in the absence of dystrophin-deficiency.

Results

Development of an inducible model for ectopic fusogen expression in myofibers

To ectopically induce expression of the muscle fusogens in the myofiber compartment, we employed a doxycycline-inducible system. Each gene was independently inserted downstream of a tetracycline response element (TRE) at the *Col1a1* locus (Fig. 1A,B). This construct also contained Cre recombinase downstream of Myomaker or Myomerger, linked by an internal ribosome entry site (IRES). These mice were crossed with a previously generated transgenic mouse which utilizes the human skeletal α-actin (HSA) promoter to drive expression of reverse tetracycline transactivator (rtTA)[315]. The resulting mouse lines, HSA^{rtTA}; $Col1a1^{TRE\text{-}Mymk\text{-}IRES\text{-}Cre}$ (iMymk) and HSA^{rtTA}; $Col1a1^{TRE\text{-}Mymg\text{-}IRES\text{-}Cre}$ (iMymg), allowed for both temporal and spatial control of Myomaker or Myomerger expression in myofiber compartments of skeletal muscle.To assess induction of the fusogens in various muscles, *Myomaker* and *Myomerger* mRNA was measured in the tibialis anterior (TA), rectus femoris, and gastrocnemius (gastroc) muscles after a three-day induction with doxycycline chow (Fig. 1C,D). Protein expression of Myomaker and Myomerger was validated in the soleus, extensor digitorum longus (EDL), TA, gastroc, and rectus femoris muscles after three days of induction (Fig. 1E,F). Controls used for 1C-F were dox-treated $Col1a1^{TRE\text{-}Mymk}$ or $Col1a1^{TRE\text{-}Mymg}$ mice, which lacked HSA^{rtTA}. We also confirmed that the mouse models did not have leaky expression of Myomaker or Myomerger in the absence of doxycycline in the gastroc (Fig. 1G,H).

Myomaker expression in myofibers leads to membrane damage and muscle pathology

Gene expression downstream of the TRE element has been validated as soon as 24 hours after induction with doxycycline with the HSA promoter[315]. Thus, we first wanted to assess the impact of short-term Myomaker expression in the myofiber compartment. Evidence of myofiber membrane damage was present as early as three days after induction, as shown by the elevated

serum creatine kinase (CK) (Fig. 2A) and increased proportion of IgM$^+$ myofibers (Fig. 2B). The proportion of IgM$^+$ myofibers in each muscle appeared to correlate with the level of Myomaker expression (Fig 1E, 2B). Previous studies report that dystrophic myofibers exhibit an altered response to an atomic force microscopy (AFM)-based indentation probe, which is generally interpretated as a reduction in myofiber stiffness[316]. Gene-mediated rescue of dystrophic myofibers restores myofiber stiffness, suggesting that a cause of disrupted myofiber architecture in dystrophic myofibers is the lack of dystrophin[317]. To determine if Myomaker in myofibers impacts the biophysical properties of myofibers, such as stiffness, we performed AFM on control and iMymk myofibers. We observed that after three days of induction, Myomaker expression within myofibers caused a reduction in myofiber stiffness (Fig. 2C). These data are consistent with the concept that Myomaker may disrupt the myofiber membrane and alter its biophysical properties.

Consistent with previous reports we also observed reduced stiffness of dystrophic myofibers (Fig. 2C), which was unexpectedly comparable to that of myofibers with ectopic Myomaker expression. Since we previously showed that a reduction of Myomaker in dystrophic myofibers leads to more stable myofiber membranes, we also wanted to evaluate stiffness in this model. We deleted Myomaker in myofibers by treating *mdx*^{4cv}; *Mymk*$^{loxP/loxP}$; HSA$^{CreERT2\ 308}$ with tamoxifen starting at two months of age. Ablating Myomaker from dystrophic myofibers resulted in a normalization of myofiber stiffness (Fig. 2C). Overall, these data reveal the deleterious effects of Myomaker in both wild-type and dystrophic myofibers.

We next wanted to evaluate the long-term effects of Myomaker expression in myofibers. We sacrificed mice twelve weeks after activation of Myomaker in myofibers and, surprisingly, we did not observe direct signs of myofiber membrane damage based on levels of creatine kinase in the serum or IgM$^+$ myofibers (Fig. 3 A,B). The lack of detectable membrane damage could be due to reduced levels of Myomaker expression after twelve weeks of induction compared to three

days (Fig. S1A,B). Despite this apparent reduction in Mymk levels, we detected central nuclei (Fig. 3C), a marker of myofiber repair, reduced muscle masses (Fig. 3D), and reduced myofiber size in the rectus femoris (Fig. 3E) after twelve weeks of Myomaker expression. Altogether, these data implicate Myomaker as a contributor to muscle pathology. We interpret the pathological effects in the long-term to be a result of Myomaker-induced membrane damage at an early stage after activation of the transgene.

Myomerger expression is damaging to skeletal myofibers causing altered muscle histology

Because myoblast fusion also requires Myomerger and this protein is likely activated in muscle pathologies, given its similar transcriptional activation to Myomaker[291,294], we wanted to test the effects of Myomerger in the myofiber compartment. No evidence of membrane damage or changes in muscle mass were observed following a three-day induction of Myomerger in myofibers (Fig. S2 A-C). Even after eight weeks of Myomerger induction within myofibers, myofiber membrane damage was absent and no changes in muscle mass or histology were observed (Fig. S2D-F).

These data conflicted with an alternative approach to assessing the impact of Myomerger within myofibers. Given its high transduction efficiency in skeletal muscle, we utilized adeno-associated virus serotype 9 (AAV9) with a CMV promoter to drive expression of Myomerger in skeletal muscle[318]. AAV9-Myomerger or AAV9-GFP (control) was intramuscularly injected in the TA of two-month-old wild-type mice and muscle was harvested two weeks after the injection. Successful transduction of AAV9-Myomerger was confirmed by western blot analysis (Fig. S3A). Despite the level of Myomerger protein, at the level of the whole muscle, being lower compared to the iMymg model (Fig. S3B), central nucleation was observed two weeks after injection of AAV9-Myomerger, suggesting damage and subsequent regeneration (Fig. S3C). To resolve why

regeneration was observed with viral transduction of skeletal muscle but not the myofiber-inducible model, despite lower levels of Myomerger in the AAV system, we performed immunofluorescence for Myomerger to determine expression at the level of individual myofibers. We found that expression on a per myofiber basis was significantly higher with AAV9-Myomerger compared to the inducible model (Fig. S3D). We interpret the Myomerger-negative myofibers in the AAV system to be derived from fusion of progenitors that were not transduced with AAV9. These data associate Myomerger with induction of regeneration and suggest that myofibers may have a threshold of Myomerger expression which they can tolerate.

We genetically increased expression of Myomerger in the myofiber with the inducible transgenic mice. Mice homozygous for the *Col1a1*$^{\text{TRE-Mymg-IRES-Cre}}$ transgene (iMymg/Mymg) had higher *Myomerger* mRNA levels compared to hemizygous mice (Fig. 4A). Myomerger protein was also elevated in the TA and rectus femoris muscles of iMymg/Mymg mice compared to iMymg mice (Fig. 4B). Several indices of myofiber membrane damage were elevated after fourteen days of induction, the earliest timepoint where changes in serum CK (Fig. 4C) and the proportion of IgM^{+} myofibers (Fig. 4D) were detected, suggesting that the higher myofiber expression of Myomerger can indeed result in myofiber membrane damage. Using the previously described AFM-based indentation approach, we found that the stiffness of these myofibers was reduced compared to the control, indicating that damage from Myomerger levels and activity may impact myofiber stiffness (Fig. 4E). Assessing if Myomerger had a long-term effect in myofibers, we observed altered pathology (Fig. 4F), but the proportion of IgM^{+} myofibers was not significantly changed compared to the control (Fig. 4G). We also observed an increase in regeneration, reduced muscle mass indices, and reduced myofiber size (Fig. 4H-J). Overall, these data reveal that accumulation of Myomerger above certain threshold levels negatively impacts myofiber membrane integrity.

Dual myomaker and myomerger expression in myofibers exacerbates muscle pathology

Thus far, we have demonstrated that Myomaker and Myomerger are individually capable of negatively impacting the myofiber membrane and leading to muscle pathology. However, they are normally expressed together as myoblasts are fusing to repair damaged myofibers or generating new myofibers[295,312]. To study the impact of Myomaker and Myomerger co-expression in myofibers, we crossed the two inducible mouse lines to generate HSA^{rtTA}; *Col1a1*$^{TRE\text{-}Mymk\text{-}IRES\text{-}Cre}$, *Col1a1*$^{TRE\text{-}Mymg\text{-}IRES\text{-}Cre}$ (iMymk/Mymg). *Myomaker* mRNA levels in the iMymk/Mymg mouse were reduced from that of the iMymk mouse (Fig. S4A), but the level of reduction was not statistically significant. Myomaker protein content also appeared slightly lower (Fig. 5A). Reduced mRNA levels were observed for *Myomerger* in iMymk/iMymg muscle compared to the iMymg mouse (Fig. S4A) but such differences were not observed at the protein level (Fig. 5A). Since we have already established that this level of Myomerger does not elicit effects on muscle pathology (Fig. S2), this model allows us to test the combinatorial effects of Myomaker and Myomerger activities. The iMymk/Mymg mouse exhibited increased levels of membrane damage compared to control mice after three days of dox-treatment (Fig. 5B,C). When assessing the stiffness of these fibers, we found that it was reduced compared to the control (Fig. 5D), similar to reductions observed in iMymk (Fig. 2C) and iMymg/Mymg (Fig. 4E) myofibers.

Downstream pathology was evident in iMymk/iMymg mice after twelve weeks of dox treatment (Fig. S4B). Due to the lack of myofiber membrane damage or downstream consequences on muscle pathology in the iMymg mouse (Fig. S2), indices of pathology in the iMymk/Mymg mice were only compared to iMymk mice. For centrally nucleated myofibers, in the iMymk mice we observed 7.2% in the TA, 18.1% in the rectus femoris, and 3.8% in the soleus (Fig. 3C), but the iMymk/Mymg mice exhibited 20% in the TA, 24% in the rectus femoris, and 19.2% in the soleus (Fig. 5E). Effects on muscle masses and myofiber sizes were also exacerbated in iMymk/iMymg mice. iMymk mice exhibited muscle mass reductions of 20% in the

TA and 24% in the rectus femoris (Fig. 3D), whereas iMymk/iMymg mice displayed reductions of 42% in the TA and 52% in the rectus femoris (Fig. 5F). The average myofiber size was reduced by 3% in the soleus, 9% in the TA, and 25% in the rectus femoris of iMymk mice (Fig. 3E) and by 18% in the soleus, 23% in the TA, and 32% in the rectus femoris of iMymk/iMymg mice (Fig. 5G). Consistent with the concept of an exacerbated phenotype when both fusogens are expressed in myofibers, we detected increased fibrosis in iMymk/iMymg muscle (Fig. 5H). These data indicate that ectopic co-expression Myomaker and Myomerger has a combinatorial impact on muscle pathology.

Discussion

In this study, we sought to evaluate the consequences of Myomaker and Myomerger within the terminally differentiated unit of skeletal muscle, the myofiber. We show that short-term induction of Myomaker in the myofiber compartment led to compromised myofiber integrity, which aligns with previous work that linked the myofiber activity of Myomaker to dystrophic pathology[308]. Acute expression of Myomerger similarly led to compromised myofiber integrity. This loss of integrity triggered increased pathology in both inducible models, including centrally nucleated fibers and reduced muscle mass indices. In sum, we postulate that expression of the fusogens in the short-term yields membrane damage causing detectable changes in muscle histology in the long-term. These data are consistent with the concept that expression of the muscle fusogens needs to be highly restricted during myoblast fusion to prevent negative outcomes within the myofiber.

Despite the unambiguous damaging consequences of Myomaker and Myomerger in the myofiber compartment, careful analysis of the data reveals that myofibers have a threshold for which they may tolerate fusogen expression. Myofiber membrane damage was not observed after induction of Myomerger until expression was genetically increased (Fig. 4). The same paradigm holds true for Myomaker, where lower levels of Myomaker after twelve weeks of expression compared to three days is not sufficient to induce detectable myofiber membrane damage (Fig. 3B). While this study implicates the fusogens as having a negative effect on myofibers, it does not definitively discount the possibility that Myomaker and Myomerger could have functional roles on the myofiber for fusion in some contexts.

When Myomaker and Myomerger were co-expressed, indices of myofiber membrane damage, muscle regeneration, and muscle atrophy were exacerbated. When Myomerger was expressed by itself, no detectable levels of serum CK or IgM^+ myofibers were observed (Fig. S2). However, when the same level of Myomerger was expressed with Myomaker, elevated levels of IgM^+ myofibers were observed in the short-term and increased centrally nucleated myofibers,

muscle atrophy, and fibrosis were observed in the long-term (Fig. 5). These data are consistent with the paradigm that these two fusogens have independent but overlapping membrane remodeling activities, which drive fusion in myoblasts but cause membrane instability in myofibers.

To evaluate the biophysical consequences of Myomaker and Myomerger in the myofiber, we utilized an AFM-based indentation approach to measure myofiber stiffness. Myofiber stiffness is primarily a function of two parameters: cytoskeletal components and plasma membrane integrity. Actin and myosin are the main cytoskeletal contributors to skeletal muscle stiffness while cholesterol and lipid saturation are membrane contributors[319,320]. Cell stiffness is associated with changes in function, increasing as myocytes differentiate to myotubes[319]. Although previous studies utilizing AFM-based indentation to quantify skeletal muscle stiffness have reported variable stiffness values for skeletal muscle, absolute stiffness values measured by AFM indentation protocols are strongly dependent on the experimental parameters, model, and method used to analyze the results[321–324]. For example, cell fixation with PFA increases the measured stiffness[325]. Despite divergent quantifications of cell stiffness, there is a general consensus that dystrophic muscle is less stiff than its wild-type counterpart[316,317,326]. The reason behind this difference has previously been attributed to the loss of structural integrity provided by dystrophin, a crucial protein linking intracellular cytoskeletal components to the basal lamina[327,328]. Here, we provide evidence that reduced myofiber stiffness in dystrophy may not be primarily caused by lack of dystrophin. The activity of the muscle fusogens, essential for myoblast fusion during regeneration, individually led to reduced myofiber stiffness and ablation of myomaker in dystrophic myofibers restored stiffness to a level comparable to that of wild-type myofibers. These data support the concept that ectopic fusogen expression and activity in myofibers may contribute to reduced stiffness in dystrophic myofibers.

Defective function of myogenic progenitors has been implicated in muscular dystrophy pathology. Although repetitive rounds of degeneration and regeneration lead to an exhausted satellite cell pool[329–331], other studies have shown that an increased number of satellite cells are present in dystrophic muscle[332,333]. Despite this discordance, it is apparent that dystrophic satellite cells exhibit impaired regeneration[334,335]. The inability to replace necrotic myofibers culminates in fibro-fatty replacement of skeletal muscle and muscle atrophy[336,337]. Collectively, those data indicate that the regenerative process goes awry in dystrophy, which could overall accelerate pathology. However, there is increasing evidence that the regenerative program has maladaptive features during skeletal muscle disease[338,339]. Indeed, ablation of satellite cells in dystrophic mouse models results in a situation where remaining myofibers exhibit increased size and stabilized membranes[340]. Based on this work, one could envision a scenario where reduction of satellite cell activity could be a valuable therapeutic approach. However, in the long-term, ablation of satellite cells or blockade of their fusogenic activity results in muscle wasting in a dystrophic setting[308]. Interestingly, attenuation of the MyoD pathway in dystrophic myofibers blunts sarcolemma instability[340], which is consistent with a maladaptive function in this setting for Myomaker and Myomerger given that these proteins are transcriptionally induced by MyoD. Thus, instead of broadly modulating satellite cells and their corresponding regenerative capacity that has beneficial consequences for long-term muscle maintenance, specifically targeting negative consequences of chronic fusion, namely persistent delivery of progenitor-derived Myomaker and Myomerger to myofibers, could be an approach to mitigate pathology in dystrophic tissue.

One limitation of our study is that we are only able to detect robust membrane damage through IgM analysis and serum CK levels. These methods of assessing myofiber membrane damage may not detect more moderate levels of damage. This is exemplified by elevated levels of centrally nucleated myofibers despite initially low proportions of IgM^+ myofibers in the iMymk soleus (Fig. 2B and 3C) and iMymg/Mymg rectus femoris (Fig. 4D,H). Thus, the analytical

pipelines used to determine loss of membrane integrity were not able to stratify the deleterious effects of the fusogens. Additionally, the nature of membrane damage caused by Myomaker and Myomerger may not be identical. Another limitation of our study is that we are unable to uncouple the potential activity of the fusogens at the plasma membrane and intracellular compartments. Previous studies have demonstrated that Myomaker not only resides at the plasma membrane but also in the Golgi and post-Golgi vesicles[341]. Additionally, Myomerger has been shown to also associate with intracellular membrane compartments[294]. Overexpression of either fusogen could accordingly have a negative impact in other membrane-bound organelles, which perhaps could explain why we observed a strong atrophy phenotype. Our study was also limited by the lack of measurements for the same muscle. For example, myofiber stiffness was not quantified for the TA or rectus femoris due to inherent challenges with isolating individual myofibers from these muscles and we did not assess long-term pathology in the EDL, which was used for AFM measurements. However, there is a consistent pattern of elevated levels of myofiber membrane damage early (measured by the proportion of IgM^+ myofibers and serum CK) and muscle pathology in the long term (elevated centrally nucleated myofibers and muscle atrophy).

In summary, this study supports a paradigm whereby Myomaker and Myomerger are essential for fusion of muscle progenitors but have deleterious consequences within myofibers. The adverse effects of Myomaker and Myomerger in myofibers could explain why their expression is so tightly restricted to the myocyte stage of the muscle lineage. Persistent and dysregulated activation of the regeneration program in skeletal muscle may lead to unintended consequences of these membrane-active fusogens disrupting the myofiber membrane, further exacerbating myofiber membrane damage in pathologic conditions, like muscular dystrophy. Down-regulation of these fusogens in myofibers may serve as a potential therapeutic option for reducing muscle damage in muscular dystrophy.

Methods

Mice

This study was performed entirely in mice using either commercially available transgenic mice or novel transgenic mice generated as described below. All mice used in this study were maintained on a *C57BL/6* background. For ectopic expression of the muscle fusogens, doxycycline-inducible transgenes, TRE3G-Myomaker-IRES2-Cre-pA and TRE3G-Myomerger-IRES2-Cre-pA, were targeted into the Col1a1 safe harbor (CaSH) locus using a CRISPR/Cas9-mediated approach developed by Transgenic Animal and Genome Editing Core at Cincinnati Children's Hospital Medical Center. The transgenes were inserted to a genetic location ~1.65kb downstream of the Col1a1 gene in a reverse orientation. This was achieved using a sgRNA (target sequence: GGGAGGAAACCTGCCCTTGG) and a donor plasmid containing the transgene flanked with the 5' and 3' homologous arms at the length of 2.5 kb and 3.0 kb, respectively. The donor plasmids were amplified and purified with the EndoFree Plasmid kit (Qiagen). The targeted transgenic mice were generated via pronuclear injection of fertilized C57BL/6 eggs with Cas9 protein (IDT, Catalog #1081059), synthetic sgRNA (Synthego), and the donor plasmid at a concentration of 40 ng/μl, 20 ng/μl, and 4 ng/μl, respectively. The injected eggs were transferred immediately into the oviductal ampulla of pseudopregnant CD-1 females for development and birth. The pups were then genotyped by long-range PCR and Sanger sequencing. These mice (*Col1a1*$^{\text{TRE-Mymk IRES-Cre}}$ and *Col1a1*$^{\text{TRE-Mymg IRES-Cre}}$) were crossed with mice carrying the HSA$^{\text{rtTA}}$ allele to drive fusogen expression in the myofiber compartment[315]. Dual expression of Myomaker and Myomerger was generated by breeding the *Col1a1*$^{\text{TRE-Mymk IRES-Cre}}$ mice with *Col1a1*$^{\text{TRE-Mymg IRES-Cre}}$ mice, followed by breeding with the HSA$^{\text{rtTA}}$ mouse. Myofiber-specific deletion of *Mymk* in the dystrophic background was accomplished by introducing an HSA$^{\text{CreERT2}}$ allele into the *Mymk*$^{\text{loxP/loxP}}$ *mdx*$^{\text{4cv}}$ mouse[308,342].

To induce fusogen expression in myofibers, 1- to 2-month-old mice were provided chow supplemented with 0.0625% doxycycline (TestDiet). Tissue was collected immediately upon completion of doxycycline treatment.

Tamoxifen (MilliporeSigma) was prepared in corn oil with 10% ethanol at a concentration of 25 mg/mL. Mice were given intraperitoneal injections of tamoxifen (0.075 mg/kg/d) for four days to induce recombination. For experiments with the $HSA^{CreERT2}$ allele, mice were then maintained on tamoxifen by injection every third day.

AAV9-GFP and AAV9-Myomerger were generated by Vigene Biosciences and intramuscularly injected (5×10^{11} genome copies/injection, diluted with sterile PBS) into the TA muscle of 2-month-old mice while under inhaled isoflurane anesthesia. The injection site was prepared by first removing hair with hair clippers and then sanitizing the area with chlorhexidine gluconate and allowing it to dry.

Muscle collection and sample preparation

Mouse hindlimb muscles were dissected, dried, and weighed. Tibias were dissected and remaining tissue was digested proteinase K (0.4 mg/mL) overnight at 55°C, after which tibia length was measured using digital calipers. Muscles were embedded in 10% tragacanth/PBS (MilliporeSigma) and frozen in 2-methylbutane cooled in liquid nitrogen. We used 10-μm sections for all histology. For RNA and immunoblot preparations, tissues were flash-frozen in liquid nitrogen immediately upon collection.

Histological analyses

Immunohistochemical studies were performed as described previously with minor modifications[308]. Briefly, sections were fixed in 1% PFA/PBS and permeabilized with 0.2% Triton X-100/PBS. Sections were blocked using 2% BSA, 1% heat-inactivated goat serum, and 0.1%

Tween-20/PBS. Primary antibodies were incubated overnight at 4°C and secondary Alexa Fluor antibodies (1:300) were applied at room temperature for 30 minutes. Anti-laminin antibody (1:300, MilliporeSigma, stock No. L9393) was used to visualize the outline of all myofibers present in each muscle section. IgM primary antibody conjugated to Texas Red (1:100, MilliporeSigma, stock No. SAB3701210) was used to highlight myofibers with compromised membrane integrity. Anti-ESGP antibody (1:100, R&D, stock No. AF4580) was used to stain Myomerger protein on muscle sections. Immunostained slides were imaged using a Nikon A1R confocal system. Centrally located myonuclei were quantified from two 10X images using ImageJ (NIH). IgM positive myofibers and myofiber size were quantified from the entire muscle section using NIS-Elements software (Nikon).

