

Thesis

Skeletal Muscle Growth and Maintenance depend on BMP Signaling

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Statutory declaration

I hereby declare that I wrote the present thesis with the topic “Skeletal Muscle Growth and Maintenance depend on BMP signaling” independently and used no other aids than those cited. For each citation, I have clearly identified the source of the passages that are taken word for word or paraphrased from other works. I also hereby declare that I have carried out my scientific work according to the principles of good scientific practice in accordance with the current „Richtlinien der Freien Universität Berlin“ [Guidelines of the Free University of Berlin] and “Charte du doctorat à l’UPMC” [Charter of PhD students at UPMC]. I, Elija Schirwis, received assistance in the writing of this thesis in respect of language, grammar, spelling and syntax, which was provided by Carmen Birchmeier and Helge Amthor.

Elija Schirwis

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Abstract

Growth factors of the TGF- β superfamily play a role in all stages of prenatal myogenesis and govern adult muscle maintenance. Bone morphogenetic proteins (BMPs) are members of the TGF- β subfamily and are key signals that regulate embryonic and fetal muscle development. This work investigates the role of BMP signaling in muscle stem cells of the postnatal muscle, the satellite cells. These cells are required for the growth of skeletal muscle at early postnatal age. Signaling molecules provide essential cues for satellite cells that regulate their activation, proliferation, self-renewal and differentiation. I showed that BMPs regulate satellite cell-dependent growth of postnatal fibers and the generation of the satellite cell pool. After inhibition of BMP signaling, I observed that myogenic precursor cells become quiescent and fail to progress towards differentiation, whereas treatment with BMP4 on its own is sufficient to reactivate the myogenic program.

BMP signaling, however, does not only affect satellite cell-dependent growth of muscle fibers, but also affects the size of the muscle in a satellite cell-independent manner. I found that BMPs provide a hypertrophic signal and protect from denervation-induced muscle atrophy. Under such condition, BMP signaling inhibits the expression of the E3 ubiquitin ligase Fbxo30. I further analyzed the interaction between myostatin and BMP signaling. Myostatin is another member of the TGF- β superfamily, but myostatin and BMPs bind to different receptors for signaling. Myostatin mutant mice display very large muscles, and work done by others had implicated myostatin in regulating adult muscle mass in a satellite cell-independent manner. I observed that muscle function of myostatin deficient mice depends on gender and age. Importantly, large muscles in absence of myostatin entirely depend on the presence of BMP signaling. Denervation of muscle in myostatin mutant mice caused a strong muscle atrophy, which was aggravated by the inhibition of BMP signaling. Therefore, the BMP pathway is a fundamental hypertrophic signal in adult muscle and is dominant over myostatin signaling.

In conclusion, I found that BMP signaling controls satellite cell-dependent growth of postnatal muscle and satellite cell-independent growth and homeostasis of adult skeletal muscle.

Résumé

Les facteurs de croissance de la superfamille TGF- β jouent un rôle dans toutes les étapes de la myogenèse prénatale et régissent l'entretien des tissus musculaires adultes. Les protéines morphogénétiques osseuses (bone morphogenetic proteins - BMPs) sont membres de la sous-famille des TGF- β et sont à l'origine de signaux clés régulant le développement musculaire embryonnaire et foetal. Cette thèse étudie le rôle de la signalisation BMP dans les cellules souches musculaires, dénommées cellules satellites, du muscle postnatal. Ces cellules sont nécessaires pour la croissance postnatale du muscle squelettique. Les molécules de signalisation fournissent des repères essentiels pour les cellules satellites en régulant leur activation, prolifération, auto-renouvellement et différenciation. J'ai montré qu'après la naissance les BMPs régulent la croissance des fibres musculaires dépendante des cellules satellites et la génération des nouvelles cellules satellites. Suite à l'inhibition de la signalisation BMP, j'ai observé que les précurseurs myogéniques deviennent quiescents et sont contraints de progresser vers la différenciation, tandis que le traitement avec BMP4 suffit pour réactiver leur programme myogénique.