Picrosirius red staining was used to quantify muscle fibrosis. Briefly, fresh-frozen sections were incubated overnight in Bouin's solution. After a five-minute wash in PBS, sections were incubated in working Weigert's hematoxylin for five minutes before a one-hour incubation in picrosirius red. Sections were dipped two times in 0.5% acetic acid and three times in ethanol. Three one-minute exchanges in xylenes were performed before mounting. Picrosirius red stained sections were imaged using an Olympus BX60 widefield microscope. Fibrosis was quantified from two 10X images using ImageJ (NIH).

Gross pathology was assessed with hematoxylin and eosin (H&E) staining. Fresh-frozen sections were incubated in 10% formalin for five minutes before washing in PBS for two minutes followed by a two-minute wash in tap water. After incubating sections in working Weigert's hematoxylin for five minutes, they were rinsed with tap water until tap water ran clear. Sections were dipped ten times in 0.7% Eosin Y, ten times in 95% ethanol, ten times in 95% ethanol, ten times in 100% ethanol, ten times in 100% ethanol, ten times in xylene, ten times in xylene, and ten times in one last xylene solution before mounting. H&E stained sections were imaged using an Olympus BX60 widefield microscope. All image analyses were performed in a blinded fashion.

RNA analysis

Total RNA was isolated from muscle samples using established TRIzol protocols (Life Technologies, stock No 15596018). cDNA was synthesized with the Superscript VILO cDNA synthesis kit (Invitrogen, Thermo Fishers Scientific, stock No. 11754250). Standard qPCR methods were used with PowerUp SYBR Green Master Mix (Applied Biosystems, Thermo Fisher Scientific) and the assay was performed on the Bio-Rad CFX96 Real-Time System with the following primers: *GAPDH*: forward, 5'-TGCGACTTCAACAGCAACTC-3'; reverse, 5'-GCCTCTCTTGCTCAGTGTCC-3', *Mymk*: forward, 5'-ATCGCTACCAAGAGGCGTT-3'; reverse, 5'-CACAGCACAGACAAACCAGG-3', *Mymx*: forward, 5'-CAGGAGGGCAAGAAGTTCAG-3'; reverse, 5'-ATGTCTTGGGAGCTCAGTCG-3'. mRNA levels were quantified using the *ΔΔ*Ct method[343].

Western blotting

After measuring the mass, muscles were homogenized in muscle lysis buffer (10 mM Tris, 1 mM EDTA, 0.5% Triton X-100, and 50 mM NaF buffer, pH 7.4) supplemented with a protease inhibitor cocktail (Sigma Aldrich, stock No. 5056489001). Solubilization was allowed to proceed on a nutator for 2 hr at 4°C. Protein lysates were prepared for SDS-PAGE analysis by heating at 95°C for 5 min in 1x Laemmli sample buffer containing 10% beta-mercaptoethanol. Proteins were resolved on discontinuous polyacrylamide gels (12% for myomaker and 15% for myomerger) and transferred to Immobilon-FL PVDF membranes (Millipore Sigma, stock No. IPFL00010). Membranes were blocked in 5% milk/TBST for 1 hr at room temperature before incubation with primary antibodies in 5% BSA/TBST against Myomaker (1:250, provided from Dr. Leonid Chernomordik laboratory), Myomerger (1:200, R&D, stock No. AF4580), and GAPDH (1:5,000, Millipore, stock No. MAB374) overnight on a nutator at 4°C. The resulting immunoblots generated

after incubation with relevant secondary antibodies (goat anti-rabbit IgG DyLight 800, Cell Signaling Technology, stock No. 5151; donkey anti-sheep IgG Alexa Fluor 680, Invitrogen Thermo Fisher Scientific, stock No. A21102; goat anti-mouse IgG DyLight 680, Cell Signaling Technology, stock No. 5470; goat anti-mouse IgG Dylight 800, Cell Signaling Technology, stock No. 5257) were scanned, imaged, and analyzed using the Odyssey CLx imaging system (LI-COR Biosciences, stock No. 9140). Protein expression was quantified using densitometric analysis tools on ImageJ (NIH). The band intensities of Myomaker and Myomerger were measured and standardized to the intensity of the housekeeping gene, GAPDH.

Serum creatine kinase

Serum creatinine kinase levels were measured using a Roche c 311 clinical chemistry analyzer per manufacturer instructions.

Atomic force microscopy

Atomic force microscopy was used to measure stiffness of single muscle fibers. For isolation of single muscle fibers, whole EDL muscles were incubated in 0.22% type I collagenase (MilliporeSigma C0130) in DMEM at 37°C for 40 minutes. Following incubation, muscles were triturated in PBS to release individual myofibers. The myofibers were subsequently washed with PBS before fixing in 4% PFA/PBS for 10 minutes at room temperature, after which the fixed fibers were washed again with PBS and stored at 4°C. Fixed myofibers were placed on double-sided tape applied to the bottom of 60 mm plates. The plates were centrifuged at 400 *g* for 10 min at room temperature to attach the myofibers to the tape[322]. Attached myofibers were submerged in 0.22 μm-filtered PBS prior to measurement by AFM. Stiffness was quantified using the contact mode of force mapping on a NanoWizard 4 XP BioScience atomic force microscope with a HybridStage (Bruker). A Nikon Eclipse Ti-U inverted microscope permitted precise positioning of

the cantilever tip above the myofiber. Before each experiment, the cantilever was calibrated while submerged in PBS in a region of the dish that did not contain a myofiber nearby. A z closed loop with constant force, 0.05 nN setpoint, 1.0 μm z length, 2.0 μm/s z speed, and 0.0 s contact time was used to make sixty-four measurements were collected from a 10 x 10 μm area of the myofiber. The calibrated spring constant of cantilever D was used to convert the photodiode signal into a force value (k_{nom} = 0.03 N/m, MLCT-BIO; Bruker). Young's modulus was extracted from each force-indentation curve using a modified Hertz model with the Bilodeau formula for a quadratic pyramidal indenter[344]:

$$F = 0.7453 \frac{E}{1-\nu^2} \delta^2 \tan \alpha,$$

where *F* is the indentation force, *E* is Young's modulus, *v* is Poisson's ratio (approximated as 0.5, the value for isotropic incompressible materials), *δ* is the indentation (vertical tip position), and *α* is the half face angle of the pyramid (17.5° for cantilever D). The equivalent radius of a contact circle was calculated as the following:

$$a_e = 0.709 \delta \tan \alpha,$$

where a_e is the equivalent radius of contact circle, *δ* is the indentation (vertical tip position), and *α* is the half face angle of the pyramid (17.5° for cantilever D). The data curve was fitted using a least squares fit with the Levenberg-Marquardt algorithm. The contact point, baseline, and Young's modulus values were all fitted simultaneously. Measurements were taken at three different locations and averaged to yield the stiffness of a given myofiber. The mean stiffness of three unique myofibers comprised the myofiber stiffness for a given mouse.

Statistics

All statistical analysis was performed using GraphPad Prism 9 software. Data are presented as mean ± standard error of the mean. Groups were assessed for normality using a Shapiro-Wilk test and analyzed using a one-way ANOVA with post hoc Tukey's for multiple

comparisons. Significant differences between two groups were determined using a two-tailed unpaired Student's *t* test. Statistical significance throughout was set at *P* values less than 0.05. Specific statistical tests are noted in the figure legends.

Figures

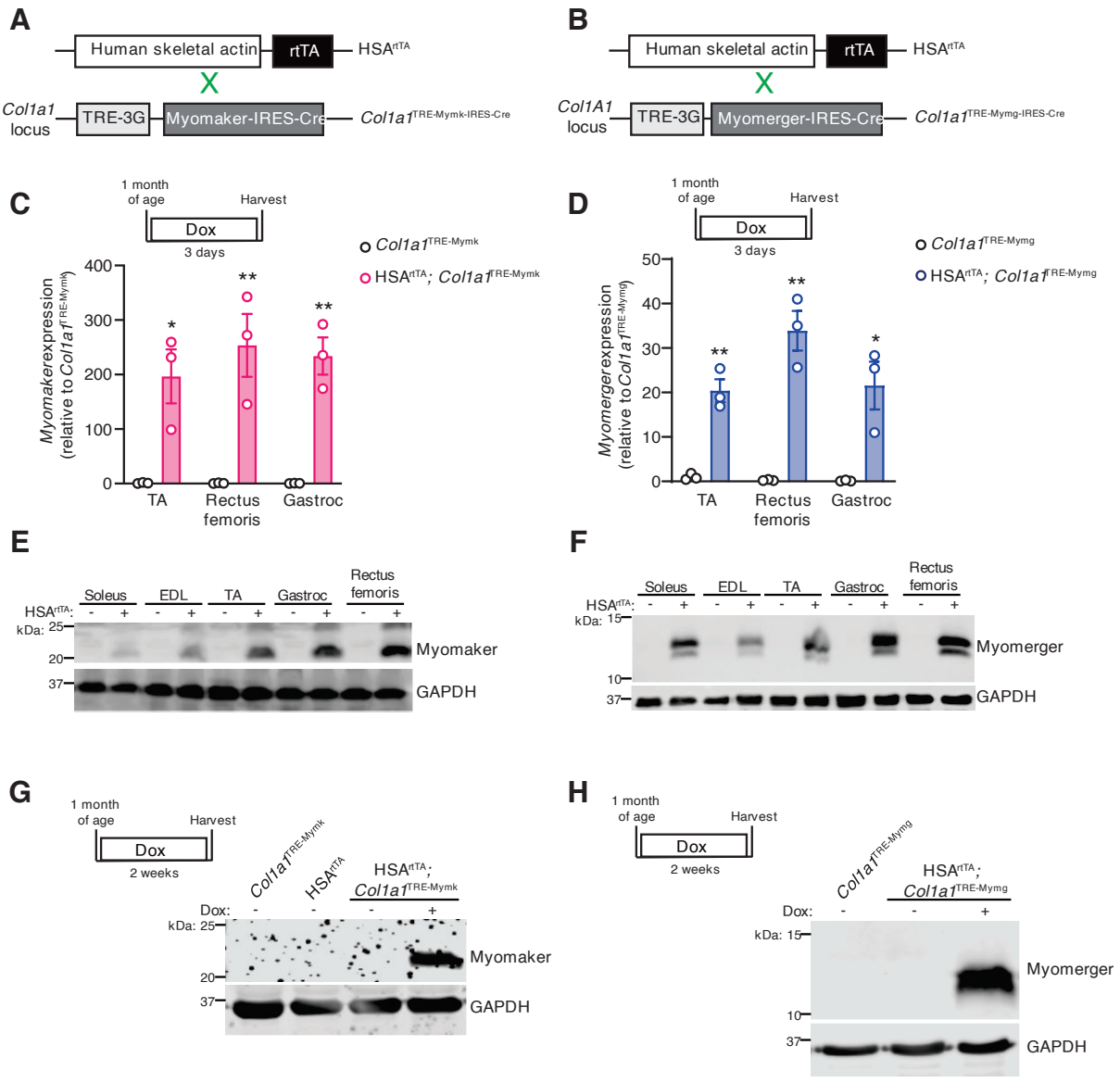

Figure 1. Validation of transgenic models to activate Myomaker and Myomerger expression in myofibers. **A** Schematic of the breeding strategy for inducible expression of Myomaker within myofibers. **B** Schematic of the breeding strategy for inducible expression of Myomerger within myofibers. **C** qPCR analysis of *Myomaker* mRNA levels from the TA, rectus femoris, and gastroc muscles after three days of dox treatment. **D** qPCR analysis for *Myomerger* mRNA levels from

the TA, rectus femoris, and gastroc muscles after three days of induction. **E** Western blot for Myomaker expression in the soleus, EDL, TA, gastroc, and rectus femoris after three days of dox treatment. **F** Western blot for Myomerger expression in the soleus, EDL, TA, gastroc, and rectus femoris after three days of dox treatment. **G** Western blot for Myomaker expression in the gastroc muscle after two weeks of induction. **H** Western blot for Myomerger in the gastroc after two weeks of induction. Statistical analyses and presentation: Data are presented as mean ± SEM; **C,D** two-tailed Student's *t* test; $*P < 0.05$, $**P < 0.01$.

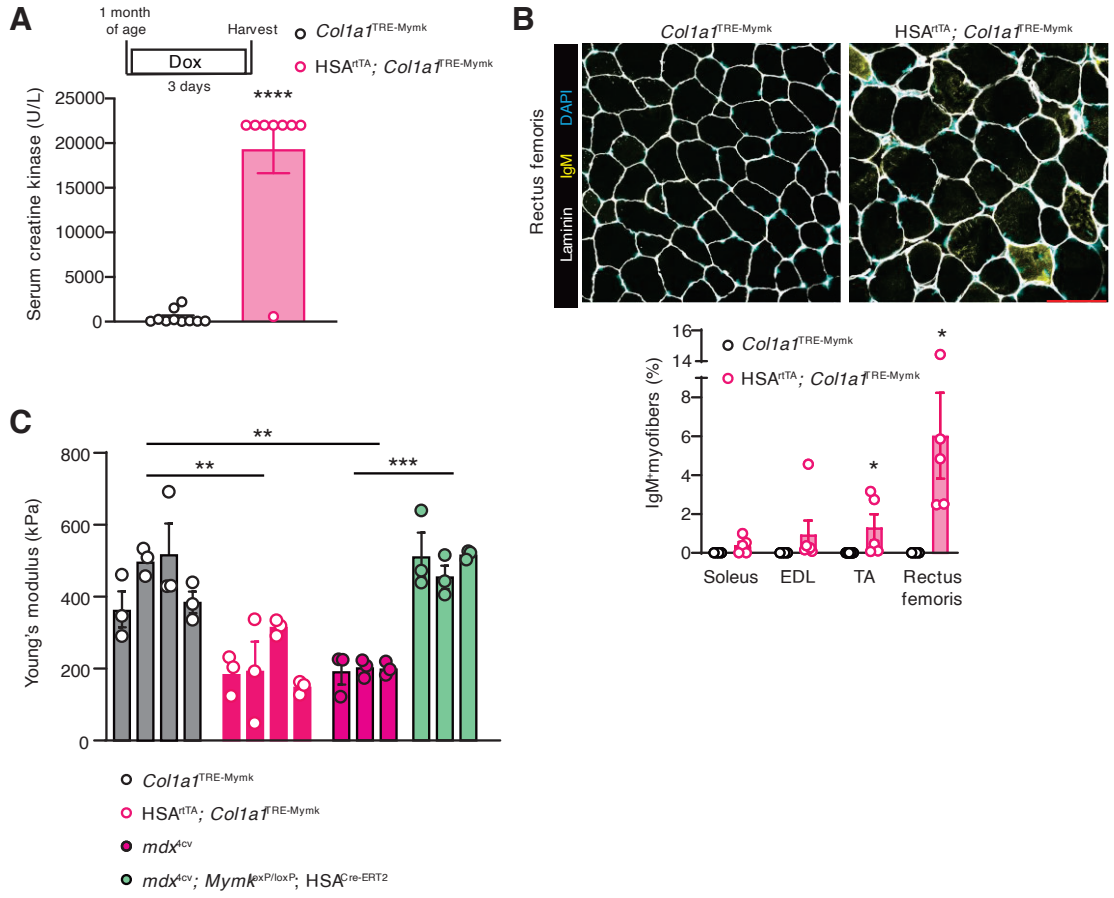

Figure 2. Myomaker expression in myofibers is associated with myofiber membrane damage and reduced myofiber stiffness. **A** Serum CK levels are elevated after three days of Myomaker induction in myofibers. **B** Representative images of immunofluorescence staining for IgM in the rectus femoris after three days of Myomaker induction reveal an elevated proportion of IgM$^+$ myofibers compared to the control. Quantification of the percentage of IgM$^+$ myofibers in the soleus, EDL, TA, and rectus femoris is shown below the images. Scale bar = 100 μm. **C** Atomic force microscopy on isolated myofibers reveals reduced myofiber stiffness with Myomaker expression in myofibers three days after induction. Myofiber stiffness in dystrophic myofibers is restored to wild-type levels after ablation of Myomaker from myofibers. Each bar represents the average stiffness of three myofibers from a given mouse. Statistical analyses and presentation: Data are presented as mean ± SEM; **A,B** two-tailed Student's *t* test; **P* < 0.05, *****P* < 0.0001; **C**

one-way ANOVA between experimental groups with a Tukey's post hoc test; $^{**}P < 0.01$, $^{***}P < 0.001$.

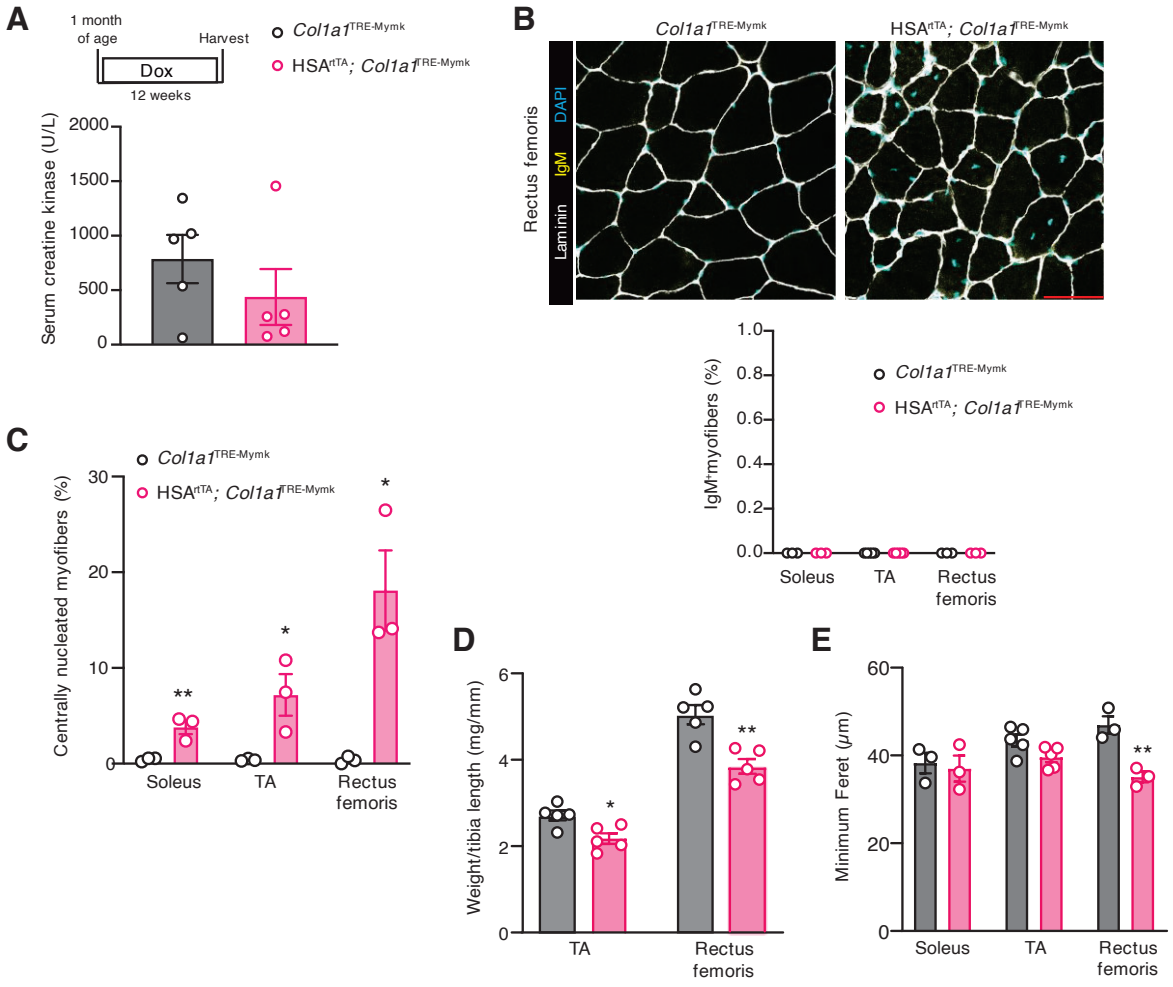

Figure 3. Long-term Myomaker expression in myofibers leads to elevated centrally nucleated myofibers and muscle atrophy. **A** Serum CK levels are not elevated after twelve weeks of Myomaker expression in myofibers. **B** Immunofluorescence staining for IgM in the rectus femoris after twelve weeks of Myomaker induction. Quantification of the percentage of IgM^+ myofibers in the soleus, TA, and rectus femoris is shown below the images. Scale bar = 100 μm. **C** Quantification of myofibers with centrally localized nuclei in the soleus, TA, and rectus femoris reveals elevated levels of regeneration with Myomaker expression in myofibers. **D** Muscle mass to tibia length ratios of the rectus femoris and TA were reduced after twelve weeks of Myomaker expression in myofibers. **E** Myofiber size, quantified by minimum Feret's diameter, was quantified

in the soleus, TA, and rectus femoris. Statistical analyses and presentation: Data are presented as mean ± SEM; **C-E** two-tailed Student's *t* test; **P* < 0.05, ***P* < 0.01.

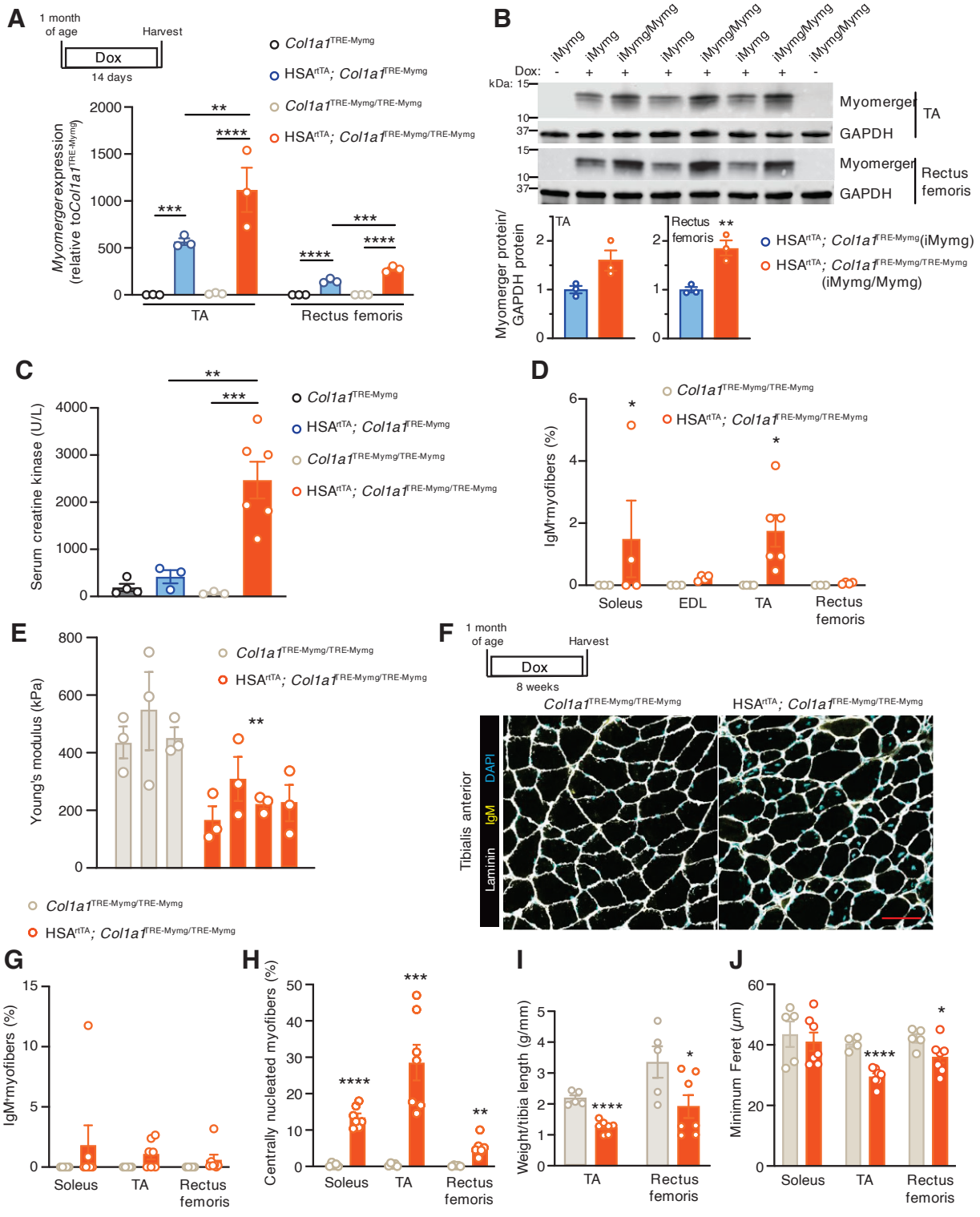

Figure 4. Muscle pathology results from elevated Myomerger expression in myofibers. **A** qPCR analysis reveals elevated levels of *Myomerger* in the TA and rectus femoris muscles of

homozygous mice compared to heterozygous mice after two weeks of induction. **B** Quantification of Western blots demonstrate elevated Myomerger protein content in the TA and rectus femoris muscles of iMymg/Mymg mice compared to iMymg mice after two weeks of induction. **C** Serum CK levels in iMymg/Mymg mice are elevated after two weeks of Myomerger expression. Levels are also significantly higher than two weeks of Myomerger expression in iMymg mice. **D** Quantification of the proportion of IgM^+ myofibers reveals elevated levels of damage in the soleus and TA muscles after two weeks of Myomerger expression. **E** Myofiber stiffness is reduced after two weeks of Myomerger expression in myofibers by atomic force microscopy. Each bar represents the average stiffness of three myofibers from a given mouse. **F** Representative sections of the TA muscle from iMymg/Mymg mice after eight weeks of Myomerger expression revealed a lack of significantly elevated proportion of IgM^+ myofibers (**G**). **H** Elevated levels of centrally nucleated myofibers were observed in the soleus, TA, and rectus femoris. Scale bar = 100 μm. **I** Muscle mass to tibia length ratios were reduced in the TA and rectus femoris after eight weeks of Myomerger expression in myofibers. **J** Myofiber size, quantified by minimum Feret's diameter, was quantified in the soleus, TA, and rectus femoris. Statistical analyses and presentation: Data are presented as mean ± SEM; **A**, **C** one-way ANOVA with a Tukey's post hoc test compared samples from the same muscle; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$; **B**, **D-E**, **G-J** two-tailed Student's *t* test, identical muscles were compared in **D**, **G-J**, stiffness values were compared between the two groups using average values from each mouse for **E**; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$.

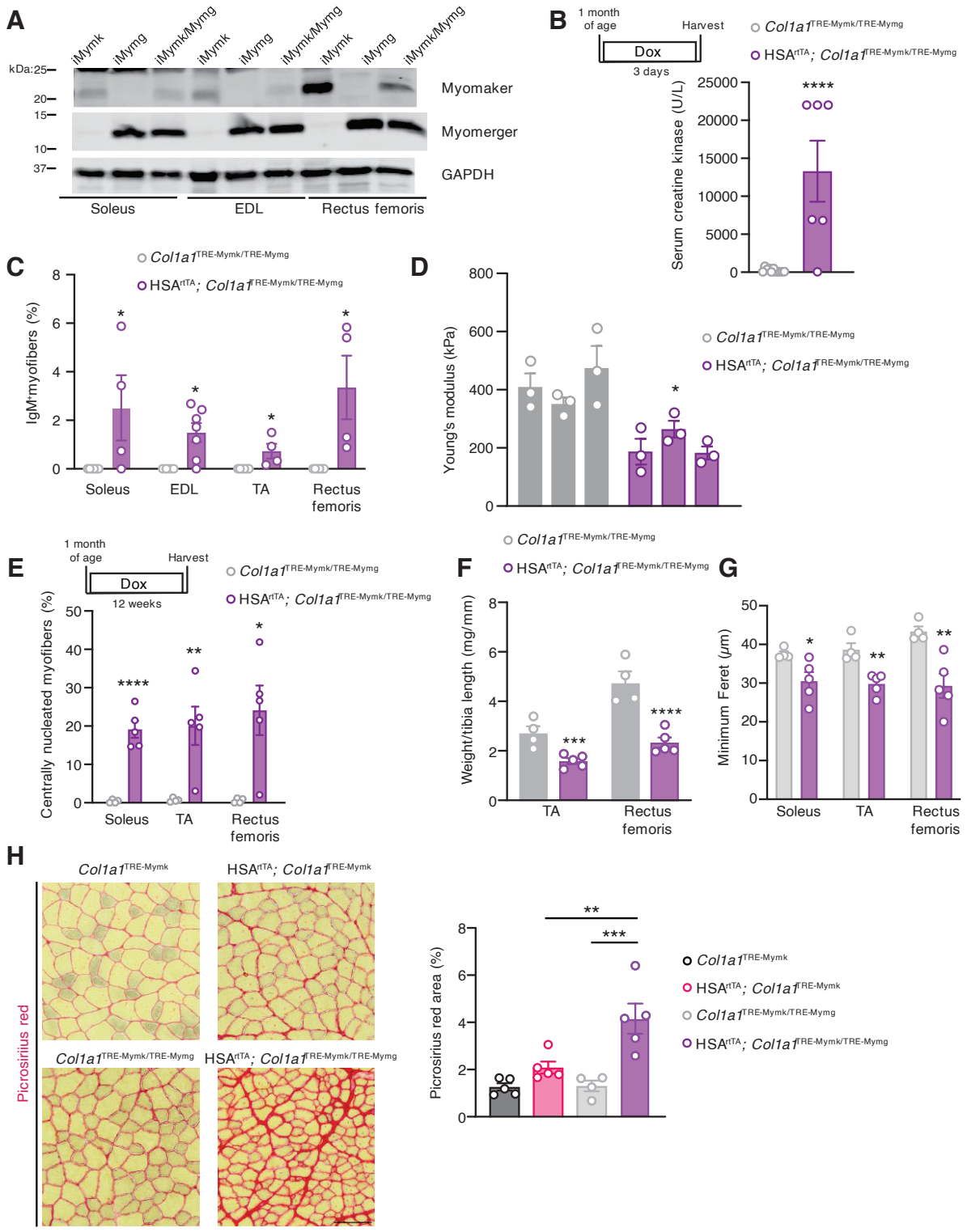

Figure 5. Expression of both Myomaker and Myomerger in myofibers results in combinatorial effects. **A** Western blots for Myomaker and Myomerger expression in the soleus,

EDL, and rectus femoris after three days of dox treatment. **B** Serum CK levels after three days of induction in myofibers. **C** Quantification of the proportion of IgM^+ myofibers in the soleus, EDL, TA, and rectus femoris after three days of induction reveal an elevated proportion of IgM^+ myofibers compared to the control in all four muscles. **D** Myofiber stiffness assessed by atomic force microscopy is reduced after three days of concurrent Myomaker and Myomerger expression. Each bar represents the average stiffness of three myofibers from a given mouse. **E** Quantification of myofibers with centrally localized nuclei in the soleus, TA, and rectus femoris. **F** Muscle mass to tibia length ratios of the TA and rectus femoris. **G** Myofiber size in the soleus, TA, and rectus femoris, quantified by Minimum Feret's diameter. **H** Picrosirius red staining revealed elevated fibrosis when both Myomaker and Myomerger are expressed in myofibers. Scale bar = 100 μm. Fibrosis is quantified as the percentage of total area staining positive for Picrosirius red. Statistical analyses and presentation: Data are presented as mean ± SEM; **B-G** two-tailed Student's *t* test, identical muscles were compared in **C**, **E-G**, stiffness values were compared between the two groups using average values from each mouse for **D**; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$. **H** one-way ANOVA with a Tukey's post hoc test; $**P < 0.01$, $***P < 0.001$.

Supplemental Figures

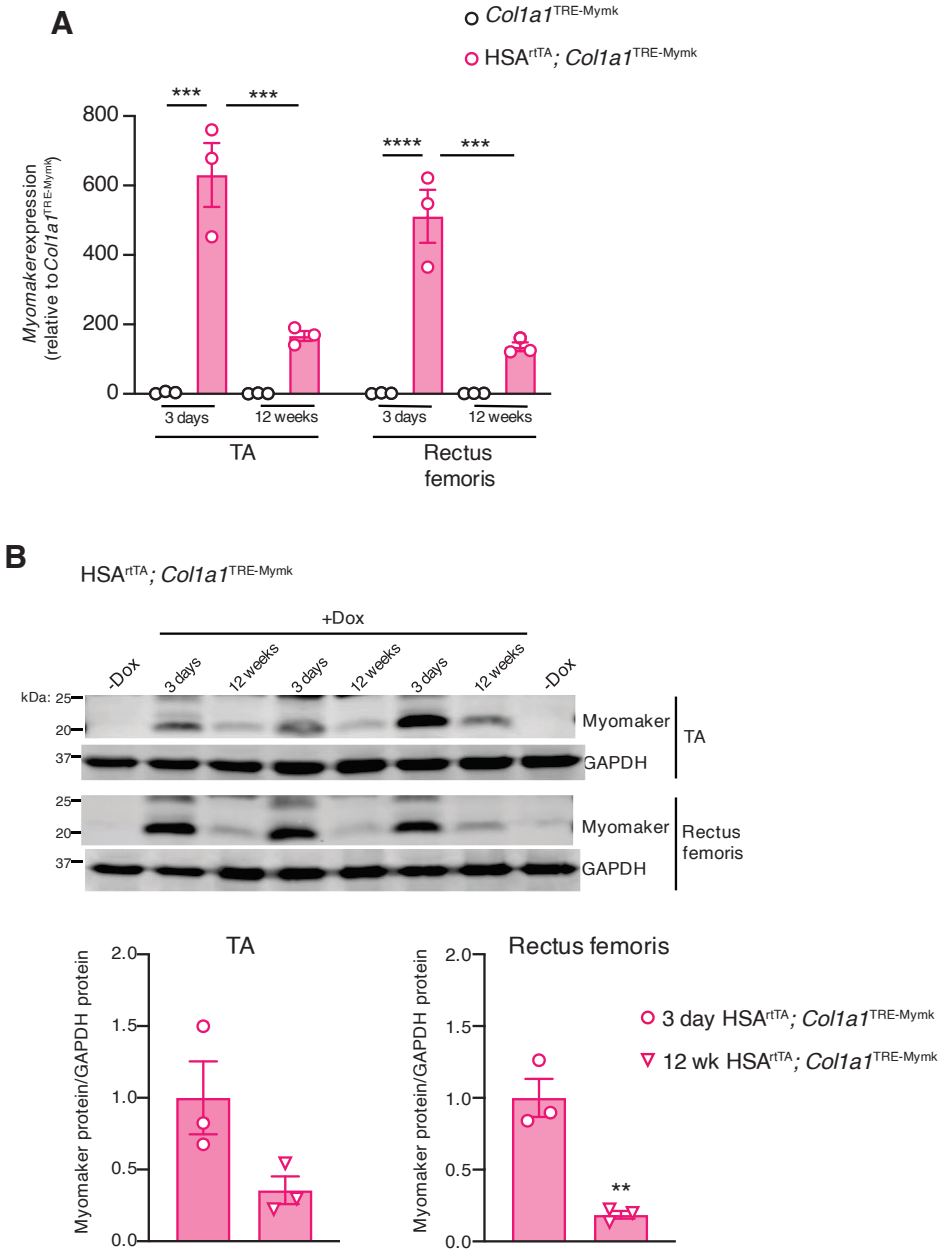

Figure S1. Myomaker expression is lower after twelve weeks of induction compared to three days of induction. **A** qPCR analysis of *Myomaker* mRNA levels from the TA and rectus femoris muscles after twelve weeks or three days of induction. **B** Quantification of Western blot for Myomaker expression in the TA and rectus femoris relative to GAPDH expression

demonstrates reduced Myomaker protein after twelve weeks of induction compared to three days of induction. Statistical analyses and presentation: Data are presented as mean ± SEM; **A** one-way ANOVA with a Tukey's post hoc test compared samples from the same muscle; ***$P < 0.001$, ****$P < 0.0001$; **B** two-tailed Student's *t* test; ** $P < 0.01$.

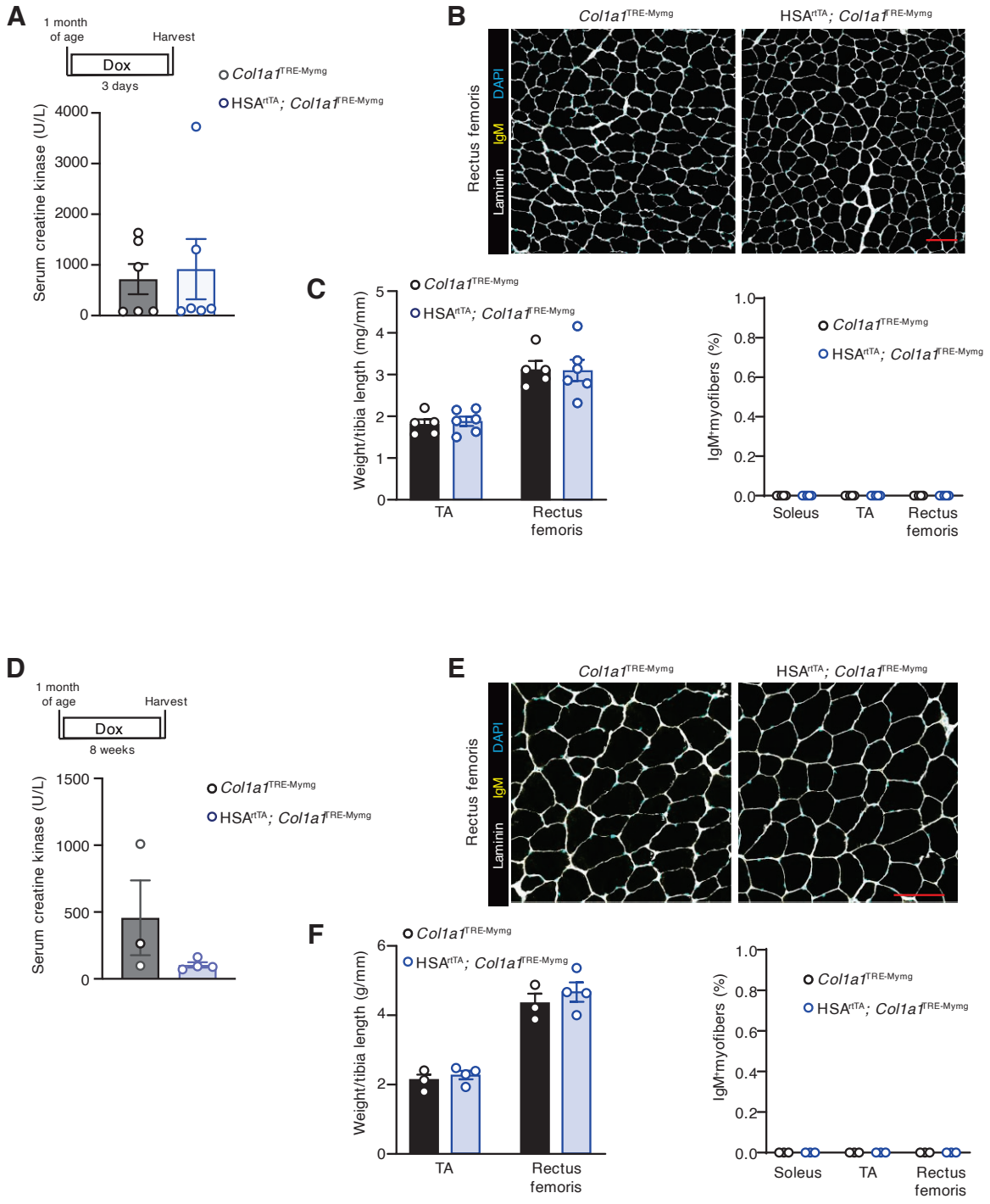

Figure S2. Myomerger expression in myofibers of iMymg mice does not lead to pathology. **A** Serum CK levels are not elevated after three days of Myomerger expression in myofibers. **B** Immunofluorescence staining for IgM after three days of Myomerger induction. Quantification of

the percentage of IgM^+ myofibers in the soleus, TA, and rectus femoris is shown below the images. Scale bar = 100 μm. **C** Muscle mass to tibia length ratios of the TA and rectus femoris are not altered after three days of Myomerger expression in myofibers. **D** Serum CK levels are not elevated after eight weeks of Myomerger expression in myofibers. **E** Immunofluorescence staining for IgM after eight weeks of Myomerger induction. Quantification of the percentage of IgM^+ myofibers in the soleus, TA, and rectus femoris is shown below the images. Scale bar = 100 μm. **F** Muscle mass to tibia length ratios of the TA and rectus femoris muscles are not altered after eight weeks of Myomerger expression in myofibers. Statistical analyses and presentation: Data are presented as mean ± SEM; **A,C,D,F** two-tailed Student's *t* test (within the same muscle for **C** and **F**).

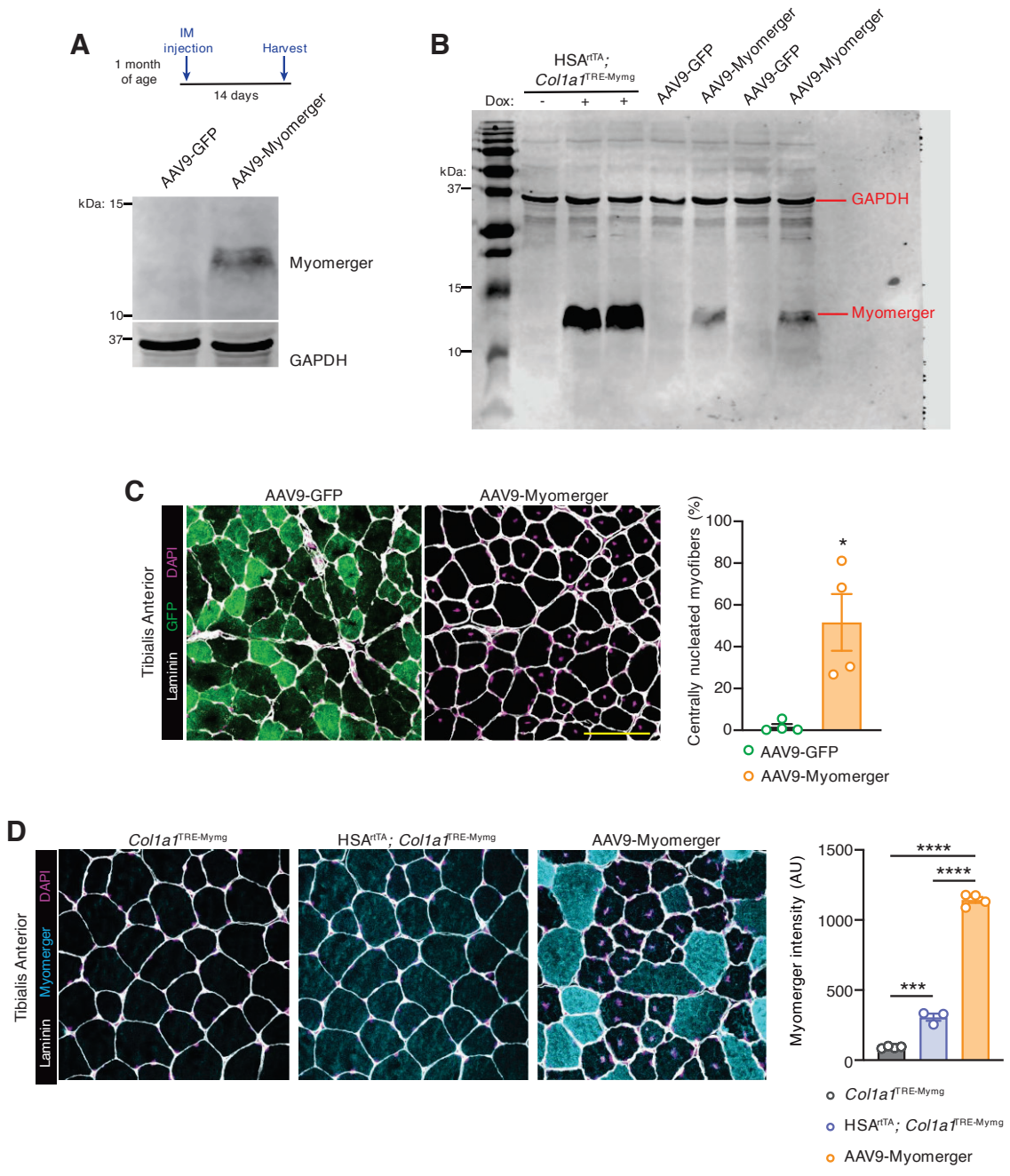

Figure S3. Elevated Myomerger expression leads to muscle regeneration. **A** Western blot for Myomerger validated transduction by AAV9-Myomerger two weeks after IM injection in the TA. **B** Western blot of Myomerger revealed higher levels of Myomerger in iMymg muscle after two weeks of dox treatment compared to AAV9-Myomerger injected muscle two weeks after IM

injection. **C** Histological analysis revealed elevated levels of centrally nucleated myofibers in the TA two weeks after IM injection with AAV9-Myomerger. Scale bar = 100 μm. **D** Myomerger immunofluorescence staining of the TA revealed elevated levels of Myomerger after AAV9-Myomerger (two weeks post-injection) compared to the myofiber inducible mouse model after two weeks of dox treatment. Fluorescence intensity of Myomerger antibody staining is quantified in arbitrary units (AU). Scale bar = 100 μm. Statistical analyses and presentation: Data are presented as mean ± SEM; **C** two-tailed Student's *t* test; **P* < 0.05; **D** one-way ANOVA with a Tukey's post hoc test; ****P* < 0.001, *****P* < 0.0001.