La signalisation BMP n'affecte pas seulement la croissance des fibres musculaires dépendante des cellules satellites, mais aussi la taille du muscle de façon indépendante des cellules satellites. J'ai observé que les BMPs fournissent un signal hypertrophique et protègent de l'atrophie musculaire suite à une dénervation. Dans les conditions précédentes, la signalisation BMP inhibe l'expression de l'ubiquitine ligase E3, Fbxo30. J'ai également analysé l'interaction entre la myostatine et la signalisation BMP. La myostatine est un autre membre de la superfamille des TGF- β , mais elle se lie à des récepteurs différents de ceux des BMPs. Les souris déficientes en myostatine ont des muscles hypertrophiés. Les travaux effectués par d'autres groupes ont mis en évidence le rôle de la myostatine dans la régulation de la masse musculaire chez adulte de manière indépendante des cellules satellites. J'ai observé que la fonction musculaire des souris déficientes en myostatine varie selon l'âge et le sexe. De manière intéressante, en l'absence de myostatine, l'hypertrophie musculaire dépend entièrement de la signalisation BMP. La dénervation musculaire chez les souris déficientes en myostatine provoque une forte atrophie musculaire, aggravée par l'inhibition de la signalisation BMP. Par conséquent, la voie BMP est un

signal hypertrophique essentiel dans le muscle adulte qui prédomine sur la signalisation de la myostatine.

En conclusion, j'ai démontré que la signalisation BMP contrôle la croissance musculaire postnatale dépendante des cellules satellites puis régule la croissance et l'homéostasie des muscles squelettiques de manière indépendante des cellules satellites chez l'adulte.

Zusammenfassung

Wachstumsfaktoren der TGF- β -Superfamilie sind an allen Phasen der pränatalen Myogenese beteiligt und sind zur Erhaltung der Muskelmasse im adulten Alter erforderlich. Morphogenetische Proteine des Knochens (bone morphogenetic proteins - BMPs) sind als Mitglieder der TGF- β -Subfamilie wichtige Signale, die die embryonale und fötale Entwicklung der Muskulatur regulieren. Die vorliegende Arbeit untersucht die Rolle der BMP-Signalkaskade in den Stammzellen des postnatalen Muskels, den sogenannten Satellitenzellen. Diese Zellen sind für das Wachstum der Skelettmuskulatur im frühen postnatalen Alter verantwortlich. Ihre Aktivierung, Proliferation, Selbsterneuerung und Differenzierung wird durch Signalmoleküle gesteuert. Ich konnte zeigen, dass die BMPs sowohl Satellitenzell-abhängiges Wachstum in postnatalen Fasern als auch die Generierung von neuen Satellitenzellen regulieren. Ich beobachtete, dass die Vorläuferzellen der Myogenese nach der Blockade des BMP-Signalweges in den Ruhezustand versetzt wurden und den Prozess der Differenzierung nicht einleiten konnten. Allerdings genügte die Zugabe von BMP4, um das Myogenese-Programm zu reaktivieren.

Der BMP-Signalweg wirkt nicht nur ausschliesslich auf Satellitenzell-abhängiges Wachstum der Muskelfasern, sondern beeinflusst die Muskelgröße auch durch einen Satellitenzell-unabhängigen Mechanismus. Ich stellte fest, dass BMPs hypertroph wirkten und vor Muskelatrophie schützten, die durch Denervierung induziert wurde. Unter solchen Bedingungen hemmen BMP-Signale die Expression der E3-Ubiquitin-Ligase Fbxo30. Im weiteren analysierte ich die Interaktion zwischen den-Signalkaskaden von Myostatin und BMP. Myostatin ist ebenfalls ein Mitglied der TGF- β -Superfamilie, das jedoch andere Rezeptoren bindet als BMPs. Mäuse mit Myostatinmutation haben sehr große Muskeln, und die Arbeit von anderen Gruppen zeigten, dass Myostatin bei der Regulierung der Muskelmasse im erwachsenen Alter in einer Satellitenzell-unabhängigen Weise agiert. Ich konnte zeigen, dass die Muskelfunktion bei Myostatin-defizienten Mäusen von Geschlecht und Alter abhängt. In Abwesenheit von Myostatin wird die Muskelhypertrophie komplett durch den BMP-Signalkaskade getragen. Denervierung von Muskeln bei Myostatin-defizienten Mäusen führte zu einer starken Muskelatrophie, die durch Hemmung der BMP-Signalkaskade verstärkt wurde. Der BMP-Signalweg ist daher ein essentielles