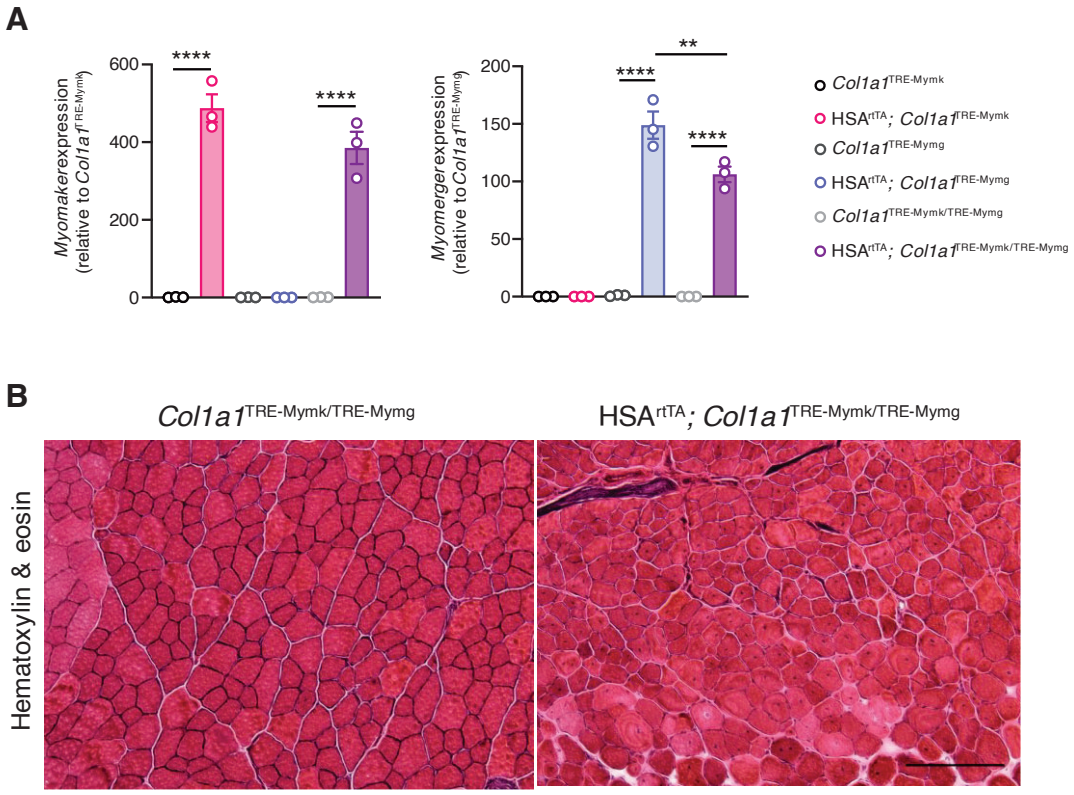

Figure S4. Myomaker and Myomerger expression in myofibers leads to muscle pathology. **A** qPCR analysis comparing *Myomaker* and *Myomerger* mRNA levels in the gastroc muscle between iMymk, iMymg, and iMymk/Mymg mice after three days of induction. **B** Representative H&E sections from the TA of *Col1a1*$^{\text{TRE-Mymk/TRE-Mymg}}$ and HSA$^{\text{rtTA}}$; *Col1a1*$^{\text{TRE-Mymk/TRE-Mymg}}$ mice. Scale bar = 200 μm. Statistical analyses and presentation: Data are presented as mean ± SEM; **A** one-way ANOVA with a Tukey's post hoc test; $^{**}P < 0.01$, $^{****}P < 0.0001$.

Acknowledgements

We thank Vikram Prasad of the Molkentin laboratory for technical assistance and Stephanie Kidder of the Molkentin laboratory for assistance with atomic force microscopy. We would also like to thank James Rose and Mindy Tepe for processing serum samples for serum CK analysis. The HSA^{rtTA} mouse was generously provided by Dr. John McCarthy (University of Kentucky). We also thank Dr. Yueh-Chiang Hu for his guidance with developing the transgenic mouse model. This project was made possible, in part, using the Cincinnati Children's Confocal Imaging Core (RRID:SCR_022628) and Transgenic Animal and Genomic Editing Core (RRID:SCR_022642).

Chapter 3: Discussion

Function of Myomaker and Myomerger in myoblast fusion

Independent functions of Myomaker and Myomerger have been shown to contribute to myoblast fusion[297]. Still, it remains unclear if these two muscle fusogens functionally interact to achieve myoblast fusion in a physiologic setting. Previous studies have revealed interactions between Myomaker and Myomerger, supporting a model whereby Myomaker and Myomerger directly interact during the fusion process[297,304]. However, these interactions were observed when the fusogens were overexpressed and labeled with an epitope. A separate study failed to detect any appreciable interaction between Myomaker and Myomerger with endogenous protein[307]. In another study, mammalian homologs of Myomaker and Myomerger were found to synergize in trans with one another[300]. Even so, it is not clear which epitopes of these fusogens could feasibly interact, given the lack of a long enough protrusion from the cell membrane for such an interaction to take place. Lack of unambiguous data demonstrating functional interactions between endogenous Myomaker and Myomerger is a major shortcoming of this direct interaction model. Less biased methods, such as proximity ligation assay or fluorescence resonance energy transfer assays, could serve to test the fidelity of this model.

Another model for myoblast fusion proposes that the independent activities of Myomaker and Myomerger contribute to myoblast fusion. Indeed, Myomaker can assist with myoblast fusion in the absence of Myomerger. Furthermore, Myomerger can advance fusion pore formation in cells which lack Myomaker[297]. However, this model fails to explain the increase in fusion observed when Myomaker and Myomerger are expressed on opposing membranes compared to the same membrane of Myomaker- and Myomerger-null human myoblasts[300]. Thus, while Myomaker and Myomerger may have independent activities, their cooperative role in cell fusion exposes gaps in this model.

A more evolved model of myoblast fusion proposes that the activities of Myomaker and Myomerger, which independently contribute to myoblast fusion, cooperate when the proteins are co-expressed, leading to an augmented fusion process. This model is supported by the trans synergy of Myomaker and Myomerger[300] as well as findings from this thesis, whereby dual expression of Myomaker and Myomerger led to increased pathology in myofibers. Given the activation of Myomerger by phosphatidylserine residues[303], it is plausible that Myomaker could catalyze changes in the plasma membrane which are permissive for Myomerger activity. The precise mechanism by which Myomaker acts, however, remains unknown. The hypothesis that Myomaker has a membrane remodeling activity is supported by its structural homology to hydrolases, such as the adiponectin receptor[298–300], which has ceramidase activity[301]. Ceramide and its derivatives have been shown to play a role in myoblast fusion[345,346]. Even if Myomaker does not possess enzymatic control over ceramide, its histidine residues may allow it to bind membrane lipids[299] and impact membrane curvature[347]. The facilitation of hemifusion or modulation of the plasma membrane environment, whether through ceramide metabolism, ceramide rearrangement, or phosphatidylserine asymmetry, could allow regulation of Myomerger activity, providing a layer of control over the fusion process.

Differential skeletal muscle susceptibilities in DMD

Despite global mutations in the dystrophin gene, skeletal muscles are differentially affected in DMD. While most research has been focused on pathogenesis within limb muscles, which seem to be distinctly susceptible[348], craniofacial muscles, such as extraocular and internal laryngeal muscles, are more or less spared in DMD[125,349–351]. The differential skeletal muscle susceptibilities seem to be linked to the embryological origin of each muscle. While the limb muscles are derived from somitic mesoderm[36], EOM originate from prechordal and cranial

paraxial mesoderm of the first pharyngeal arch[352,353] and intrinsic laryngeal muscle originates from occipital somites[353,354].

There are several proposed explanations for the observed differences in pathology between different skeletal muscle groups. First, the regenerative capacity of EOM stem cells has been shown to be higher than their limb muscle counterparts[125,348]. Although EOM satellite cells engraft better into host muscle compared to limb satellite cells[130], they lose some components of their distinct regenerative identity[32]. Thus, an alternative explanation for the differential pathology is that the stem cell niche within EOM is inherently unique. Indeed, a distinct population of progenitors, including a portion of myogenic progenitors, express a cell stress-mediator *PW1/Peg3*. This progenitor population, which is maintained in EOMs but not in limb muscles throughout life, may foster a promyogenic environment for satellite cells through paracrine interactions[129]. Another mononuclear population, defined by the cell markers $CD34^{+}$/$Sca\text{-}1^{-}$/$CD45^{-}$/$CD31^{-}$/M-$cadherin^{-}$, is maintained in EOMs but not limb muscles over time. This population, which is resistant to elevated levels of oxidative stress and toxins and acutely proliferates throughout life, may also stave off pathology in EOM[127].

Not only does the EOM niche foster a divergence in regenerative capacity but also the transcriptional programs activated by EOM satellite cells. Proteomic analysis of EOM satellite cells has revealed that these stem cells may handle cellular stress better[356]. Their more robust qualities could be conferred by the altered myosin heavy chain expression or metabolic adaptations[357]. When comparing the transcriptome of EOM and limb muscle satellite cells, cell signaling pathways demonstrate the greatest divergence[126]. Among the cell signaling pathways, activation of adenylyl cyclase downstream of the thyroid-stimulating hormone receptor was recently purported as an explanation for the enhanced regenerative capacity of EOM satellite cells[358].

Given these unique differences in niche and progenitor phenotype, it is unclear if altered regulation of the fusogens plays a role in the reduced susceptibility to DMD pathology of EOM muscles. *Mdx* EOM has dystrophin-competent and dystrophin-deficient myofibers, which pattern similarly to fiber type, based on utrophin expression. Despite these differences in dystrophin retention, both groups of myofibers are spared in *mdx*, suggesting an alternative model from the prevailing mechanical stabilization hypothesis for the DGC in skeletal muscle[359]. Indeed, sarcolemmal disruption was insufficient to elicit changes in EOM phenotype, supporting the concept that factors other than the DGC stability may spare the EOM from pathology in DMD[360]. Rather, altered regulation of calcium-handling proteins may play a role in EOM protection[132,361].

While it is apparent that Myomaker and Myomerger play a role in limb muscle DMD pathogenesis, further work is needed to clarify if altered activity or regulation of the fusogens impacts the susceptibility of other muscles, such as craniofacial muscles, in disease. Comparing Myomaker or Myomerger expression within dystrophic EOM fibers to dystrophic limb myofibers could provide insight into differential regulation of these fusogens. It is possible that the enhanced regenerative capabilities of the satellite cells within EOM can better regulate fusogen expression, downregulating it more promptly after fusion to the myofiber. Additionally, ectopically expressing Myomaker and Myomerger within EOM fibers may shed light on the susceptibility of these fibers to fusogen activity. This analysis could be performed on the transgenic mice generated in the aforementioned studies in Chapter 2. If they are more resilient to membrane damage compared to their limb muscle counterparts, there could be several different explanations. First, the EOM fibers might be inherently less susceptible to the activities of Myomaker and Myomerger. This reduced susceptibility could be conferred by a distinct plasma membrane composition or organization which is more resilient to the membrane activities or Myomaker or Myomerger. Alternatively, EOM fibers may express a unique set of proteins which counteract the maladaptive behaviors of Myomaker and Myomerger within the myofiber. Another reason to explain potentially

reduced susceptibility to fusogen activity within EOM myofibers could be the extracellular environment. Unique properties of the EOM niche may confer additional benefits to myogenic regeneration[129]. This is supported by the fact that EOM satellite cells phenocopy their surrounding myofibers when transplanted in limb muscle, failing to express MyHC isoforms and metabolic components more characteristic of EOM[32].

Alternative contributors to DMD pathogenesis

There is a growing body of evidence implicating the MyoD regenerative program in contributing to progression of dystrophic pathology. Irradiating a dystrophic limb to deplete satellite cells reduced muscle damage, albeit at the expense of regenerative capacity, compared to the contralateral, nonirradiated limb[338]. A separate study found that silencing *Nfix*, a transcription factor which regulates fetal myogenesis, led to reduced pathology in two dystrophic mouse models, α-sarcoglycan- and dystrophin-deficient[339]. More recently, ablation of the satellite cell pool in δ-sarcoglycan- and dystrophin-deficient dystrophic mouse models led to reduced pathology and increased sarcolemmal stability[340]. These observations are in concordance with an overactive MyoD-centric regenerative program triggering expression of Myomaker and Myomerger, both of which may be activated by MyoD[340,362]. Indeed, stem cell-derived myomaker has been implicated in reducing sarcolemmal stability in dystrophic mice[308].

While ablation of Myomaker from the myofiber compartment of dystrophic skeletal muscle may enhance sarcolemmal stability, this experiment was performed in a mouse model which does not fully phenocopy the human disease. The *mdx* mouse in the C57 background can compensate for the loss of dystrophin through upregulation of utrophin[363,364]. To overcome this limitation, alternative mouse models were developed to better model human DMD. A double knockout mouse lacking dystrophin and utrophin was developed which has similar degeneration of limb and diaphragm muscles and premature death[365]. Additionally, the C57 *mdx* mouse was bred onto

the DBA/2J background to generate D2.*mdx* mice[366]. In order to test the hypothesis that Myomaker and Myomerger membrane activities play a major role in DMD pathogenesis, downregulation of these proteins in more representative DMD mouse models is needed.

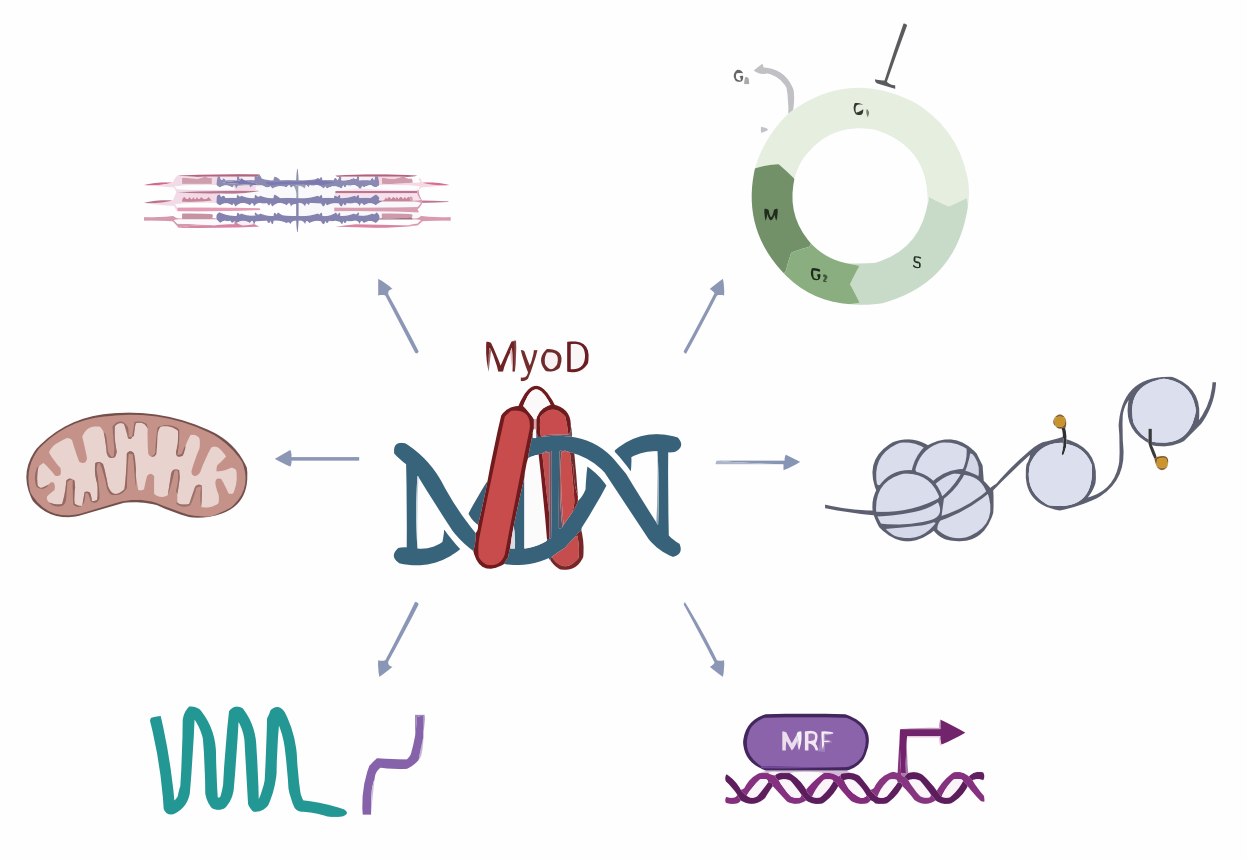

Figure 1: Pathways regulated by MyoD. MyoD, one of the four MRFs, regulates an array of pathways, not limited to the cell cycle, chromatin accessibility and recruitment of RNA polymerase, activation of other transcription factors, such as Myogenin, myoblast fusion, metabolism, and cytoskeletal proteins.

If downregulation of the muscle fusogens is not sufficient to reduce disease progression in the more representative DMD mouse models, alternative pathways stimulated by an overactive MyoD regenerative program may be culpable. MyoD resides at a nexus of pathways involved in,

but not limited to, chromatin accessibility, metabolism, cell fusion, cytoskeletal structure, and regulation of other transcription factors. In light of the fact that long term attenuation of satellite cell regeneration may be maladaptive for skeletal muscle as a whole[308] and satellite cell exhaustion may be permissive to DMD pathogenesis[331,367–369], specifically targeting the maladaptive features of the MyoD regenerative program may provide more viable therapeutic opportunities to reduce disease progression.

A central question probed in this study is the impact of excessive regeneration within skeletal muscle. Myofibers need to be repaired following damage in order to prevent the onset of necrosis and subsequent degradation. An inability to replace damaged myofibers leads to muscle atrophy[180] and increased fibrosis in disease[308]. Counterintuitively, downregulation of the regenerative program reduces pathology and muscle damage in muscular dystrophy[338–340]. Such studies juxtapose the beneficial effects of skeletal muscle regeneration following exercise[370,371]. A potential explanation for this conundrum is the fact that dystrophic muscle may respond differently to regeneration than wild-type muscle. A “2-hit” model in muscular dystrophy has been proposed, whereby the first hit is the underlying genetic defect and the subsequent initiation of the regenerative program is the second hit[340]. Another possibility is that regeneration is inherently different in disease compared to exercise. Indeed, numerous studies in animal models and humans have suggested that exercise itself may provide benefits to dystrophic skeletal muscle[372–377]. Altogether, it is apparent that regeneration benefits dystrophic muscle. Identifying how regeneration differs between exercise and dystrophy may provide alternative therapeutic avenues for patients with muscular dystrophy.

Given that downregulation of Myomaker in dystrophic myofibers reduces pathology[308], it remains unclear if dystrophic myofibers are more susceptible to the activities of the fusogens compared to their wild-type counterparts. Myomaker and Myomerger expression is lower in *mdx* muscle compared to the inducible model muscle. However, there is a greater level of myofiber

membrane damage in dystrophy. There are several explanations for this phenomenon. First, wild-type myofibers may be more resistant to injury compared to dystrophic myofibers, especially because dystrophic myofibers lack dystrophin, a critical structural protein connecting the inner cytoskeleton to extracellular laminin. Next, activation of the regenerative program may not only activate fusogen expression but also expression of other cytoskeletal components, some of which could be maladaptive. Further studies are needed to validate if dystrophic muscle adapts as the disease progresses and how such adaptations make it less susceptible to the activities of Myomaker and Myomerger.

A key challenge assessed in the discussion section of the previous chapter is the ability to detect myofiber membrane damage. Although the tools we utilized are considered to be sensitive markers of myofiber membrane damage (serum creatine kinase and the proportion of IgM^+ myofibers), these markers may not be sensitive enough to detect more minute damage to the sarcolemma. For example, myofiber membrane damage after induction of fusogen expression in myofibers, quantified by the proportion of IgM^+ myofibers, generally seemed to peak around 2%, similar to another study which assessed the impact of suppressing the regenerative program in dystrophic muscle[340]. Inability of this method to detect myofibers with lower levels of membrane damage significantly impedes the potential resolution of this method. Conversely, elevated serum creatine kinase values sometimes could not be accurately quantified due to instrumental limitations. As a result, this method lacked the ability to appropriately stratify myofiber membrane damage. These limitations in the quantification of myofiber membrane damage hampered our ability to accurately assess the impact of Myomaker and Myomerger on sarcolemmal integrity.

In addition to limitations in detection of myofiber membrane damage, the manner in which myofiber stiffness was assessed could have been more physiological relevant. Myofiber membrane stiffness was quantified perpendicular to the plane of the myofiber in a compressive fashion. However, muscle fibers primarily contract and relax along the longitudinal axis during the

cross-bridge cycle. Thus, assessing the tensile properties of myofibers rather than compressive properties might provide more insight into the impact of the fusogens within myofibers.

In sum, we sought to test the hypothesis that expression of the muscle fusogens in myofibers was deleterious. Our data support this hypothesis, explaining why expression of Myomaker and Myomerger is so tightly restricted to myoblasts during development and regeneration. Downregulation of the fusogens within myofibers may provide an alternative therapeutic avenue for patients with muscular dystrophy. Because myofiber membrane damage was exacerbated when both Myomaker and Myomerger were expressed in myofibers, this suggests that these fusogens may have cooperative functions during myoblast fusion. However, it remains unclear if these fusogens directly or indirectly interact during this process.

References

1. Gentsch GE, Monteiro RS, Smith JC. *Cooperation between T-Box Factors Regulates the Continuous Segregation of Germ Layers during Vertebrate Embryogenesis*. Vol 122. 1st ed. Elsevier Inc.; 2017. doi:10.1016/bs.ctdb.2016.07.012

2. Tajbakhsh S, Cossu G. Establishing myogenic identity during somitogenesis. *Curr Opin Genet Dev*. 1997;7(5):634-641. doi:10.1016/S0959-437X(97)80011-1

3. Kinder SJ, Tsang TE, Quinlan GA, Hadjantonakis AK, Nagy A, Tam PP. The orderly allocation of mesodermal cells to the extraembryonic structures and the anteroposterior axis during gastrulation of the mouse embryo. *Development*. 1999;126(21):4691-4701. doi:10.1242/dev.126.21.4691

4. Lawson KA, Meneses JJ, Pedersen RA. Clonal analysis of epiblast fate during germ layer formation in the mouse embryo. *Development*. 1991;113(3):891-911. doi:10.1242/dev.113.3.891

5. Tam PPL, Parameswaran M, Kinder SJ, Weinberger RP. The allocation of epiblast cells to the embryonic heart and other mesodermal lineages: The role of ingression and tissue movement during gastrulation. *Development*. 1997;124(9):1631-1642. doi:10.1242/dev.124.9.1631

6. Tzouanacou E, Wegener A, Wymeersch FJ, Wilson V, Nicolas JF. Redefining the progression of lineage segregations during mammalian embryogenesis by clonal analysis. *Dev Cell*. 2009;17(3):365-376. doi:10.1016/j.devcel.2009.08.002

7. Selleck MAJ, Stern CD. Fate mapping and cell lineage analysis of Hensen's node in the

chick embryo. *Development*. 1991;112(2):615-626. doi:10.1242/dev.112.2.615

8. Zorn AM, Wells JM. Vertebrate endoderm development and organ formation. *Annu Rev Cell Dev Biol*. 2009;25:221-251. doi:10.1146/annurev.cellbio.042308.113344

9. Hamidi S, Nakaya Y, Nagai H, et al. Mesenchymal-epithelial transition regulates initiation of pluripotency exit before gastrulation. *Development*. 2020;147(3). doi:10.1242/dev.184960

10. Kimmel CB, Warga RM, Schilling TF. Origin and organization of the zebrafish fate map. *Development*. 1990;108(4):581-594. doi:10.1242/dev.108.4.581

11. Warga RM, Nüsslein-Volhard C. Origin and development of the zebrafish endoderm. *Development*. 1999;126(4):827-838. doi:10.1242/dev.126.4.827

12. Weston MJD, Kato K, Gurdon JB. A community effect is required for amphibian notochord differentiation. *Roux's Arch Dev Biol*. 1994;203(5):250-253. doi:10.1007/BF00360520

13. Solnica-Krezel L, Sepich DS. Gastrulation: Making and shaping germ layers. *Annu Rev Cell Dev Biol*. 2012;28:687-717. doi:10.1146/annurev-cellbio-092910-154043

14. Arnold SJ, Robertson EJ. Making a commitment: Cell lineage allocation and axis patterning in the early mouse embryo. *Nat Rev Mol Cell Biol*. 2009;10(2):91-103. doi:10.1038/nrm2618

15. Tam PPL, Behringer RR. Mouse gastrulation: The formation of a mammalian body plan. *Mech Dev*. 1997;68(1-2):3-25. doi:10.1016/S0925-4773(97)00123-8

16. Rohde LA, Oates AC, Ho RK. A crucial interaction between embryonic red blood cell progenitors and paraxial mesoderm revealed in spadetail embryos. *Dev Cell*. 2004;7(2):251-262. doi:10.1016/j.devcel.2004.07.010

17. Hernández-Hernández JM, García-González EG, Brun CE, Rudnicki MA. The myogenic regulatory factors, determinants of muscle development, cell identity and regeneration. *Semin Cell Dev Biol*. 2017;72:10-18. doi:10.1016/j.semcdb.2017.11.010

18. Zammit PS. Function of the myogenic regulatory factors Myf5, MyoD, Myogenin and MRF4 in skeletal muscle, satellite cells and regenerative myogenesis. *Semin Cell Dev Biol*. 2017;72:19-32. doi:10.1016/j.semcdb.2017.11.011

19. Christ B, Ordahl CP. Early stages of chick somite development. *Anat Embryol (Berl)*. 1995;191(5):381-396. doi:10.1007/BF00304424

20. Kassar-Duchossoy L, Gayraud-Morel B, Gomès D, et al. Mrf4 determines skeletal muscle identity in Myf5:Myod double-mutant mice. *Nature*. 2004;431(7007):466-471. doi:10.1038/nature02876

21. Summerbell D, Halai C, Rigby PWJ. Expression of the myogenic regulatory factor Mrf4 precedes or is contemporaneous with that of Myf5 in the somitic bud. *Mech Dev*. 2002;117(1-2):331-335. doi:10.1016/S0925-4773(02)00208-3

22. Smith JL, Schoenwolf GC. Neurulation: Coming to closure. *Trends Neurosci*. 1997;20(11):510-517. doi:10.1016/S0166-2236(97)01121-1

23. Christ B, Huang R, Scaal M. Formation and differentiation of the avian sclerotome. *Anat Embryol (Berl)*. 2004;208(5):333-350. doi:10.1007/s00429-004-0408-z

24. Scaal M, Christ B. Formation and differentiation of the avian dermomyotome. *Anat Embryol (Berl)*. 2004;208(6):411-424. doi:10.1007/s00429-004-0417-y

25. Tzahor E. *Head Muscle Development*. Vol 56. (Brand-Saberi B, Richter D, Tiedge H, eds.). Springer US; 2015. doi:10.1007/978-3-662-44608-9_4

26. Tajbakhsh S. Skeletal muscle stem cells in developmental versus regenerative myogenesis. *J Intern Med*. 2009;266(4):372-389. doi:10.1111/j.1365-2796.2009.02158.x

27. Theis S, Patel K, Valasek P, et al. The occipital lateral plate mesoderm is a novel source for vertebrate neck musculature. *Development*. 2010;137(17):2961-2971. doi:10.1242/dev.049726

28. Yamane A. Embryonic and postnatal development of masticatory and tongue muscles. *Cell Tissue Res*. 2005;322(2):183-189. doi:10.1007/s00441-005-0019-x

29. Tajbakhsh S, Rocancourt D, Cossu G, Buckingham M. Redefining the genetic hierarchies controlling skeletal myogenesis: Pax- 3 and Myf-5 act upstream of MyoD. *Cell*. 1997;89(1):127-138. doi:10.1016/S0092-8674(00)80189-0

30. Shih HP, Gross MK, Kioussi C. Muscle development: Forming the head and trunk muscles. *Acta Histochem*. 2008;110(2):97-108. doi:10.1016/j.acthis.2007.08.004

31. Sambasivan R, Tajbakhsh S. Skeletal muscle stem cell birth and properties. *Semin Cell Dev Biol*. 2007;18(6):870-882. doi:10.1016/j.semcdb.2007.09.013

32. Sambasivan R, Gayraud-Morel B, Dumas G, et al. Distinct regulatory cascades govern extraocular and pharyngeal arch muscle progenitor cell fates. *Dev Cell*. 2009;16(6):810-

821. doi:10.1016/j.devcel.2009.05.008

33. Rudnicki MA, Schnegelsberg PNJ, Stead RH, Braun T, Arnold HH, Jaenisch R. MyoD or Myf-5 is required for the formation of skeletal muscle. *Cell*. 1993;75(7):1351-1359. doi:10.1016/0092-8674(93)90621-V

34. Tajbakhsh S. Stem cells to tissue: Molecular, cellular and anatomical heterogeneity in skeletal muscle. *Curr Opin Genet Dev*. 2003;13(4):413-422. doi:10.1016/S0959-437X(03)00090-X

35. Relaix F, Rocancourt D, Mansouri A, Buckingham M. A Pax3/Pax7-dependent population of skeletal muscle progenitor cells. *Nature*. 2005;435(7044):948-953. doi:10.1038/nature03594

36. Dumont NA, Bentzinger CF, Sincennes MC, Rudnicki MA. Satellite cells and skeletal muscle regeneration. *Compr Physiol*. 2015;5(3):1027-1059. doi:10.1002/cphy.c140068

37. Harel I, Nathan E, Tirosh-Finkel L, et al. Distinct Origins and Genetic Programs of Head Muscle Satellite Cells. *Dev Cell*. 2009;16(6):822-832. doi:10.1016/j.devcel.2009.05.007

38. Biressi S, Molinaro M, Cossu G. Cellular heterogeneity during vertebrate skeletal muscle development. *Dev Biol*. 2007;308(2):281-293. doi:10.1016/j.ydbio.2007.06.006

39. Stockdale FE. Myogenic cell lineages. *Dev Biol*. 1992;154(2):284-298. doi:10.1016/0012-1606(92)90068-R

40. Babai F, Musevi-Aghdam J, Schurch W, Royal A, Gabbiani G. Coexpression of α-sarcomeric actin, α-smooth muscle actin and desmin during myogenesis in rat and

mouse embryos I. Skeletal muscle. *Differentiation*. 1990;44(2):132-142. doi:10.1111/j.1432-0436.1990.tb00546.x

41. Furst DO, Osborn M, Weber K. Myogenesis in the mouse embryo: Differential onset of expression of myogenic proteins and the involvement of titin in myofibril assembly. *J Cell Biol*. 1989;109(2):517-527. doi:10.1083/jcb.109.2.517

42. Lyons GE, Buckingham ME, Mannherz HG. A-Actin Proteins and Gene Transcripts Are Colocalized in Embryonic Mouse Muscle. *Development*. 1991;111(2):451-454. doi:10.1242/dev.111.2.451

43. Lyons GE, Ontell M, Cox R, Sassoon D, Buckingham M. The expression of myosin genes in eeveloping skeletal muscle in the mouse embryo. *JCB*. 1990;111(4):1465-1476. doi:10.1083/jcb.111.4.1465

44. Sassoon DA, Garner I, Buckingham M. Transcripts of α-cardiac and α-skeletal actins are early markers for myogenesis in the mouse embryo. *Development*. 1988;104(1):155-164. doi:10.1242/dev.104.1.155

45. Condon K, Silberstein L, Blau HM, Thompson WJ. Development of muscle fiber types in the prenatal rat hindlimb. *Dev Biol*. 1990;138(2):256-274. doi:10.1016/0012-1606(90)90196-P

46. Lyons GE, Muhlebach S, Moser A, Masood R, Paterson BM, Buckingham Perriard MEJC. Developmental regulation of creatine kinase gene expression by myogenic factors in embryonic mouse and chick skeletal muscle. *Development*. 1991;113(3):1017-1029. doi:10.1242/dev.113.3.1017

47. Tweedie S, Morrison K, Charlton J, Edwards YH. CAIII a marker for early myogenesis: Analysis of expression in cultured myogenic cells. *Somat Cell Mol Genet*. 1991;17(3):215-228. doi:10.1007/BF01232818

48. Christ B, Jacob M, Jacob HJ. On the origin and development of the ventrolateral abdominal muscles in the avian embryo. *Anat Embryol (Berl)*. 1983;166:87-101. doi:10.1007/BF00317946

49. Denetclaw WF, Christ B, Ordahl CP. Location and growth of epaxial myotome precursor cells. *Development*. 1997;124(8):1601-1610. doi:10.1242/dev.124.8.1601

50. Gros J, Serralbo O, Marcelle C. WNT11 acts as a directional cue to organize the elongation of early muscle fibres. *Nature*. 2009;457(7229):589-593. doi:10.1038/nature07564

51. Gros J, Scaal M, Marcelle C. A two-step mechanism for myotome formation in chick. *Dev Cell*. 2004;6(6):875-882. doi:10.1016/j.devcel.2004.05.006

52. Sieiro-Mosti D, De La Celle M, Pelé M, Marcelle C. A dynamic analysis of muscle fusion in the chick embryo. *Development*. 2014;141(18):3605-3611. doi:10.1242/dev.114546

53. Kassar-Duchossoy L, Giacone E, Gayraud-Morel B, Jory A, Gomès D, Tajbakhsh S. Pax3/Pax7 mark a novel population of primitive myogenic cells during development. *Genes Dev*. 2005;19(12):1426-1431. doi:10.1101/gad.345505

54. Gros J, Manceau M, Thomé V, Marcelle C. A common somitic origin for embryonic muscle progenitors and satellite cells. *Nature*. 2005;435(7044):954-958. doi:10.1038/nature03572

55. Kahane N, Cinnamon Y, Bachelet I, Kalcheim C. The third wave of myotome colonization by mitotically competent progenitors: Regulating the balance between differentiation and proliferation during muscle development. *Development*. 2001;128(12):2187-2198. doi:10.1242/dev.128.12.2187

56. Cossu G, Kelly R, Di Donna S, Vivarelli E, Buckingham M. Myoblast differentiation during mammalian somitogenesis is dependent upon a community effect. *Proc Natl Acad Sci U S A*. 1995;92(6):2254-2258. doi:10.1073/pnas.92.6.2254

57. Kato K, Gurdon JB. Single-cell transplantation determines the time when Xenopus muscle precursor cells acquire a capacity for autonomous differentiation. *Proc Natl Acad Sci U S A*. 1993;90(4):1310-1314. doi:10.1073/pnas.90.4.1310

58. Chevallier A. Role of the somitic mesoderm in the development of the thorax in bird embryos. *Development*. 1979;49(1):73-88. doi:10.1242/dev.49.1.73

59. Jacob M, Christ B, Jacob HJ. The migration of myogenic cells from the somites into the leg region of avian embryos - An ultrastructural study. *Anat Embryol (Berl)*. 1979;157(3):291-309. doi:10.1007/BF00304995

60. Berkes CA, Tapscott SJ. MyoD and the transcriptional control of myogenesis. *Semin Cell Dev Biol*. 2005;16(4-5):585-595. doi:10.1016/j.semcdb.2005.07.006

61. Pownall ME, Gustafsson MK, Emerson CP. Myogenic regulatory factors and the specification of muscle progenitors in vertebrate embryos. *Annu Rev Cell Dev Biol*. 2002;18:747-783. doi:10.1146/annurev.cellbio.18.012502.105758

62. Tapscott SJ. The circuitry of a master switch: Myod and the regulation of skeletal muscle

gene transcription. *Development*. 2005;132(12):2685-2695. doi:10.1242/dev.01874

63. Hasty P, Bradley A, Morris JH, et al. Muscle deficiency and neonatal death in mice with a targeted mutation in the myogenin gene. *Nature*. 1993;364:501-506. doi:10.1038/364501a0

64. Nabeshlma Y, Hanaokat K, Hayasakat M, et al. Myogenin gene disruption results in perinatal lethality because of severe muscle defect. *Nature*. 1993;364(August):532-535. doi:10.1038/364532a0

65. Venuti JM, Morris JH, Vivian JL, Olson EN, Klein WH. Myogenin is required for late but not early aspects of myogenesis during mouse development. *J Cell Biol*. 1995;128(4):563-576. doi:10.1083/jcb.128.4.563

66. Chal J, Pourquié O. Making muscle: Skeletal myogenesis in vivo and in vitro. *Development*. 2017;144(12):2104-2122. doi:10.1242/dev.151035

67. Fougerousse F, Edom-Vovard F, Merkulova T, et al. The muscle-specific enolase is an early marker of human myogenesis. *J Muscle Res Cell Motil*. 2001;22(6):535-544. doi:10.1023/a:1015008208007

68. Keller A, Ott M, Lamand N, et al. Activation of the gene encoding the glycolytic enzyme beta-enolase during early myogenesis precedes an increased expression during fetal muscle development. *Mech Dev*. 1992;38(1):41-54. doi:10.1016/0925-4773(92)90037-k

69. Kelly RG, Zammit PS, Schneider A, Alonso S, Biben C, Buckingham ME. Embryonic and fetal myogenic programs act through separate enhancers at the MLC1F/3F locus. *Dev Biol*. 1997;187(2):183-199. doi:10.1006/dbio.1997.8577

70. Messina G, Biressi S, Monteverde S, et al. Nfix regulates fetal-specific transcription in developing skeletal muscle. *Cell*. 2010;140(4):554-566. doi:10.1016/j.cell.2010.01.027

71. White RB, Biérinx A, Gnocchi VF, Zammit PS. Dynamics of muscle fibre growth during postnatal mouse development. *BMC Dev Biol*. 2010;10(21). doi:10.1186/1471-213X-10-21

72. Gokhin DS, Ward SR, Bremner SN, Lieber RL. Quantitative analysis of neonatal skeletal muscle functional improvement in the mouse. *J Exp Biol*. 2008;211(6):837-843. doi:10.1242/jeb.014340

73. Sparrow JC, Schöck F. The initial steps of myofibril assembly: integrins pave the way. *Nat Rev Mol Cell Biol*. 2009;10:293-298. doi:10.1038/nrm2634

74. Schiaffino S, Reggiani C. Fiber types in mammalian skeletal muscles. *Physiol Rev*. 2011;91(4):1447-1531. doi:10.1152/physrev.00031.2010

75. Talbot J, Maves L. Skeletal muscle fiber type: using insights from muscle developmental biology to dissect targets for susceptibility and resistance to muscle disease. *Wiley Interdiscip Rev Dev Biol*. 2016;5(4):518-534. doi:10.1002/wdev.230

76. Grifone R, Laclef C, Spitz F, et al. Six1 and Eya1 expression can reprogram adult muscle from the slow-twitch phenotype into the fast-twitch phenotype. *Mol Cell Biol*. 2004;24(14):6253-6267. doi:10.1128/MCB.24.14.6253-6267.2004

77. Richard A, Demignon J, Sakakibara I, et al. Genesis of muscle fiber-type diversity during mouse embryogenesis relies on Six1 and Six4 gene expression. *Dev Biol*. 2011;359(2):303-320. doi:10.1016/j.ydbio.2011.08.010

78. Lemke SB, Schnorrer F. Mechanical forces during muscle development. *Mech Dev*. 2017;144(Pt A):92-101. doi:10.1016/j.mod.2016.11.003

79. Bowman W. On the minute structure and movements of voluntary muscle. *Philos Trans R Soc London*. 1840;130:457-501. http://www.jstor.org/stable/108230.

80. Sanes JR. The basement membrane/basal lamina of skeletal muscle. *J Biol Chem*. 2003;278(15):12601-12604. doi:10.1074/jbc.R200027200

81. Rahimov F, Kunkel LM. The cell biology of disease: cellular and molecular mechanisms underlying muscular dystrophy. *J Cell Biol*. 2013;201(4):499-510. doi:10.1083/jcb.201212142

82. Flucher BE. Structural analysis of muscle development: transverse tubules, sarcoplasmic reticulum, and the triad. *Dev Biol*. 1992;154(2):245-260. doi:10.1016/0012-1606(92)90065-o

83. Flucher BE, Phillips JL, Powell JA, Andrews SB, Daniels MP. Coordinated development of myofibrils, sarcoplasmic reticulum and transverse tubules in normal and dysgenic mouse skeletal muscle, in vivo and in vitro. *Dev Biol*. 1992;150(2):266-280. doi:10.1016/0012-1606(92)90241-8

84. Flucher BE, Andrews SB, Fleischer S, Marks AR, Caswell A, Powell JA. Triad formation: organization and function of the sarcoplasmic reticulum calcium release channel and triadin in normal and dysgenic muscle in vitro. *J Cell Biol*. 1993;123(5):1161-1174. doi:10.1083/jcb.123.5.1161

85. Lepper C, Fan C. Inducible lineage tracing of Pax7-descendant cells reveals embryonic

origin of adult satellite cells. *Genesis*. 2010;48(7):424-436. doi:10.1002/dvg.20630

86. Seale P, Sabourin LA, Girgis-Gabardo A, Mansouri A, Gruss P, Rudnicki MA. Pax7 is required for the specification of myogenic satellite cells. *Cell*. 2000;102(6):777-786. doi:10.1016/s0092-8674(00)00066-0

87. Relaix F, Rocancourt D, Mansouri A, Buckingham M. Divergent functions of murine Pax3 and Pax7 in limb muscle development. *Genes Dev*. 2004;18(9):1088-1105. doi:10.1101/gad.301004

88. Vasyutina E, Lenhard DC, Birchmeier C. Notch function in myogenesis. *Cell cycle*. 2007;6(12):1451-1454. doi:10.4161/cc.6.12.4372

89. Kanisicak O, Mendez JJ, Yamamoto S, Yamamoto M, Goldhamer DJ. Progenitors of skeletal muscle satellite cells express the muscle determination gene, MyoD. *Dev Biol*. 2009;332(1):131-141. doi:10.1016/j.ydbio.2009.05.554

90. Allbrook DB, Han MF, Hellmuth AE. Population of muscle satellite cells in relation to age and mitotic activity. *Pathology*. 1971;3(3):223-243. doi:10.3109/00313027109073739

91. Cardasis CA, Cooper GW. An analysis of nuclear numbers in individual muscle fibers during differentiation and growth: A satellite cell-muscle fiber growth unit. *J Exp Zool*. 1975;191(3):347-357. doi:10.1002/jez.1401910305

92. Schmalbruch H, Hellhammer U. The number of satellite cells in normal human muscle. *Anat Rec*. 1976;185(3):279-287. doi:10.1002/ar.1091850303

93. Mukund K, Subramaniam S. Co-expression network approach reveals functional

similarities among diseases affecting human skeletal muscle. *Front Physiol*. 2017;8:1-13. doi:10.3389/fphys.2017.00980

94. Ryder S, Leadley RM, Armstrong N, et al. The burden, epidemiology, costs and treatment for Duchenne muscular dystrophy: An evidence review. *Orphanet J Rare Dis*. 2017;12(1):1-21. doi:10.1186/s13023-017-0631-3

95. Chang RF, Mubarak SJ. Pathomechanics of Gowers' sign: A video analysis of a spectrum of Gowers' maneuvers. *Clin Orthop Relat Res*. 2012;470(7):1987-1991. doi:10.1007/s11999-011-2210-6

96. Pradhan S. Valley sign in duchenne muscular dystrophy: Importance in patients with inconspicuous calves. *Neurol India*. 2002;50(2):184-186.

97. Mercuri E, Bönnemann CG, Muntoni F. Muscular dystrophies. *Lancet*. 2019;394(10213):2025-2038. doi:10.1016/S0140-6736(19)32910-1

98. Kieny P, Chollet S, Delalande P, et al. Evolution of life expectancy of patients with Duchenne muscular dystrophy at AFM Yolaine de Kepper centre between 1981 and 2011. *Ann Phys Rehabil Med*. 2013;56(6):443-454. doi:10.1016/j.rehab.2013.06.002

99. Landfeldt E, Thompson R, Sejersen T, McMillan HJ, Kirschner J, Lochmüller H. Life expectancy at birth in Duchenne muscular dystrophy: a systematic review and meta-analysis. *Eur J Epidemiol*. 2020;35(7):643-653. doi:10.1007/s10654-020-00613-8

100. Van Ruiten HJA, Marini Bettolo C, Cheetham T, et al. Why are some patients with Duchenne muscular dystrophy dying young: An analysis of causes of death in North East England. *Eur J Paediatr Neurol*. 2016;20(6):904-909. doi:10.1016/j.ejpn.2016.07.020

101. Wittlieb-Weber CA, Knecht KR, Villa CR, et al. Risk Factors for Cardiac and Non-cardiac Causes of Death in Males with Duchenne Muscular Dystrophy. *Pediatr Cardiol*. 2020;41(4):764-771. doi:10.1007/s00246-020-02309-y

102. Villa C, Auerbach SR, Bansal N, et al. Current practices in treating cardiomyopathy and heart failure in Duchenne muscular dystrophy (DMD): Understanding care practices in order to optimize DMD heart failure through ACTION. *Pediatr Cardiol*. 2022;43(5):977-985. doi:10.1007/s00246-021-02807-7

103. McNally EM, Kaltman JR, Woodrow Benson D, et al. Contemporary cardiac issues in Duchenne muscular dystrophy. *Circulation*. 2015;131(18):1590-1598. doi:10.1161/CIRCULATIONAHA.114.015151

104. Ricotti V, Mandy WPL, Scoto M, et al. Neurodevelopmental, emotional, and behavioural problems in Duchenne muscular dystrophy in relation to underlying dystrophin gene mutations. *Dev Med Child Neurol*. 2016;58(1):77-84. doi:10.1111/dmcn.12922

105. Battini R, Chieffo D, Bulgheroni S, et al. Cognitive profile in Duchenne muscular dystrophy boys without intellectual disability: The role of executive functions. *Neuromuscul Disord*. 2018;28(2):122-128. doi:10.1016/j.nmd.2017.11.018

106. Hoffman EP. The discovery of dystrophin, the protein product of the Duchenne muscular dystrophy gene. *FEBS J*. 2020;287(18):3879-3887. doi:10.1111/febs.15466

107. Jacobs PA, Hunt PA, Mayer M, Bart RD. Duchenne muscular dystrophy (DMD) in a female with an X/autosome translocation: further evidence that the DMD locus is at Xp21. *Am J Hum Genet*. 1981;33(4):513-518. http://www.ncbi.nlm.nih.gov/pubmed/7258185.