hypertrophes Signal im erwachsenen Muskel und dominiert die Myostatin-Signalkaskade.

Zusammenfassend konnte ich zeigen, dass der BMP-Signalweg ein Satellitenzell-abhängiges und -unabhängiges Muskelwachstum steuert, als auch die Erhaltung der Skelettmuskulatur im Adulten kontrolliert.

List of abbreviations

AAV	adeno-associated virus
ADP	adenosine diphosphate
Alk	activin-like kinase
ATP	adenosine triphosphate
BMP	bone morphogenetic protein
Cdc42	cell division control protein 42
CD34	cluster of differentiation protein 34
DAPI	4',6-diamidin-2-phenylindol
DMD	Duchenne muscular dystrophy
E	day in embryonic development
EDL	<i>extensor digitorum longus</i> muscle
Fast-1	forkhead activin signal transducer 1
Fbxo30	F-box protein 30
FoxO	forkhead box proteins of the class O
GDF	growth and differentiation factor
i.e.	for example (latin : <i>it est</i>)
IGF	insulin-like growth factor
IL-6	interleukin-6
LAP	latency associated peptide
MAFbx	muscle atrophy F-box protein
MAPK	mitogen-activated protein kinase
Mdx	muscular dystrophy X-chromosome linked
MHC	myosin heavy chain
mTOR	mammalian target of rapamycin
Mstn	myostatin
Murf1	muscle RING-finger protein 1
MUSA1	muscle ubiquitin-ligase of SCF complex in atrophy-1
Myf5	myogenic factor 5
MyoD	myoblast determination protein
NF-kB	nuclear factor kappa-light-chain-enhancer of activated B cells

Pax	paired box transcription factor
PBS	phosphate buffered saline
Ras	rat sarcoma protein
RBP-J	recombination signal binding protein for immunoglobulin kappa J region
sActR2B-Fc	soluble activin receptor 2 B
SCs	satellite cells
SCF	Skp1/Cul1/F-box complex of E3 ubiquitin ligases
s.e.m.	standard error of mean
siRNA	small interfering ribonucleic acid
SLC	small latent complex
Smad	homolog of proteins “small body size” (SMA) and “mothers against decapentaplegic” (MAD)
TA	<i>tibialis anterior</i> muscle
TB	<i>triceps brachii</i> muscle
TGF-β	transforming growth factor beta
Wnt	Wingless-related integration site

1. Introduction

1.1. Muscle tissue

1.1.1. Anatomy

Skeletal muscle is essential to provide locomotor activity for the organism. Myofibers, the basic cellular units of muscle capable of contraction, are formed during embryonic and fetal development and in some species even in postnatal life (Condon et al., 1990; Harris et al., 1989; Ontell et al., 1988; Ross et al., 1987; Van Swearingen and Lance-Jones, 1995; Walro and Kucera, 1999; Wigmore and Dunglison, 1998). In adults, they are regenerated following muscle damage (Charge and Rudnicki, 2004). Myofibers are syncytia, i.e. multinuclear cells that are postmitotic. Bundles of around 150 myofibers form fasciculi and are covered by the perimysium (Figure 1). Several fasciculi together form a single skeletal muscle, which is covered by the epimysium and attached to the bone by tendons, which are formed by collagen-rich connective tissue (Tedesco et al., 2010). The arrangement of muscle fibers between tendons allows force transduction from the muscle to the bone (Lieber and Friden, 2000).