108. Hoffman EP, Monaco AP, Feener CC, Kunkel LM. Conservation of the Duchenne muscular dystrophy gene in mice and humans. *Science (80-)*. 1987;238(4825):347-350. doi:10.1126/science.3659917

109. Koenig M, Hoffman EP, Bertelson CJ, Monaco AP, Feener C, Kunkel LM. Complete cloning of the duchenne muscular dystrophy (DMD) cDNA and preliminary genomic organization of the DMD gene in normal and affected individuals. *Cell*. 1987;50(3):509-517. doi:10.1016/0092-8674(87)90504-6

110. Koenig M, Monaco AP, Kunkel LM. The complete sequence of dystrophin predicts a rod-shaped cytoskeletal protein. *Cell*. 1988;53(2):219-228. doi:10.1016/0092-8674(88)90383-2

111. Hoffman EP, Brown RH, Kunkel LM. Dystrophin: The protein product of the duchenne muscular dystrophy locus. *Cell*. 1987;51(6):919-928. doi:10.1016/0092-8674(87)90579-4

112. Gao QQ, McNally EM. The dystrophin complex: structure, function, and implications for therapy. *Compr Physiol*. 2015;5(3):1223-1239. doi:10.1002/cphy.c140048

113. Briggs DC, Yoshida-Moriguchi T, Zheng T, et al. Structural basis of laminin binding to the LARGE glycans on dystroglycan. *Nat Chem Biol*. 2016;12(10):810-814. doi:10.1038/nchembio.2146

114. Dreyfus J-C, Schapira G, Schapira F. Serum enzymes in the physiopathology of muscle. *Ann N Y Acad Sci*. 1958;75(1):235-249. doi:10.1111/j.1749-6632.1958.tb36870.x

115. Badalamente MA, Stracher A. Delay of muscle degeneration and necrosis in mdx mice by calpain inhibition. *Muscle Nerve*. 2000;23(1):106. doi:10.1002/(sici)1097-

4598(200001)23:1<106::aid-mus14>3.3.co;2-4

116. Menazza S, Blaauw B, Tiepolo T, et al. Oxidative stress by monoamine oxidases is causally involved in myofiber damage in muscular dystrophy. *Hum Mol Genet*. 2010;19(21):4207-4215. doi:10.1093/hmg/ddq339

117. Evans NP, Misyak SA, Robertson JL, Bassaganya-Riera J, Grange RW. Immune-mediated mechanisms potentially regulate the disease time-course of Duchenne muscular dystrophy and provide targets for therapeutic intervention. *PM R*. 2009;1(8):755-768. doi:10.1016/j.pmrj.2009.04.010

118. Shoji E, Sakurai H, Nishino T, et al. Early pathogenesis of Duchenne muscular dystrophy modelled in patient-derived human induced pluripotent stem cells. *Sci Rep*. 2015;5(August):1-13. doi:10.1038/srep12831

119. Iwata Y, Katanosaka Y, Arai Y, Shigekawa M, Wakabayashi S. Dominant-negative inhibition of Ca2+ influx via TRPV2 ameliorates muscular dystrophy in animal models. *Hum Mol Genet*. 2009;18(5):824-834. doi:10.1093/hmg/ddn408

120. Millay DP, Goonasekera SA, Sargent MA, Maillet M, Aronow BJ, Molkentin JD. Calcium influx is sufficient to induce muscular dystrophy through a TRPC-dependent mechanism. *Proc Natl Acad Sci U S A*. 2009;106(45):19023-19028. doi:10.1073/pnas.0906591106

121. Vandebrouck C, Martin D, Schoor MC Van, Debaix H, Gailly P. Involvement of TRPC in the abnormal calcium influx observed in dystrophic (mdx) mouse skeletal muscle fibers. *J Cell Biol*. 2002;158(6):1089-1096. doi:10.1083/jcb.200203091

122. Constantin B, Sebille S, Cognard C. New insights in the regulation of calcium transfers by

muscle dystrophin-based cytoskeleton: Implications in DMD. *J Muscle Res Cell Motil*. 2006;27(5-7):375-386. doi:10.1007/s10974-006-9085-2

123. Harisseh R, Chatelier A, Magaud C, Déliot N, Constantin B. Involvement of TRPV2 and SOCE in calcium influx disorder in DMD primary human myotubes with a specific contribution of α1-syntrophin and PLC/PKC in SOCE regulation. *Am J Physiol - Cell Physiol*. 2013;304(9). doi:10.1152/ajpcell.00182.2012

124. Allen DG, Whitehead NP. Duchenne muscular dystrophy - What causes the increased membrane permeability in skeletal muscle? *Int J Biochem Cell Biol*. 2011;43(3):290-294. doi:10.1016/j.biocel.2010.11.005

125. Emery AEH. The muscular dystrophies. *Lancet*. 2002;359(9307):687-695. doi:10.1016/S0140-6736(02)07815-7

126. Porter JD, Israel S, Gong B, et al. Distinctive morphological and gene/protein expression signatures during myogenesis in novel cell lines from extraocular and hindlimb muscle. *Physiol Genomics*. 2006;24(3):264-275. doi:10.1152/physiolgenomics.00234.2004

127. Kallestad KM, Hebert SL, McDonald AA, Daniel ML, Cu SR, McLoon LK. Sparing of extraocular muscle in aging and muscular dystrophies: A myogenic precursor cell hypothesis. *Exp Cell Res*. 2011;317(6):873-885. doi:10.1016/j.yexcr.2011.01.018

128. Pacheco-Pinedo EC, Budak MT, Zeiger U, et al. Transcriptional and functional differences in stem cell populations isolated from extraocular and limb muscles. *Physiol Genomics*. 2009;37(1):35-42. doi:10.1152/physiolgenomics.00051.2008

129. Formicola L, Marazzi G, Sassoon DA. The extraocular muscle stem cell niche is resistant

to ageing and disease. *Front Aging Neurosci*. 2014;6(DEC):328. doi:10.3389/fnagi.2014.00328

130. Stuelsatz P, Shearer A, Li Y, et al. Extraocular muscle satellite cells are high performance myo-engines retaining efficient regenerative capacity in dystrophin deficiency. *Dev Biol*. 2015;397(1):31-44. doi:10.1016/j.ydbio.2014.08.035

131. Kunert-Keil CH, Gredes T, Lucke S, Botzenhart U, Dominiak M, Gedrange T. Differential expression of genes involved in the calcium homeostasis in masticatory muscles of mdx mice. *J Physiol Pharmacol*. 2014;65(2):317-324.

132. Khurana TS, Prendergast RA, Alameddine HS, et al. Absence of extraocular muscle pathology in Duchenne's muscular dystrophy: Role for calcium homeostasis in extraocular muscle sparing. *J Exp Med*. 1995;182(2):467-475. doi:10.1084/jem.182.2.467

133. Porter JD, Merriam AP, Leahy P, Gong B, Khanna S. Dissection of temporal gene expression signatures of affected and spared muscle groups in dystrophin-deficient (mdx) mice. *Hum Mol Genet*. 2003;12(15):1813-1821. doi:10.1093/hmg/ddg197

134. Bothe I, Dietrich S. The molecular setup of the avian head mesoderm and Its implication for craniofacial myogenesis. *Dev Dyn*. 2006;235(10):2845-2860. doi:10.1002/dvdy.20903

135. Nathan E, Monovich A, Tirosh-Finkel L, et al. The contribution of Islet1-expressing splanchnic mesoderm cells to distinct branchiomeric muscles reveals significant heterogeneity in head muscle development. *Development*. 2008;135(4):647-657. doi:10.1242/dev.007989

136. Tirosh-Finkel L, Elhanany H, Rinon A, Tzahor E. Mesoderm progenitor cells of common

origin contribute to the head musculature and the cardiac outflow tract. *Development*. 2006;133(10):1943-1953. doi:10.1242/dev.02365

137. Dong F, Sun X, Liu W, et al. Pitx2 promotes development of splanchnic mesoderm-derived branchiomeric muscle. *Development*. 2006;133(24):4891-4899. doi:10.1242/dev.02693

138. Verzi MP, McCulley DJ, De Val S, Dodou E, Black BL. The right ventricle, outflow tract, and ventricular septum comprise a restricted expression domain within the secondary/anterior heart field. *Dev Biol*. 2005;287(1):134-145. doi:10.1016/j.ydbio.2005.08.041

139. Grifone R, Kelly RG. Heartening news for head muscle development. *Trends Genet*. 2007;23(8):365-369. doi:10.1016/j.tig.2007.05.002

140. Tzahor E. Heart and craniofacial muscle development: A new developmental theme of distinct myogenic fields. *Dev Biol*. 2009;327(2):273-279. doi:10.1016/j.ydbio.2008.12.035

141. Tzahor E, Evans SM. Pharyngeal mesoderm development during embryogenesis: Implications for both heart and head myogenesis. *Cardiovasc Res*. 2011;91(2):196-202. doi:10.1093/cvr/cvr116

142. Duan D, Goemans N, Takeda S, Mercuri E, Aartsma-Rus A. Duchenne muscular dystrophy. *Nat Rev Dis Prim*. 2021;7(13):1-19. doi:10.1201/b13434

143. Angelini C, Peterle E. Old and new therapeutic developments in steroid treatment in Duchenne muscular dystrophy. *Acta Myol*. 2012;31(MAY):9-15.

144. Matthews E, Brassington R, Kuntzer T, Jichi F, Manzur AY. Corticosteroids for the treatment of Duchenne muscular dystrophy. *Cochrane Database Syst Rev*. 2016;2016(5). doi:10.1002/14651858.CD003725.pub4

145. Guglieri M, Clemens PR, Perlman SJ, et al. Efficacy and Safety of Vamorolone vs Placebo and Prednisone among Boys with Duchenne Muscular Dystrophy: A Randomized Clinical Trial. *JAMA Neurol*. 2022;79(10):1005-1014. doi:10.1001/jamaneurol.2022.2480

146. Elhalag RH, Motawea KR, Talat NE, Rouzan SS, Shah J. Efficacy of vamorolone in treatment of Duchene muscle dystrophy. A meta-analysis. *Front Neurol*. 2023;14(1):1-9. doi:10.3389/fneur.2023.1107474

147. Partridge TA, Grounds M, Sloper JC. Evidence of fusion between host and donor myoblasts in skeletal muscle grafts. *Nature*. 1978;273(5660):306-308. doi:10.1038/273306a0

148. Partridge TA, Morgan JE, Coulton GR, Hoffman EP, Kunkel LM. Conversion of mdx myofibres from dystrophin-negative to -positive by injection of normal myoblasts. *Nature*. 1989;337(6203):176-179. doi:10.1038/337176a0

149. Briggs D, Morgan JE. Recent progress in satellite cell/myoblast engraftment - Relevance for therapy. *FEBS J*. 2013;280(17):4281-4293. doi:10.1111/febs.12273

150. Péault B, Rudnicki M, Torrente Y, et al. Stem and progenitor cells in skeletal muscle development, maintenance, and therapy. *Mol Ther*. 2007;15(5):867-877. doi:10.1038/mt.sj.6300145

151. Galvez BG, Sampaolesi M, Brunelli S, et al. Complete repair of dystrophic skeletal muscle by mesoangioblasts with enhanced migration ability. *J Cell Biol*. 2006;174(2):231-243. doi:10.1083/jcb.200512085

152. Sampaolesi M, Blot S, D'Antona G, et al. Mesoangioblast stem cells ameliorate muscle function in dystrophic dogs. *Nature*. 2006;444(7119):574-579. doi:10.1038/nature05282

153. Tedesco FS, Dellavalle A, Diaz-manera J, Messina G, Cossu G. Repairing skeletal muscle: regenerative potential of skeletal muscle stem cells. *J Clin Invest*. 2010;120(1):11-19. doi:10.1172/JCI40373.and

154. Li S, Kimura E, Fall BM, et al. Stable transduction of myogenic cells with lentiviral vectors expressing a minidystrophin. *Gene Ther*. 2005;12(14):1099-1108. doi:10.1038/sj.gt.3302505

155. Odom GL, Gregorevic P, Allen JM, Chamberlain JS. Gene therapy of mdx mice with large truncated dystrophins generated by recombination using rAAV6. *Mol Ther*. 2011;19(1):36-45. doi:10.1038/mt.2010.205

156. Ragot T, Vincent N, Chafey P, et al. Efficient adenovirus-mediated transfer of a human minidystrophin gene to skeletal muscle of mdx mice. *Nature*. 1993;361(6413):647-650. doi:10.1038/361647a0

157. Wang B, Li J, Xiao X. Adeno-associated virus vector carrying human minidystrophin genes effectively ameliorates muscular dystrophy in mdx mouse model. *Proc Natl Acad Sci U S A*. 2000;97(25):13714-13719. doi:10.1073/pnas.240335297

158. Xin C, Chu X, Wei W, et al. Combined gene therapy via VEGF and mini-dystrophin

synergistically improves pathologies in temporalis muscle of dystrophin/utrophin double knockout mice. *Hum Mol Genet*. 2021;30(14):1349-1359. doi:10.1093/hmg/ddab120

159. Chu X, Li J, Qiao C, et al. Long-term effect of human mini-dystrophin in transgenic mdx mice improves muscle physiological function. *FASEB J*. 2021;35(6):1-12. doi:10.1096/fj.202100057RR

160. Kimura E, Li S, Gregorevic P, Fall BM, Chamberlain JS. Dystrophin delivery to muscles of mdx mice using lentiviral vectors leads to myogenic progenitor targeting and stable gene expression. *Mol Ther*. 2010;18(1):206-213. doi:10.1038/mt.2009.253

161. Shin JH, Pan X, Hakim CH, et al. Microdystrophin ameliorates muscular dystrophy in the canine model of duchenne muscular dystrophy. *Mol Ther*. 2013;21(4):750-757. doi:10.1038/mt.2012.283

162. Yoshimura M, Sakamoto M, Ikemoto M, et al. AAV vector-mediated microdystrophin expression in a relatively small percentage of mdx myofibers improved the mdx phenotype. *Mol Ther*. 2004;10(5):821-828. doi:10.1016/j.ymthe.2004.07.025

163. Piepho AB, Lowe J, Cumby LR, et al. Micro-dystrophin gene therapy demonstrates long-term cardiac efficacy in a severe Duchenne muscular dystrophy model. *Mol Ther - Methods Clin Dev*. 2023;28(March):344-354. doi:10.1016/j.omtm.2023.02.001

164. Birch SM, Lawlor MW, Conlon TJ, et al. Assessment of systemic AAV-microdystrophin gene therapy in the GRMD model of Duchenne muscular dystrophy. *Sci Transl Med*. 2023;15(677). doi:10.1126/scitranslmed.abo1815

165. Wasala N, Yue Y, Hu B, et al. Life-long outcomes of systemic AAV micro-dystrophin gene

therapy in a murine Duchenne muscular dystrophy model. *Hum Gene Ther*. 2022:1-26. doi:10.1089/hum.2022.181

166. Li D, Mastaglia FL, Fletcher S, Wilton SD. Precision medicine through antisense oligonucleotide-mediated exon skipping. *Trends Pharmacol Sci*. 2018;39(11):982-994. doi:10.1016/j.tips.2018.09.001

167. Patterson G, Conner H, Groneman M, Blavo C, Parmar MS. Duchenne muscular dystrophy: Current treatment and emerging exon skipping and gene therapy approach. *Eur J Pharmacol*. 2023;947(March):175675. doi:10.1016/j.ejphar.2023.175675

168. Wilton-Clark H, Yokota T. Recent trends in antisense therapies for duchenne muscular dystrophy. *Pharmaceutics*. 2023;15(3):1-12. doi:10.3390/pharmaceutics15030778

169. Mendell JR, Campbell K, Rodino-Klapac L, et al. Dystrophin immunity in Duchenne's muscular dystrophy. *N Engl J Med*. 2010;363(15):1429-1437. doi:10.1056/nejmoa1000228

170. Vila MC, Novak JS, Benny Klimek M, et al. Morpholino-induced exon skipping stimulates cell-mediated and humoral responses to dystrophin in mdx mice. *J Pathol*. 2019;248(3):339-351. doi:10.1002/path.5263

171. Gilchrist SC, Ontell MP, Kochanek S, Clemens PR. Immune response to full-length dystrophin delivered to Dmd muscle by a high-capacity adenoviral vector. *Mol Ther*. 2002;6(3):359-368. doi:10.1006/mthe.2002.0675

172. Flanigan KM, Campbell K, Viollet L, et al. Anti-dystrophin T cell responses in duchenne muscular dystrophy: Prevalence and a glucocorticoid treatment effect. *Hum Gene Ther*.

2013;24(9):797-806. doi:10.1089/hum.2013.092

173. Eghtesad S, Jhunjhunwala S, Little SR, Clemens PR. Effect of rapamycin on immunity induced by vector-mediated dystrophin expression in mdx skeletal muscle. *Sci Rep*. 2012;2:2-7. doi:10.1038/srep00399

174. Farini A, Sitzia C, Cassani B, et al. Therapeutic potential of immunoproteasome inhibition in duchenne muscular dystrophy. *Mol Ther*. 2016;24(11):1898-1912. doi:10.1038/mt.2016.162

175. Li N, Parkes JE, Spathis R, et al. The effect of immunomodulatory treatments on anti-dystrophin immune response after AAV gene therapy in dystrophin deficient mdx mice. *J Neuromuscul Dis*. 2021;8(s2):S325-S340. doi:10.3233/JND-210706

176. Günther S, Kim J, Kostin S, Lepper C, Fan CM, Braun T. Myf5-positive satellite cells contribute to Pax7-dependent long-term maintenance of adult muscle stem cells. *Cell Stem Cell*. 2013;13(5):590-601. doi:10.1016/j.stem.2013.07.016

177. Von Maltzahn J, Jones AE, Parks RJ, Rudnicki MA. Pax7 is critical for the normal function of satellite cells in adult skeletal muscle. *Proc Natl Acad Sci U S A*. 2013;110(41):16474-16479. doi:10.1073/pnas.1307680110

178. Lepper C, Partridge TA, Fan CM. An absolute requirement for pax7-positive satellite cells in acute injury-induced skeletal muscle regeneration. *Development*. 2011;138(17):3639-3646. doi:10.1242/dev.067595

179. Murphy MM, Lawson JA, Mathew SJ, Hutcheson DA, Kardon G. Satellite cells, connective tissue fibroblasts and their interactions are crucial for muscle regeneration.

Development. 2011;138(17):3625-3637. doi:10.1242/dev.064162

180. Sambasivan R, Yao R, Kissenpfennig A, et al. Pax7-expressing satellite cells are indispensable for adult skeletal muscle regeneration. *Development*. 2011;138(17):3647-3656. doi:10.1242/dev.067587

181. Yin H, Price F, Rudnicki MA. Satellite cells and the muscle stem cell niche. *Physiol Rev*. 2013;93(1):23-67. doi:10.1152/physrev.00043.2011

182. Olguin HC, Yang Z, Tapscott SJ, Olwin BB. Reciprocal inhibition between Pax7 and muscle regulatory factors modulates myogenic cell fate determination. *J Cell Biol*. 2007;177(5):769-779. doi:10.1083/jcb.200608122

183. Bentzinger CF, Wang YX, Rudnicki MA. Building muscle: Molecular regulation of myogenesis. *Cold Spring Harb Perspect Biol*. 2012;4(2). doi:10.1101/cshperspect.a008342

184. Cheung TH, Rando TA. Molecular regulation of stem cell quiescence. *Nat Rev Mol Cell Biol*. 2013;14(6):329-340. doi:10.1038/nrm3591

185. Fukada S, Uezumi A, Ikemoto M, et al. Molecular signature of quiescent satellite cells in adult skeletal muscle. *Stem Cells*. 2007;25(10):2448-2459. doi:10.1634/stemcells.2007-0019

186. Irintchev A, Zeschnigk M, Starzinski-Powitz A, Wernig A. Expression pattern of M-cadherin in normal, denervated, and regenerating mouse muscles. *Dev Dyn*. 1994;199(4):326-337. doi:10.1002/aja.1001990407

187. Pallafacchina G, François S, Regnault B, et al. An adult tissue-specific stem cell in its niche: A gene profiling analysis of in vivo quiescent and activated muscle satellite cells. *Stem Cell Res*. 2010;4(2):77-91. doi:10.1016/j.scr.2009.10.003

188. Koch U, Lehal R, Radtke F. Stem cells living with a Notch. *Development*. 2013;140(4):689-704. doi:10.1242/dev.080614

189. Fujimaki S, Seko D, Kitajima Y, et al. Notch1 and Notch2 Coordinately Regulate Stem Cell Function in the Quiescent and Activated States of Muscle Satellite Cells. *Stem Cells*. 2018;36(2):278-285. doi:10.1002/stem.2743

190. Philippos M, Sambasivan R, Castel D, Rocheteau P, Bizzarro V, Tajbakhsh S. A critical requirement for notch signaling in maintenance of the quiescent skeletal muscle stem cell state. *Stem Cells*. 2012;30(2):243-252. doi:10.1002/stem.775

191. Bjornson CRR, Cheung TH, Liu L, Tripathi P V., Steeper KM, Rando TA. Notch signaling is necessary to maintain quiescence in adult muscle stem cells. *Stem Cells*. 2012;30(2):232-242. doi:10.1002/stem.773

192. Wen Y, Bi P, Liu W, Asakura A, Keller C, Kuang S. Constitutive Notch activation upregulates Pax7 and promotes the self-renewal of skeletal muscle satellite cells. *Mol Cell Biol*. 2012;32(12):2300-2311. doi:10.1128/mcb.06753-11

193. Buas MF, Kabak S, Kadesch T. The notch effector Hey1 associates with myogenic target genes to repress myogenesis. *J Biol Chem*. 2010;285(2):1249-1258. doi:10.1074/jbc.M109.046441

194. Dahlqvist C, Blokzijl A, Chapman G, et al. Functional Notch signaling is required for

BMP4-induced inhibition of myogenic differentiation. *Development*. 2003;130(24):6089-6099. doi:10.1242/dev.00834

195. Gustafsson M V, Zheng X, Pereira T, et al. Hypoxia requires notch signaling to maintain the undifferentiated cell state. *Dev Cell*. 2005;9(5):617-628. doi:10.1016/j.devcel.2005.09.010

196. Brack AS, Conboy IM, Conboy MJ, Shen J, Rando TA. A temporal switch from notch to Wnt signaling in muscle stem cells is necessary for normal adult myogenesis. *Cell Stem Cell*. 2008;2(1):50-59. doi:10.1016/j.stem.2007.10.006

197. DiMario J, Buffinger N, Yamada S, Strohman RC. Fibroblast growth factor in the extracellular matrix of dystrophic (mdx) mouse muscle. *Science (80-)*. 1989;244(4905):688-690. doi:10.1126/science.2717945

198. Kästner S, Elias MC, Rivera AJ, Yablonka-Reuveni Z. Gene expression patterns of the fibroblast growth factors and their receptors during myogenesis of rat satellite cells. *J Histochem Cytochem*. 2000;48(8):1079-1096. doi:10.1177/002215540004800805

199. Cornelison DDW, Wilcox-Adelman SA, Goetinck PF, Rauvala H, Rapraeger AC, Olwin BB. Essential and separable roles for Syndecan-3 and Syndecan-4 in skeletal muscle development and regeneration. *Genes Dev*. 2004;18(18):2231-2236. doi:10.1101/gad.1214204

200. Liu Y, Schneider MF. FGF2 activates TRPC and Ca2+ signaling leading to satellite cell activation. *Front Physiol*. 2014;5:1-10. doi:10.3389/fphys.2014.00038

201. Jones NC, Tyner KJ, Nibarger L, et al. The p38α/β MAPK functions as a molecular switch

to activate the quiescent satellite cell. *J Cell Biol*. 2005;169(1):105-116. doi:10.1083/jcb.200408066

202. Maher P. p38 mitogen-activated protein kinase activation is required for fibroblast growth factor-2-stimulated cell proliferation but not differentiation. *J Biol Chem*. 1999;274(25):17491-17498. doi:10.1074/jbc.274.25.17491

203. Tatsumi R, Anderson JE, Nevoret CJ, Halevy O, Allen RE. HGF/SF is present in normal adult skeletal muscle and is capable of activating satellite cells. *Dev Biol*. 1998;194(1):114-128. doi:10.1006/dbio.1997.8803

204. Allen RE, Sheehan SM, Taylor RG, Kendall TL, Rice GM. Hepatocyte growth factor activates quiescent skeletal muscle satellite cells in vitro. *J Cell Physiol*. 1995;165(2):307-312. doi:10.1002/jcp.1041650211

205. Anderson JE. A role for nitric oxide in muscle repair: Nitric oxide-mediated activation of muscle satellite cells. *Mol Biol Cell*. 2000;11(5):1859-1874. doi:10.1091/mbc.11.5.1859

206. Tatsumi R. Mechano-biology of skeletal muscle hypertrophy and regeneration: Possible mechanism of stretch-induced activation of resident myogenic stem cells. *Anim Sci J*. 2010;81(1):11-20. doi:10.1111/j.1740-0929.2009.00712.x

207. Musarò A, McCullagh K, Paul A, et al. Localized Igf-1 transgene expression sustains hypertrophy and regeneration in senescent skeletal muscle. *Nat Genet*. 2001;27(2):195-200. doi:10.1038/84839

208. Perrone CE, Fenwick-Smith D, Vandenburgh HH. Collagen and stretch modulate autocrine secretion of insulin-like growth factor-1 and insulin-like growth factor binding

proteins differentiated skeletal muscle cells. *J Biol Chem*. 1995;270(5):2099-2106. doi:10.1074/jbc.270.5.2099

209. Yoshida T, Delafontaine P. Mechanisms of IGF-1-Mediated Regulation of Skeletal Muscle Hypertrophy and Atrophy. *Cells*. 2020;9(9):1-25. doi:10.3390/cells9091970

210. Machida S, Spangenburg EE, Booth FW. Forkhead transcription factor FoxO1 transduces insulin-like growth factor's signal to p27Kip1 in primary skeletal muscle satellite cells. *J Cell Physiol*. 2003;196(3):523-531. doi:10.1002/jcp.10339

211. Li YP. TNF-α is a mitogen in skeletal muscle. *Am J Physiol - Cell Physiol*. 2003;285(2 54-2):370-376. doi:10.1152/ajpcell.00453.2002

212. Acharyya S, Sharma SM, Cheng AS, et al. TNF inhibits notch-1 in skeletal muscle cells by Ezh2 and DNA methylation mediated repression: Implications in Duchenne muscular dystrophy. *PLoS One*. 2010;5(8). doi:10.1371/journal.pone.0012479

213. Nagata Y, Kobayashi H, Umeda M, et al. Sphingomyelin levels in the plasma membrane correlate with the activation state of muscle satellite cells. *J Histochem Cytochem*. 2006;54(4):375-384. doi:10.1369/jhc.5A6675.2006

214. Nagata Y, Partridge TA, Matsuda R, Zammit PS. Entry of muscle satellite cells into the cell cycle requires sphingolipid signaling. *J Cell Biol*. 2006;174(2):245-253. doi:10.1083/jcb.200605028

215. Becciolini L, Meacci E, Donati C, Cencetti F, Rapizzi E, Bruni P. Sphingosine 1-phosphate inhibits cell migration in C2C12 myoblasts. *Biochim Biophys Acta*. 2006;1761(1):43-51. doi:10.1016/j.bbalip.2006.01.006

216. Donati C, Meacci E, Nuti F, Becciolini L, Farnararo M, Bruni P. Sphingosine 1-phosphate regulates myogenic differentiation: a major role for S1P 2 receptor. *FASEB J*. 2005;19(3):1-22. doi:10.1096/fj.04-1780fje

217. Calise S, Blescia S, Cencetti F, Bernacchioni C, Donati C, Bruni P. Sphingosine 1-phosphate stimulates proliferation and migration of satellite cells. Role of S1P receptors. *Biochim Biophys Acta*. 2012;1823(2):439-450. doi:10.1016/j.bbamcr.2011.11.016

218. Zammit PS, Partridge TA, Yablonka-Reuveni Z. The skeletal muscle satellite cell: The stem cell that came in from the cold. *J Histochem Cytochem*. 2006;54(11):1177-1191. doi:10.1369/jhc.6R6995.2006

219. Watt DJ, Morgan JE, Clifford MA, Partridge TA. The movement of muscle precursor cells between adjacent regenerating muscles in the mouse. *Anat Embryol (Berl)*. 1987;175(4):527-536. doi:10.1007/BF00309688

220. Siegel AL, Atchison K, Fisher KE, Davis GE, Cornelison DDW. 3D timelapse analysis of muscle satellite cell motility. *Stem Cells*. 2009;27(10):2527-2538. doi:10.1002/stem.178

221. Jockusch H, Voigt S. Migration of adult myogenic precursor cells as revealed by GFP/nLacZ labelling of mouse transplantation chimeras. *J Cell Sci*. 2003;116(8):1611-1616. doi:10.1242/jcs.00364

222. Sacco A, Doyonnas R, Kraft P, Vitorovic S, Blau HM. Self-renewal and expansion of single transplanted muscle stem cells. *Nature*. 2008;456(7221):502-506. doi:10.1038/nature07384

223. Alfaro LAS, Dick SA, Siegel AL, et al. CD34 promotes satellite cell motility and entry into

proliferation to facilitate efficient skeletal muscle regeneration. *Stem Cells*. 2011;29(12):2030-2041. doi:10.1002/stem.759

224. Orian-Rousseau V, Sleeman J. CD44 is a multidomain signaling platform that integrates extracellular matrix cues with growth factor and cytokine signals. *Adv Cancer Res*. 2014;123:231-254. doi:10.1016/B978-0-12-800092-2.00009-5

225. Mylona E, Jones KA, Mills ST, Pavlath GK. CD44 regulates myoblast migration and differentiation. *J Cell Physiol*. 2006;209(2):314-321. doi:10.1002/jcp.20724

226. Carmeli E, Moas M, Reznick AZ, Coleman R. Matrix metalloproteinases and skeletal muscle: A brief review. *Muscle and Nerve*. 2004;29(2):191-197. doi:10.1002/mus.10529

227. Nishimura T, Nakamura K, Kishioka Y, Kato-Mori Y, Wakamatsu JI, Hattori A. Inhibition of matrix metalloproteinases suppresses the migration of skeletal muscle cells. *J Muscle Res Cell Motil*. 2008;29(1):37-44. doi:10.1007/s10974-008-9140-2

228. Bellayr I, Holden K, Mu X, Pan H, Li Y. Matrix metalloproteinase inhibition negatively affects muscle stem cell behavior. *Int J Clin Exp Pathol*. 2013;6(2):124-141.

229. Kaar JL, Li Y, Blair HC, et al. Matrix metalloproteinase-1 treatment of muscle fibrosis. *Acta Biomater*. 2008;4(5):1411-1420. doi:10.1016/j.actbio.2008.03.010

230. Kherif S, Lafuma C, Dehaupas M, et al. Expression of matrix metalloproteinases 2 and 9 in regenerating skeletal muscle: A study in experimentally injured and mdx muscles. *Dev Biol*. 1999;205(1):158-170. doi:10.1006/dbio.1998.9107

231. Morgan J, Rouche A, Bausero P, et al. MMP-9 overexpression improves myogenic cell

migration and engraftment. *Muscle Nerve*. 2010;42(4):584-595. doi:10.1002/mus.21737

232. Smith LR, Kok HJ, Zhang B, et al. Matrix metalloproteinase 13 from satellite cells is required for efficient muscle growth and regeneration. *Cell Physiol Biochem*. 2020;54(3):333-353. doi:10.33594/000000223

233. Alameddine HS, Morgan JE. Matrix metalloproteinases and tissue inhibitor of metalloproteinases in inflammation and fibrosis of skeletal muscles. *J Neuromuscul Dis*. 2016;3(4):455-473. doi:10.3233/JND-160183

234. Arpino V, Brock M, Gill SE. The role of TIMPs in regulation of extracellular matrix proteolysis. *Matrix Biol*. 2015;44-46:247-254. doi:10.1016/j.matbio.2015.03.005

235. Bischoff R. Chemotaxis of skeletal muscle satellite cells. *Dev Dyn*. 1997;208(4):505-515. doi:10.1002/(SICI)1097-0177(199704)208:4<505::AID-AJA6>3.0.CO;2-M

236. Neuhaus P, Oustanina S, Loch T, et al. Reduced mobility of fibroblast growth factor (FGF)-deficient myoblasts might contribute to dystrophic changes in the musculature of FGF2/FGF6/mdx triple-mutant mice. *Mol Cell Biol*. 2003;23(17):6037-6048. doi:10.1128/mcb.23.17.6037-6048.2003

237. Bentzinger CF, von Maltzahn J, Dumont NA, et al. Wnt7a stimulates myogenic stem cell motility and engraftment resulting in improved muscle strength. *J Cell Biol*. 2014;205(1):97-111. doi:10.1083/jcb.201310035

238. Lafreniere JF, Mills P, Bouchentouf M, Tremblay JP. Interleukin-4 improves the migration of human myogenic precursor cells in vitro and in vivo. *Exp Cell Res*. 2006;312(7):1127-1141. doi:10.1016/j.yexcr.2006.01.002

239. Stark DA, Karvas RM, Siege AL, Cornelison DDW. Eph/ephrin interactions modulate muscle satellite cell motility and patterning. *Development*. 2011;138(24):5279-5289. doi:10.1242/dev.068411

240. Schultz E, Jaryszak DL, Valliere CR. Response of satellite cells to focal skeletal muscle injury. *Muscle Nerve*. 1985;8(3):217-222. doi:10.1002/mus.880080307

241. Jones NC, Fedorov Y V., Rosenthal RS, Olwin BB. ERK1/2 is required for myoblast proliferation but is dispensable for muscle gene expression and cell fusion. *J Cell Physiol*. 2001;186(1):104-115. doi:10.1002/1097-4652(200101)186:1<104::AID-JCP1015>3.0.CO;2-0

242. Yablonka-Reuveni Z, Rivera AJ. Influence of PDGF-BB on proliferation and transition through the MyoD-myogenin-MEF2A expression program during myogenesis in mouse C2 myoblasts. *Growth factors*. 1997;15(1):1-27. doi:10.3109/08977199709002109

243. Luo G, Hershko DD, Robb BW, Wray CJ, Hasselgren PO. IL-1β stimulates IL-6 production in cultured skeletal muscle cells through activation of MAP kinase signaling pathway and NF-κB. *Am J Physiol - Regul Integr Comp Physiol*. 2003;284(5):1249-1254. doi:10.1152/ajpregu.00490.2002

244. Prelovsek O, Mars T, Jevsek M, Podbregar M, Grubic Z. High dexamethasone concentration prevents stimulatory effects of TNF-α and LPS on IL-6 secretion from the precursors of human muscle regeneration. *Am J Physiol - Regul Integr Comp Physiol*. 2006;291(6):1651-1656. doi:10.1152/ajpregu.00020.2006

245. Cantini M, Massimino ML, Rapizzi E, et al. Human satellite cell proliferation in vitro is regulated by autocrine secretion of IL-6 stimulated by a soluble factor(s) released by

activated monocytes. *Biochem Biophys Res Commun*. 1995;216(1):49-53. doi:10.1006/bbrc.1995.2590

246. Wang X, Wu H, Zhang Z, et al. Effects of interleukin-6, leukemia inhibitory factor, and ciliary neurotrophic factor on the proliferation and differentiation of adult human myoblasts. *Cell Mol Neurobiol*. 2008;28(1):113-124. doi:10.1007/s10571-007-9247-9

247. Sun L, Ma K, Wang H, et al. JAK1-STAT1-STAT3, a key pathway promoting proliferation and preventing premature differentiation of myoblasts. *J Cell Biol*. 2007;179(1):129-138. doi:10.1083/jcb.200703184

248. Cardinalli B, Castellani L, Fasanaro P, et al. Microrna-221 and microrna-222 modulate differentiation and maturation of skeletal muscle cells. *PLoS One*. 2009;4(10). doi:10.1371/journal.pone.0007607

249. Chen JF, Mandel EM, Thomson JM, et al. The role of microRNA-1 and microRNA-133 in skeletal muscle proliferation and differentiation. *Nat Genet*. 2006;38(2):228-233. doi:10.1038/ng1725

250. Wei L, Zhou W, Croissant JD, et al. RhoA signaling via serum response factor plays an obligatory role in myogenic differentiation. *J Biol Chem*. 1998;273(46):30287-30294. doi:10.1074/jbc.273.46.30287

251. Ganassi M, Badodi S, Wanders K, Zammit PS. Myogenin is an essential regulator of adult myofibre growth and muscle stem cell homeostasis. *Elife*. 2020;9:1-23. doi:10.7554/eLife.60445

252. Wang YX, Rudnicki MA. Satellite cells, the engines of muscle repair. *Nat Rev Mol Cell*

Biol. 2012;13(2):127-133. doi:10.1038/nrm3265

253. Cornelison DDW, Olwin BB, Rudnicki MA, Wold BJ. MyoD(-/-) satellite cells in single-fiber culture are differentiation defective and MRF4 deficient. *Dev Biol*. 2000;224(2):122-137. doi:10.1006/dbio.2000.9682

254. Megeney LA, Kablar B, Garrett K, Anderson JE, Rudnicki MA. MyoD is required for myogenic stem cell function in adult skeletal muscle. *Genes Dev*. 1996;10(10):1173-1183. doi:10.1101/gad.10.10.1173

255. Sabourin LA, Girgis-Gabardo A, Seale P, Asakura A, Rudnicki MA. Reduced differentiation potential of primary MyoD-/- myogenic cells derived from adult skeletal muscle. *J Cell Biol*. 1999;144(4):631-643. doi:10.1083/jcb.144.4.631

256. Halevy O, Novitch BG, Spicer DB, et al. Correlation of terminal cell cycle arrest of skeletal muscle with induction of p21 by MyoD. *Science (80-)*. 1995;267(5200):1018-1021. doi:10.1126/science.7863327

257. De la Serna IL, Roy K, Carlson KA, Imbalzano AN. MyoD can induce cell cycle arrest but not muscle differentiation in the presence of dominant negative SWI/SNF chromatin remodeling enzymes. *J Biol Chem*. 2001;276(44):41486-41491. doi:10.1074/jbc.M107281200

258. Guo K, Wang J, Andrés V, Smith RC, Walsh K. MyoD-induced expression of p21 inhibits cyclin-dependent kinase activity upon myocyte terminal differentiation. *Mol Cell Biol*. 1995;15(7):3823-3829. doi:10.1128/mcb.15.7.3823

259. Hollenberg SM, Pei Feng Cheng, Weintraub H. Use of a conditional MyoD transcription

factor in studies of MyoD transactivation and muscle determination. *Proc Natl Acad Sci U S A*. 1993;90(17):8028-8032. doi:10.1073/pnas.90.17.8028

260. Davie JK, Cho JH, Meadows E, Flynn JM, Knapp JR, Klein WH. Target gene selectivity of the myogenic basic helix-loop-helix transcription factor myogenin in embryonic muscle. *Dev Biol*. 2007;311(2):650-664. doi:10.1016/j.ydbio.2007.08.014

261. Myer A, Olson EN, Klein WH. MyoD cannot compensate for the absence of myogenin during skeletal muscle differentiation in murine embryonic stem cells. *Dev Biol*. 2001;229(2):340-350. doi:10.1006/dbio.2000.9985

262. Zhu Z, Miller JB. MRF4 can substitute for myogenin during early stages of myogenesis. *Dev Dyn*. 1997;209(2):233-241. doi:10.1002/(SICI)1097-0177(199706)209:2<233::AID-AJA9>3.0.CO;2-J

263. Lamarche É, AlSudais H, Rajgara R, Fu D, Omaiche S, Wiper-Bergeron N. SMAD2 promotes myogenin expression and terminal myogenic differentiation. *Development*. 2021;148(3). doi:10.1242/dev.195495

264. Molkentin JD, Black BL, Martin JF, Olson EN. Cooperative activation of muscle gene expression by MEF2 and myogenic bHLH proteins. *Cell*. 1995;83(7):1125-1136. doi:10.1016/0092-8674(95)90139-6

265. Cserjesi P, Olson EN. Myogenin induces the myocyte-specific enhancer binding factor MEF-2 independently of other muscle-specific gene products. *Mol Cell Biol*. 1991;11(10):4854-4862. doi:10.1128/mcb.11.10.4854-4862.1991

266. Kaushal S, Schneider JW, Nadal-Ginard B, Mahdavi V. Activation of the myogenic

lineage by MEF2A, a factor that induces and cooperates with MyoD. *Science (80-)*. 1994;266(5188):1236-1240. doi:10.1126/science.7973707

267. Liu N, Nelson BR, Bezprozvannaya S, et al. Requirement of MEF2A, C, and D for skeletal muscle regeneration. *Proc Natl Acad Sci U S A*. 2014;111(11):4109-4114. doi:10.1073/pnas.1401732111

268. Wu Z, Woodring PJ, Bhakta KS, et al. p38 and extracellular signal-regulated kinases regulate the myogenic program at multiple steps. *Mol Cell Biol*. 2000;20(11):3951-3964. doi:10.1128/mcb.20.11.3951-3964.2000

269. Perdiguero E, Ruiz-Bonilla V, Serrano AL, Muñoz-Cánoves P. Genetic deficiency of p38α reveals its critical role in myoblast cell cycle exit: The p38α-JNK connection. *Cell Cycle*. 2007;6(11):1298-1303. doi:10.4161/cc.6.11.4315

270. Lluís F, Ballestar E, Suelves M, Esteller M, Muñoz-Cánoves P. E47 phosphorylation by p38 MAPK promotes MyoD/E47 association and muscle-specific gene transcription. *EMBO J*. 2005;24(5):974-984. doi:10.1038/sj.emboj.7600528

271. Robertson TA, Grounds MD, Mitchell CA, Papadimitriou JM. Fusion between myogenic cells in Vivo: An ultrastructural study in regenerating murine skeletal muscle. *J Struct Biol*. 1990;105(1-3):170-182. doi:10.1016/1047-8477(90)90111-O

272. Grounds MD, Yablonka-Reuveni Z. Molecular and cell biology of skeletal muscle regeneration. *Mol Cell Biol Hum Dis Ser*. 1993;3:210-256. doi:10.1007/978-94-011-1528-5_9

273. Rochlin K, Yu S, Roy S, Baylies MK. Myoblast fusion: When it takes more to make one.

Dev Biol. 2010;341(1):66-83. doi:10.1016/j.ydbio.2009.10.024

274. Ruiz-Gómez M, Coutts N, Price A, Taylor M V., Bate M. Drosophila dumbfounded: A myoblast attractant essential for fusion. *Cell*. 2000;102(2):189-198. doi:10.1016/S0092-8674(00)00024-6

275. Strünkelnberg M, Bonengel B, Moda LM, et al. Rst and its paralogue kirre act redundantly during embryonic muscle development in Drosophila. *Development*. 2001;128(21):4229-4239. doi:10.1242/dev.128.21.4229

276. Zhang R, Lee DM, Jimah JR, et al. Dynamin regulates the dynamics and mechanical strength of the actin cytoskeleton as a multifilament actin-bundling protein. *Nat Cell Biol*. 2020;22(6):674-688. doi:10.1038/s41556-020-0519-7

277. Rushton E, Drysdale R, Abmayr SM, Michelson AM, Bate M. Mutations in a navel gene, myoblast city, provide evidence in support of the founder cell hypothesis for Drosophila muscle development. *Development*. 1995;121(7):1979-1988. doi:10.1242/dev.121.7.1979

278. Artero RD, Castanon I, Baylies MK. The immunoglobulin-like protein hibris functions as a dose-dependent regulator of myoblast fusion and is differentially controlled by Ras and Notch signaling. *Development*. 2001;128(21):4251-4264. doi:10.1242/dev.128.21.4251

279. Dworak HA, Charles MA, Pellerano LB, Sink H. Characterization of Drosophila hibris, a gene related to human nephrin. *Development*. 2001;128(21):4265-4276. doi:10.1242/dev.128.21.4265

280. Gildor B, Schejter ED, Shilo BZ. Bidirectional Notch activation represses fusion competence in swarming adult Drosophila myoblasts. *Development*. 2012;139(21):4040-

4050. doi:10.1242/dev.077495

281. Abmayr SM, Pavlath GK. Myoblast fusion: Lessons from flies and mice. *Development*. 2012;139(4):641-656. doi:10.1242/dev.068353

282. Sohn RL, Huang P, Kawahara G, et al. A role for nephrin, a renal protein, in vertebrate skeletal muscle cell fusion. *Proc Natl Acad Sci U S A*. 2009;106(23):9274-9279. doi:10.1073/pnas.0904398106

283. Önel SF, Renkawitz-Pohl R. FuRMAS: Triggering myoblast fusion in Drosophila. *Dev Dyn*. 2009;238(6):1513-1525. doi:10.1002/dvdy.21961