Besides myofibers, skeletal muscle contains also other cellular components that support and regulate muscle function. Among these is the vascular system containing endothelial cells, pericytes and blood cells (Bentzinger et al., 2012). The vascular system ensures a supply of nutrients, oxygen and removal of metabolites and carbon dioxide. Cells of the immune system help to clear apoptotic cells after muscle damage. Nerves innervate the muscle, most importantly motoneurons that form the neuromuscular junction essential for muscle contractility. In addition, skeletal muscle contains fibroblasts that provide extracellular matrix and a stem cell population that contributes to muscle regeneration (Kuang and Rudnicki, 2008; Page et al., 2011).

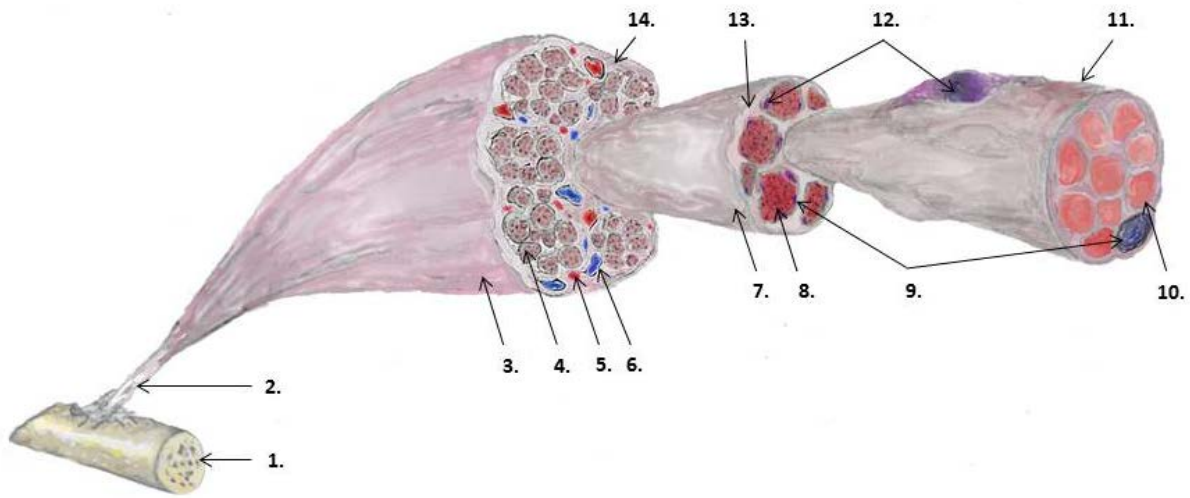


Figure 1: Scheme illustrating components of a muscle: **1.** Bone; **2.** Tendon; **3.** Muscle belly covered by epimysium; **4.** Fasciculus (cross section view); **5.** Arteriole; **6.** Venule; **7.** Fasciculus; **8.** Myofiber; **9.** Myonuclei; **10.** Myofibril; **11.** Endomysium; **12.** Muscle precursor cells; **13.** Perimysium; **14.** Epimysium. Modified according to (Baechle et al., 2008)

1.1.2. Contractile system

Contraction of skeletal muscle can cause shortening (concentric contraction), lengthening (eccentric contraction), or no change (isometric contraction) of muscle length. Concentric contractions initiate movements, and eccentric contractions slow them. During eccentric contraction, the opposing force exceeds the force generated by the muscle, leading to its elongation (Tabebordbar et al., 2013). Sarcomeres are the structural units of the myofiber that are responsible for contractions. A sarcomere consists of actin filaments that attach to the Z-disk by their plus-ends, and that bind filaments formed by myosin-II motor molecules (Figure 2). The borders of the sarcomeres are formed by the Z-disk, whereas the central structural element is the M-line.

Contraction of muscle fibers is controlled by an electrical impulse, the so-called action potential that is provided by motoneurons. The action potential triggers calcium release from an intracellular muscle organelle, the sarcoplasmic reticulum. Calcium and ATP regulate the binding of myosin heads to the actin filament. The myosin

filaments slide along the actin filaments towards the plus ends, and myosin filaments form and break cross-bridges to the actin filaments break in an ATP-dependent manner. This allows the relative movement of these two filament types. The movement of the thin myofilaments (actin) and thick myofilaments (myosin) filaments relative to one another produces a force according to the sliding filament theory (Smith, 1990). In addition to the active contraction system, there are passive elastic elements affecting the dynamics of a sarcomere. Sarcomere elasticity results from elements like the Z-disc and from titin, a molecule that extends through the whole length of a sarcomere and prevents it from falling apart upon stretching (Günther and Kruse, 2007). Myofilaments of skeletal muscle, when visualized by light microscopy, have a striated appearance, i.e. light isotropic (I-band) and dark anisotropic bands (A-band) that correspond to the zone of actin filaments and the zone of overlapping actin and myosin filaments, respectively.

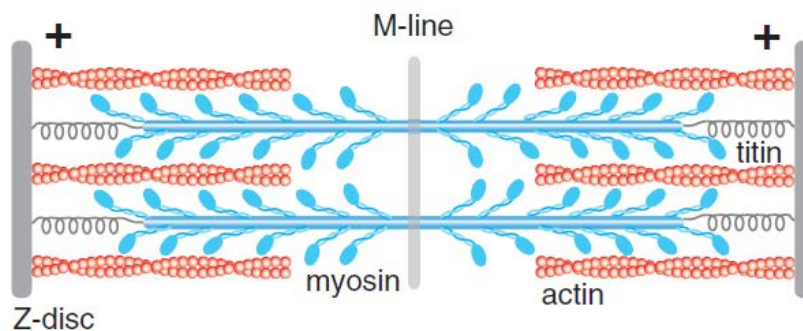


Figure 2: Scheme illustrating muscle sarcomere. Attached with their plus ends to the Z-discs, actin filaments are depicted in red. Bipolar myosin-II filaments are localized in-between are. The central structural element is the M-line. The titin molecules are represented as springs that go through the hollow myosin filaments. Adapted from (Günther and Kruse, 2007).

Two main molecules provide energy for muscle contraction, ATP and creatine phosphate (Hill, 1924; Lipmann, 2006). Creatine phosphate serves as a high-energy phosphate reservoir for the rapid regeneration of ATP by creatine kinase. It represents a fast ATP regeneration process that possesses limited capacity. The metabolic processes that result in generation of ATP and/or creatine phosphate are (i) glycolysis and (ii) mitochondrial oxidative phosphorylation. (i) Glycolysis degrades glucose to pyruvate or lactate and represents a fast source of ATP. (ii) Mitochondrial oxidation utilizes acetyl-CoA as a substrate which is generated from pyruvate and

fatty acids. This process is slower than glycolysis but has a larger capacity for ATP regeneration (Schiaffino and Reggiani, 2011).

1.1.3 Fiber type composition

Based on myosin composition and the levels of oxidative enzymes, fibers are classified as slow and fast (Barnard et al., 1971; Pette and Staron, 2000). In slow muscle, ATP generation depends on the slow oxidative mitochondrial process, whereas fast muscles rely on rapid glycolysis. The common method to identify muscle fiber types is based on specific isoforms of myosin heavy chain expressed in the different fiber types. The following fiber types exist: the slow type that expresses myosin heavy chain type 1 (MHCI), and three fast types, namely fibers that express myosin heavy chain type 2A, 2X or 2B (MHCIIa, MHCIIx and MHCIIb respectively) (Figure 3) (Schiaffino and Reggiani, 2011). The various fiber types possess distinct contractile velocity and use various amounts of energy for contraction. Type 1 fibers are slow and their energy cost is low, Type 2A and 2X fibers are intermediary, and Type 2B fibers are very fast and use high amounts of energy for contraction (Bottinelli et al., 1994). The identity of a myofiber is not static and can alter under various conditions, e.g. aging. Thus, fiber type transitions are reversible and conversion from Type 1 \leftrightarrow Type 2A \leftrightarrow Type 2X \leftrightarrow Type 2B is possible (Ciciliot et al., 2013).