284. Kesper DA, Stute C, Buttgereit D, et al. Myoblast fusion in Drosophila melanogaster is mediated through a fusion-restricted myogenic-adhesive structure (FuRMAS). *Dev Dyn*. 2007;236(2):404-415. doi:10.1002/dvdy.21035

285. Jeong J, Conboy IM. Phosphatidylserine directly and positively regulates fusion of myoblasts into myotubes. *Biochem Biophys Res Commun*. 2011;414(1):9-13. doi:10.1016/j.bbrc.2011.08.128

286. Sun KX, Jiang XY, Li X, et al. Deletion of phosphatidylserine flippase β-subunit Tmem30a in satellite cells leads to delayed skeletal muscle regeneration. *Zool Res*. 2021;42(5):650-659. doi:10.24272/J.ISSN.2095-8137.2021.195

287. Hamoud N, Tran V, Croteau LP, Kania A, Côté JF. G-protein coupled receptor BAI3 promotes myoblast fusion in vertebrates. *Proc Natl Acad Sci U S A*. 2014;111(10):3745-3750. doi:10.1073/pnas.1313886111

288. Stradal TEB, Rottner K, Disanza A, Confalonieri S, Innocenti M, Scita G. Regulation of actin dynamics by WASP and WAVE family proteins. *Trends Cell Biol*. 2004;14(6):303-311. doi:10.1016/j.tcb.2004.04.007

289. Bothe I, Deng S, Baylies M. PI(4,5)P2 regulates myoblast fusion through Arp2/3 regulator localization at the fusion site. *Development*. 2014;141(11):2289-2301. doi:10.1242/dev.100743

290. Sens KL, Zhang S, Jin P, et al. An invasive podosome-like structure promotes fusion pore formation during myoblast fusion. *J Cell Biol*. 2010;191(5):1013-1027. doi:10.1083/jcb.201006006

291. Millay DP, O'Rourke JR, Sutherland LB, et al. Myomaker is a membrane activator of myoblast fusion and muscle formation. *Nature*. 2013;499(7458):301-305. doi:10.1038/nature12343

292. Millay DP, Sutherland LB, Bassel-Duby R, Olson EN. Myomaker is essential for muscle regeneration. *Genes Dev*. 2014;28(15):1641-1646. doi:10.1101/gad.247205.114

293. Luo W, Li E, Nie Q, Zhang X. Myomaker, regulated by MYOD, MYOG and miR-140-3P, promotes chicken myoblast fusion. *Int J Mol Sci*. 2015;16(11):26186-26201. doi:10.3390/ijms161125946

294. Quinn ME, Goh Q, Kurosaka M, et al. Myomerger induces fusion of non-fusogenic cells and is required for skeletal muscle development. *Nat Commun*. 2017;8:1-9. doi:10.1038/ncomms15665

295. Bi P, McAnally JR, Shelton JM, Sánchez-Ortiz E, Bassel-Duby R, Olson EN. Fusogenic

micropeptide Myomixer is essential for satellite cell fusion and muscle regeneration. *Proc Natl Acad Sci U S A*. 2018;115(15):3864-3869. doi:10.1073/pnas.1800052115

296. Takei D, Nishi M, Fukada S ichiro, et al. Gm7325 is MyoD-dependently expressed in activated muscle satellite cells. *Biomed Res*. 2017;38(3):215-219. doi:10.2220/biomedres.38.215

297. Leikina E, Gamage DG, Prasad V, et al. Myomaker and Myomerger work independently to control distinct steps of membrane remodeling during myoblast fusion. *Dev Cell*. 2018;46(6):767-780. doi:10.1016/j.devcel.2018.08.006

298. Pei J, Millay DP, Olson EN, Grishin N V. CREST - a large and diverse superfamily of putative transmembrane hydrolases. *Biol Direct*. 2011;6:1-17. doi:10.1186/1745-6150-6-37

299. Millay DP. Regulation of the myoblast fusion reaction for muscle development, regeneration, and adaptations. *Exp Cell Res*. 2022;415(2):113134. doi:10.1016/j.yexcr.2022.113134

300. Zhang H, Shang R, Kim K, et al. Evolution of a chordate-specific mechanism for myoblast fusion. *Sci Adv*. 2022;8(35):1-15. doi:10.1126/sciadv.add2696

301. Vasiliauskaité-Brooks I, Sounier R, Rochaix P, et al. Structural insights into adiponectin receptors suggest ceramidase activity. *Nature*. 2017;544(7648):120-123. doi:10.1038/nature21714

302. Golani G, Leikina E, Melikov K, et al. Myomerger promotes fusion pore by elastic coupling between proximal membrane leaflets and hemifusion diaphragm. *Nat Commun*.

2021;12(1):1-18. doi:10.1038/s41467-020-20804-x

303. Gamage DG, Melikov K, Munoz-Tello P, et al. Phosphatidylserine orchestrates Myomerger membrane insertions to drive myoblast fusion. *Proc Natl Acad Sci U S A*. 2022;119(38):1-12. doi:10.1073/pnas.2202490119

304. Bi P, Ramirez-Martinez A, Li H, et al. Control of muscle formation by the fusogenic micropeptide myomixer. *Science (80-)*. 2017;356(6335):323-327. doi:10.1126/science.aam9361.Control

305. Südhof TC, Rothman JE. Membrane fusion: Grappling with SNARE and SM proteins. *Science (80-)*. 2009;323(5913):474-477. doi:10.1126/science.1161748

306. Millay DP, Gamage DG, Quinn ME, et al. Structure–function analysis of myomaker domains required for myoblast fusion. *Proc Natl Acad Sci*. 2016;113(8):2116-2121. doi:10.1073/PNAS.1600101113

307. Zhang Q, Vashisht AA, O'Rourke J, et al. The microprotein Minion controls cell fusion and muscle formation. *Nat Commun*. 2017;8. doi:10.1038/ncomms15664

308. Petrany MJ, Song T, Sadayappan S, Millay DP. Myocyte-derived Myomaker expression is required for regenerative fusion but exacerbates membrane instability in dystrophic myofibers. *JCI Insight*. 2020;5(9). doi:10.1172/jci.insight.136095

309. Zhang W, Xu Y, Zhang L, et al. Synergistic effects of TGFβ2, WNT9a, and FGFR4 signals attenuate satellite cell differentiation during skeletal muscle development. *Aging Cell*. 2018;17(4):1-13. doi:10.1111/acel.12788

310. Cornelison DDW. *"Known Unknowns": Current Questions in Muscle Satellite Cell Biology*. Vol 126. 1st ed. Elsevier Inc.; 2018. doi:10.1016/bs.ctdb.2017.08.006

311. McKellar DW, Walter LD, Song LT, et al. Large-scale integration of single-cell transcriptomic data captures transitional progenitor states in mouse skeletal muscle regeneration. *Commun Biol*. 2021;4(1):1-12. doi:10.1038/s42003-021-02810-x

312. Millay DP, Sutherland LB, Bassel-Duby R, Olson EN. Myomaker is essential for muscle regeneration. *Genes Dev*. 2014;28(15):1641-1646. doi:10.1101/gad.247205.114

313. Goh Q, Millay DP. Requirement of myomaker-mediated stem cell fusion for skeletal muscle hypertrophy. *Elife*. 2017;6:1-19. doi:10.7554/eLife.20007

314. Hernández JM, Podbilewicz B. The hallmarks of cell-cell fusion. *Dev*. 2017;144(24):4481-4495. doi:10.1242/dev.155523

315. Iwata M, Englund DA, Wen Y, et al. A novel tetracycline-responsive transgenic mouse strain for skeletal muscle-specific gene expression. *Skelet Muscle*. 2018;8(1):1-8. doi:10.1186/s13395-018-0181-y

316. Van Zwieten RW, Puttini S, Lekka M, et al. Assessing dystrophies and other muscle diseases at the nanometer scale by atomic force microscopy. *Nanomedicine*. 2014;9(4):393-406. doi:10.2217/nnm.12.215

317. Puttini S, Lekka M, Dorchies OM, et al. Gene-mediated restoration of normal myofiber elasticity in dystrophic muscles. *Mol Ther*. 2009;17(1):19-25. doi:10.1038/mt.2008.239

318. Muraine L, Bensalah M, Dhiab J, et al. Transduction Efficiency of Adeno-Associated Virus

Serotypes after Local Injection in Mouse and Human Skeletal Muscle. *Hum Gene Ther*. 2020;31(3-4):233-240. doi:10.1089/hum.2019.173

319. Collinsworth AM, Zhang S, Kraus WE, Truskey GA. Apparent elastic modulus and hysteresis of skeletal muscle cells throughout differentiation. *Am J Physiol - Cell Physiol*. 2002;283(4 52-4):1219-1227. doi:10.1152/ajpcell.00502.2001

320. Chakraborty S, Doktorova M, Molugu TR, et al. How cholesterol stiffens unsaturated lipid membranes. *Proc Natl Acad Sci U S A*. 2020;117(36):21896-21905. doi:10.1073/pnas.2004807117

321. Lacraz G, Rouleau AJ, Couture V, et al. Increased stiffness in aged skeletal muscle impairs muscle progenitor cell proliferative activity. *PLoS One*. 2015;10(8):1-13. doi:10.1371/journal.pone.0136217

322. Trensz F, Lucien F, Couture V, et al. Increased microenvironment stiffness in damaged myofibers promotes myogenic progenitor cell proliferation. *Skelet Muscle*. 2015;5(1):1-16. doi:10.1186/s13395-015-0030-1

323. Kammoun M, Ternifi R, Dupres V, et al. Development of a novel multiphysical approach for the characterization of mechanical properties of musculotendinous tissues. *Sci Rep*. 2019;9(1):1-12. doi:10.1038/s41598-019-44053-1

324. Harris AR, Charras GT. Experimental validation of atomic force microscopy-based cell elasticity measurements. *Nanotechnology*. 2011;22(34):1-10. doi:10.1088/0957-4484/22/34/345102

325. Kim SO, Kim J, Okajima T, Cho NJ. Mechanical properties of paraformaldehyde-treated

individual cells investigated by atomic force microscopy and scanning ion conductance microscopy. *Nano Converg*. 2017;4(1):1-8. doi:10.1186/s40580-017-0099-9

326. Iyer PS, Mavoungou LO, Ronzoni F, et al. Autologous Cell Therapy Approach for Duchenne Muscular Dystrophy using PiggyBac Transposons and Mesoangioblasts. *Mol Ther*. 2018;26(4):1093-1108. doi:10.1016/j.ymthe.2018.01.021

327. Pasternak C, Wong S, Elson EL. Mechanical function of dystrophin in muscle cells. *J Cell Biol*. 1995;128(3):355-361. doi:10.1083/jcb.128.3.355

328. Ervasti JM, Campbell KP. A role for the dystrophin-glycoprotein complex as a transmembrane linker between laminin and actin. *J Cell Biol*. 1993;122(4):809-823. doi:10.1083/jcb.122.4.809

329. Blau HM, Webster C, Pavlath GK. Defective myoblasts identified in Duchenne muscular dystrophy. *Proc Natl Acad Sci U S A*. 1983;80(15 I):4856-4860. doi:10.1073/pnas.80.15.4856

330. Heslop L, Morgan JE, Partridge TA. Evidence for a myogenic stem cell that is exhausted in dystrophic muscle. *J Cell Sci*. 2000;113(12):2299-2308. doi:10.1242/jcs.113.12.2299

331. Sacco A, Mourkioti F, Tran R, et al. Short Telomeres and Stem Cell Exhaustion Model Duchenne Muscular Dystrophy in mdx / mTR Mice. *Cell*. 2010;143(7):1059-1071. doi:10.1016/j.cell.2010.11.039

332. Kottlors M, Kirschner J. Elevated satellite cell number in Duchenne muscular dystrophy. *Cell Tissue Res*. 2010;340(3):541-548. doi:10.1007/s00441-010-0976-6

333. Boldrin L, Zammit PS, Morgan JE. Satellite cells from dystrophic muscle retain regenerative capacity. *Stem Cell Res*. 2015;14(1):20-29. doi:10.1016/j.scr.2014.10.007

334. Dumont NA, Wang YX, Von Maltzahn J, et al. Dystrophin expression in muscle stem cells regulates their polarity and asymmetric division. *Nat Med*. 2015;21(12):1455-1463. doi:10.1038/nm.3990

335. Jr AFR, Souza LS, Almeida CF, et al. Muscle satellite cells and impaired late stage regeneration in different murine models for muscular dystrophies. *Sci Rep*. 2019;9(11842):1-11. doi:10.1038/s41598-019-48156-7

336. Cros D, Harnden P, Pellissier JF, Serratrice G. Muscle hypertrophy in Duchenne muscular dystrophy - A pathological and morphometric study. *J Neurol*. 1989;236(1):43-47. doi:10.1007/BF00314217

337. Massalska D, Zimowski JG, Bijok J, et al. Prenatal diagnosis of congenital myopathies and muscular dystrophies. *Clin Genet*. 2016;90(3):199-210. doi:10.1111/cge.12801

338. Granata AL, Vecchi C, Graciotti L, Fulgenzi G, Maggi S, Corsi A. Gamma irradiation can reduce muscle damage in mdx dystrophic mice. *Acta Neuropathol*. 1998;96(6):564-568. doi:10.1007/s004010050936

339. Rossi G, Bonfanti C, Antonini S, et al. Silencing Nfix rescues muscular dystrophy by delaying muscle regeneration. *Nat Commun*. 2017;8(1):1-12. doi:10.1038/s41467-017-01098-y

340. Boyer JG, Huo J, Han S, et al. Depletion of skeletal muscle satellite cells attenuates pathology in muscular dystrophy. *Nat Commun*. 2022;13(1). doi:10.1038/s41467-022-

30619-7

341. Gamage DG, Leikina E, Quinn ME, Ratinov A, Chernomordik L V., Millay DP. Insights into the localization and function of myomaker during myoblast fusion. *J Biol Chem*. 2017;292(42):17272-17289. doi:10.1074/jbc.M117.811372

342. Schuler M, Ali F, Metzger E, Chambon P, Metzger D. Temporally Controlled Targeted Somatic Mutagenesis in Skeletal Muscles of the Mouse. *Genesis*. 2005;41(4):165-170. doi:10.1002/gene.20107

343. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. *Methods*. 2001;25(4):402-408. doi:10.1006/meth.2001.1262

344. Bilodeau GG. Regular pyramid punch problem. *J Appl Mech*. 1992;59(3):519-523. doi:10.1115/1.2893754

345. Mebarek S, Komati H, Naro F, et al. Inhibition of de novo ceramide synthesis upregulates phospholipase D and enhances myogenic differentation. *J Cell Sci*. 2007;120(3):407-416. doi:10.1242/jcs.03331

346. Bernacchioni C, Cencetti F, Kwon MJ, et al. The sphingosine kinase activator K6PC-5 stimulates C2C12 myoblast differentiation. *Int J Immunopathol Pharmacol*. 2011;24(1):55-62. doi:10.1177/039463201102400107

347. Alonso A, Goñi FM. The physical properties of ceramides in membranes. *Annu Rev Biophys*. 2018;47:633-654. doi:10.1146/annurev-biophys-070317-033309

348. Zhang SZ, Xie HQ, Xu Y, et al. Regulation of cell proliferation by fast myosin light chain 1 in myoblasts derived from extraocular muscle, diaphragm and gastrocnemius. *Exp Biol Med*. 2008;233(11):1374-1384. doi:10.3181/0804-RM-134

349. Kaminski HJ, Al-Hakim M, Leigh RJ, Bashar MK, Ruff RL. Extraocular muscles are spared in advanced Duchenne dystrophy. *Ann Neurol*. 1992;32(4):586-588. doi:10.1002/ana.410320418

350. Marques MJ, Ferretti R, Vomero VU, Minatel E, Neto HS. Intrinsic laryngeal muscles are spared from myonecrosis in the mdx mouse model of Duchenne muscular dystrophy. *Muscle and Nerve*. 2007;35(3):349-353. doi:10.1002/mus.20697

351. Shinonaga C, Fukuda M, Suzuki Y, et al. Evaluation of swallowing function in Duchenne muscular dystrophy. *Dev Med Child Neurol*. 2008;50(6):478-480. doi:10.1111/j.1469-8749.2008.00478.x

352. Couly GF, Coltey PM, Le Douarin NM. The developmental fate of the cephalic mesoderm in quail-chick chimeras. *Development*. 1992;114(1):1-15. doi:10.1242/dev.114.1.1

353. Noden DM, Francis-West P. The differentiation and morphogenesis of craniofacial muscles. *Dev Dyn*. 2006;235(5):1194-1218. doi:10.1002/dvdy.20697

354. Noden DM, Marcucio R, Borycki AG, Emerson CP. Differentiation of avian craniofacial muscles: I. Patterns of early regulatory gene expression and myosin heavy chain synthesis. *Dev Dyn*. 1999;216(2):96-112. doi:10.1002/(SICI)1097-0177(199910)216:2<96::AID-DVDY2>3.0.CO;2-6

355. McDonald AA, Kunz MD, McLoon LK. Dystrophic changes in extraocular muscles after

gamma irradiation in mdx:utrophin+/- mice. *PLoS One*. 2014;9(1). doi:10.1371/journal.pone.0086424

356. Lewis C, Ohlendieck K. Proteomic profiling of naturally protected extraocular muscles from the dystrophin-deficient mdx mouse. *Biochem Biophys Res Commun*. 2010;396(4):1024-1029. doi:10.1016/j.bbrc.2010.05.052

357. Gargan S, Dowling P, Zweyer M, et al. Mass spectrometric profiling of extraocular muscle and proteomic adaptations in the mdx-4cv model of Duchenne muscular dystrophy. *Life*. 2021;11(7). doi:10.3390/life11070595

358. Taglietti V, Kefi K, Rivera L, et al. Thyroid-stimulating hormone receptor signaling restores skeletal muscle stem cell regeneration in rats with muscular dystrophy. *Sci Transl Med*. 2023;15(685):eadd5275. doi:10.1126/scitranslmed.add5275

359. Porter JD, Merriam AP, Khanna S, et al. Constitutive properties, not molecular adaptations, mediate extraocular muscle sparing in dystrophic mdx mice. *FASEB J*. 2003;17(8):893-895. doi:10.1096/fj.02-0810fje

360. Porter JD, Merriam AP, Hack AA, Andrade FH, McNally EM. Extraocular muscle is spared despite the absence of an intact sarcoglycan complex in γ- or δ-sarcoglycan-deficient mice. *Neuromuscul Disord*. 2001;11(2):197-207. doi:10.1016/S0960-8966(00)00171-1

361. Pertille A, De Carvalho CLT, Matsumura CY, Neto HS, Marques MJ. Calcium-binding proteins in skeletal muscles of the mdx mice: Potential role in the pathogenesis of Duchenne muscular dystrophy. *Int J Exp Pathol*. 2010;91(1):63-71. doi:10.1111/j.1365-2613.2009.00688.x

362. McCormack NM, Villalón E, Viollet C, et al. Survival motor neuron deficiency slows myoblast fusion through reduced myomaker and myomixer expression. *J Cachexia Sarcopenia Muscle*. 2021:1-19. doi:10.1002/jcsm.12740

363. Amenta AR, Yilmaz A, Bogdanovich S, et al. Biglycan recruits utrophin to the sarcolemma and counters dystrophic pathology in mdx mice. *Proc Natl Acad Sci U S A*. 2011;108(2):762-767. doi:10.1073/pnas.1013067108

364. Banks GB, Combs AC, Odom GL, Bloch RJ, Chamberlain JS. Muscle Structure Influences Utrophin Expression in mdx Mice. *PLoS Genet*. 2014;10(6). doi:10.1371/journal.pgen.1004431

365. Deconinck AE, Rafael JA, Skinner JA, et al. Utrophin-dystrophin-deficient mice as a model for Duchenne muscular dystrophy. *Cell*. 1997;90(4):717-727. doi:10.1016/S0092-8674(00)80532-2

366. Coley WD, Bogdanik L, Vila MC, et al. Effect of genetic background on the dystrophic phenotype in mdx mice. *Hum Mol Genet*. 2016;25(1):130-145. doi:10.1093/hmg/ddv460

367. Webster C, Blau HM. Accelerated age-related decline in replicative life-span of Duchenne muscular dystrophy myoblasts: Implications for cell and gene therapy. *Somat Cell Mol Genet*. 1990;16(6):557-565. doi:10.1007/BF01233096

368. Decary S, Ben Hamida C, Mouly V, Barbet JP, Hentati F, Butler-Browne GS. Shorter telomeres in dystrophic muscle consistent with extensive regeneration in young children. *Neuromuscul Disord*. 2000;10(2):113-120. doi:10.1016/S0960-8966(99)00093-0

369. Morgan JE, Zammit PS. Direct effects of the pathogenic mutation on satellite cell function

in muscular dystrophy. *Exp Cell Res*. 2010;316(18):3100-3108. doi:10.1016/j.yexcr.2010.05.014

370. Chen J, Zhou R, Feng Y, Cheng L. Molecular mechanisms of exercise contributing to tissue regeneration. *Signal Transduct Target Ther*. 2022;7(1). doi:10.1038/s41392-022-01233-2

371. McGee SL, Hargreaves M. Exercise adaptations: molecular mechanisms and potential targets for therapeutic benefit. *Nat Rev Endocrinol*. 2020;16(9):495-505. doi:10.1038/s41574-020-0377-1

372. Zelikovich AS, Quattrocelli M, Salamone IM, Kuntz NL, McNally EM. Moderate exercise improves function and increases adiponectin in the mdx mouse model of muscular dystrophy. *Sci Rep*. 2019;9(1):1-10. doi:10.1038/s41598-019-42203-z

373. Kilroy EA, Ignacz AC, Brann KL, et al. Beneficial impacts of neuromuscular electrical stimulation on muscle structure and function in the zebrafish model of Duchenne muscular dystrophy. *Elife*. 2022;11:1-30. doi:10.7554/eLife.62760

374. Jansen M, Van Alfen N, Geurts ACH, De Groot IJM. Assisted bicycle training delays functional deterioration in boys with Duchenne muscular dystrophy: The randomized controlled trial “no use is disuse.” *Neurorehabil Neural Repair*. 2013;27(9):816-827. doi:10.1177/1545968313496326

375. Alemdaroğlu I, Karaduman A, Yilmaz ÖT, Topaloğlu H. Different types of upper extremity exercise training in Duchenne muscular dystrophy: Effects on functional performance, strength, endurance, and ambulation. *Muscle and Nerve*. 2015;51(5):697-705. doi:10.1002/mus.24451

376. Sveen ML, Jeppesen TD, Hauerslev S, Køber L, Krag TO, Vissing J. Endurance training improves fitness and strength in patients with Becker muscular dystrophy. *Brain*. 2008;131(11):2824-2831. doi:10.1093/brain/awn189

377. Pedrazzani PS, Araújo TOP, Sigoli E, et al. Twenty-one days of low-intensity eccentric training improve morphological characteristics and function of soleus muscles of mdx mice. *Sci Rep*. 2021;11(1):1-11. doi:10.1038/s41598-020-79168-3