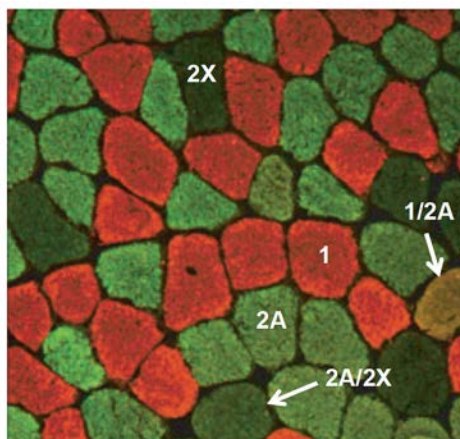


Figure 3: Fibre types in mouse skeletal muscle. Section of mouse soleus muscle stained with an antibody to MYH-slow, visualized in red, and an antibody to MYH-2A, visualized in green. In addition to type 1 and 2A fibres, note the presence of unstained fibres, corresponding to the minor proportion of type 2X fibres present in mouse soleus, and of hybrid 1-2A and 2A-2X fibres. Adapted from (Schiaffino, 2010).

1.2. Formation of skeletal muscle

Skeletal muscles develop from paraxial mesoderm during embryogenesis (Tajbakhsh, 2003; Tajbakhsh and Buckingham, 2000). Through a process of segmentation and condensation, the paraxial mesoderm generates epithelial ball-like structures, the somites, which are located on either side of the neural tube (Figure 4) (Pourquie, 2001). As the response to the signals derived from the neural tube, notochord and dorsal ectoderm, the somites differentiate into dermomyotome or sclerotome (Parker et al., 2003). The dermomyotome gives mainly rise to the skeletal muscle of the trunk and limbs, whereas the sclerotome develops into the cartilage and bone of the vertebrae (Cossu et al., 1996; Dockter and Ordahl, 1998; Gros et al., 2005; Pirskanen et al., 2000). The ventrolateral dermomyotome delaminates a population of muscle progenitors that migrate to the developing limb musculature (Birchmeier and Brohmann, 2000).

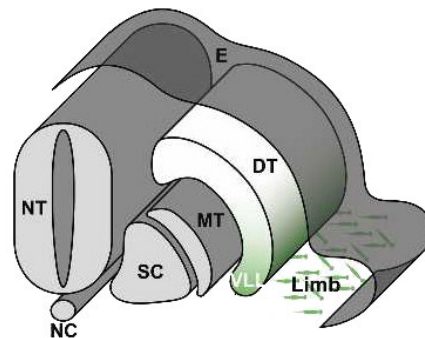


Figure 4: Schematic outlining the development of the somite from the paraxial mesoderm. Paired somites develop on either side of the notochord in a rostral–caudal direction. In response to signals or cues from adjacent lineages (i.e., notochord, neural tube, surface ectoderm), segmentation of the somite occurs into the dermomyotome, myotome, and sclerotome that are coordinated by the regulation of gene expression. Dermomyotomal cells migrate in a dorsal fashion to give rise to the epaxial myotome and in a ventral fashion to give rise to the hypaxial myotome. (E) Surface ectoderm; (DT) dermomyotome; (MT) myotome; (SC) sclerotome; (NC) notochord; (NT) neurotube, (VLL) ventrolateral lip. Adapted from (Shi and Garry, 2006).

The embryonic origin of muscle precursor cells was studied extensively using chimeric organisms, i.e. cell engraftments in avian models (Armand et al., 1983; Brand-Saberi et al., 1996; Dockter and Ordahl, 1998; Huang et al., 1997; Le Lievre and Le Douarin, 1975). Such experiments demonstrated that all precursors of trunk and limb muscles derive from the dermomyotome, and that the dermomyotome

contributes in addition to endothelial cells and pericytes of the blood vessels (Gros et al., 2005; Kassam-Duchossoy et al., 2005; Relaix et al., 2005). Differentiation and proliferation of muscle progenitor cells are regulated by different signaling pathways. Dependent on the developmental stage and the position of the myogenic progenitor cell, they receive signals that regulate and coordinate the balance between proliferation, specification and differentiation (Sambasivan and Tajbakhsh, 2007).

The first differentiated skeletal muscle appears in the myotome. During the early fetal development (E10.5- E14.5 in mice), primary myotubes form by fusion of embryonic muscle precursors. Subsequently (E14.5-18.5), fetal muscle grows by formation of secondary myotubes with different functional qualities (Figure 5) (Biressi et al., 2007; Bonner and Hauschka, 1974; Cossu et al., 1988; Matsakas et al., 2010; Miller and Stockdale, 1986; Pin and Merrifield, 1993). Around E16, a basal membrane forms around myotubes. Muscle growth continues during the late fetal and postnatal development by fusion with muscle precursors, and the number of nuclei in fibers increases. After P21, the amount of nuclei in the fibers remains stable but the fiber size continues to increase due to continuous protein synthesis (White et al., 2010).

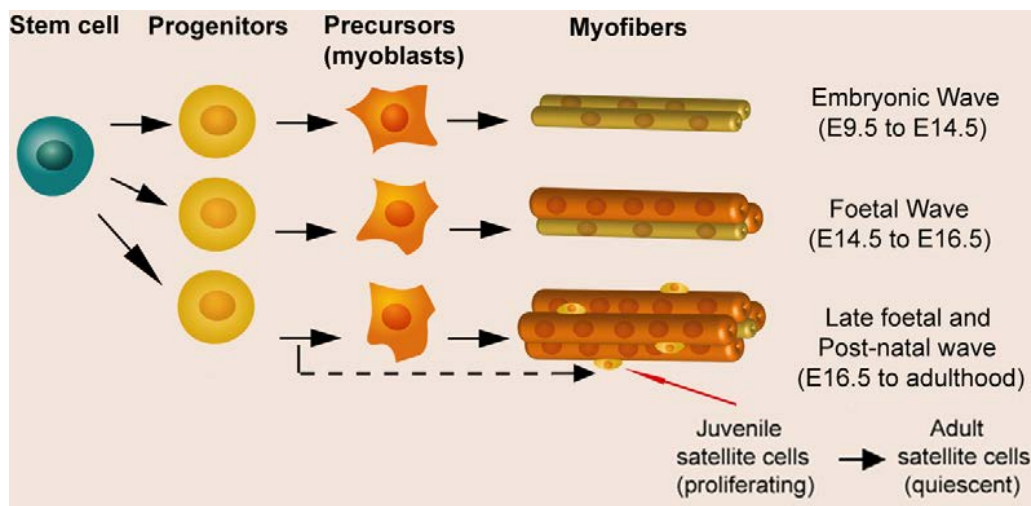


Figure 5: Illustration of lineage progression and the multiple waves of developmental myogenesis in the mouse embryo. Around E16.5, proliferating progenitor cells appear in a satellite cell position (see also chapter 1.3). A subset of these cells will become the future adult quiescent satellite cells. Adapted from (Sambasivan and Tajbakhsh, 2007)

1.3 Muscle stem cells

1.3.1. *Molecular signature of muscle stem cells*

During embryonic development, a population of muscle progenitor cells is established, which is maintained throughout embryogenesis. Muscle progenitors express Pax3 and/or Pax7, two related paired-box transcription factors that provide useful markers for the identification of these cells (Maroto et al., 1997; Relaix et al., 2004, 2005; Seale et al., 2000). Late in fetal development, these Pax3+/Pax7+ cells give rise to the pool of satellite cells, the stem cells of the adult muscle, which are characterized by their anatomical position, as cells are wedged between the myofiber and the basal lamina (Figure 1, 3) (Mauro, 1961). These cells actively proliferate in the early postnatal phase but at least a subpopulation becomes quiescent after P21 (Chakkalakal et al., 2012; Le Grand and Rudnicki, 2007).

Satellite cells express receptors on the cell surface, cytoskeletal proteins and transcription factors that can be used to identify them. The expression of these proteins depends on the physiological conditions and state of activation. In particular, quiescent and activated satellite cells express different sets of markers (Table 1). In addition to Pax3 and Pax7, quiescent and activated satellite cells express the cell adhesion protein M-cadherin, the tyrosine receptor kinase c-Met and CD34 (cluster of differentiation protein), a surface protein present in many stem cell types (Beauchamp et al., 2000; Cornelison and Wold, 1997). Furthermore, the proteoglycans syndecans-3 and -4 are expressed on the surface of quiescent and activated satellite cell. The myogenic regulatory factors Myf5 and MyoD are present in activated but not in quiescent satellite cells (Beauchamp et al., 2000; Cornelison and Wold, 1997). Many of these markers of satellite cells are of functional importance. In the absence of Pax3 and Pax7, myogenesis is arrested during vertebrate development (Relaix et al., 2005). (Gunther et al., 2013; Lepper et al., 2009). Various lines of evidence indicate that skeletal muscle regeneration depends on satellite cells (Gunther et al., 2013; Lepper et al., 2011; Sambasivan et al., 2011).

Table 1: Satellite cell markers. VCAM-1, vascular cell adhesion molecule-1; NCAM, neural cell adhesion molecule; MNF, myocyte nuclear factor. Adapted from (Charge and Rudnicki, 2004)

Molecular Markers	Satellite Cell Expression		Protocol
	Quiescent	Proliferating	
Cell surface			
M-cadherin	+/-	+	<i>in vitro/in vivo</i>
Syndecan-3	+	+	<i>in vitro/in vivo</i>
Syndecan-4	+	+	<i>in vitro/in vivo</i>
c-met	+	+	<i>in vitro/in vivo</i>
VCAM-1	+	+	<i>in vivo</i>
NCAM	+	+	<i>in vivo</i>
CD34	+/-	+	<i>in vitro</i>
Cytoskeletal			
Desmin	-	+	<i>in vitro/in vivo</i>
Transcription			
Pax3/7	+	+	<i>in vitro/in vivo</i>
Myf5	-	+	<i>in vitro/in vivo</i>
MyoD	-	+	<i>in vitro/in vivo</i>
MNF	+	+	<i>in vitro/in vivo</i>
Myostatin	+	+/-	<i>in vitro/in vivo</i>

1.3.4. Myogenic program of satellite cell

The stem cells of the muscle, the satellite cells, provide a cellular source for muscle growth and regeneration. Satellite cells can self-renew and differentiate, i.e. give rise to myoblasts. (Zammit and Beauchamp, 2001). The self-renewal potential of satellite cells was first proven by transplantation of single myofibers into a regenerating mouse muscle (Darabi et al., 2011). When a damaged muscle is depleted of its resident satellite cells by irradiation, it cannot regenerate. However, the transplantation of a single myofiber with its associated satellite cells stimulates regeneration, and the satellite cells give rise to hundreds of new satellite cells and thousands of myonuclei (Collins et al., 2005).

Key transcription factors guide the myogenic program of satellite cells, which progress from quiescence to activation and proliferation towards differentiation or self-renewal. Thus, an individual satellite cell can form a syncytial contractile myofiber. In adult muscle satellite cells (Pax7⁺) are normally mitotically quiescent, do not express MyoD and myogenin (MyoG) and reside in a sublaminar niche (Figure 6).

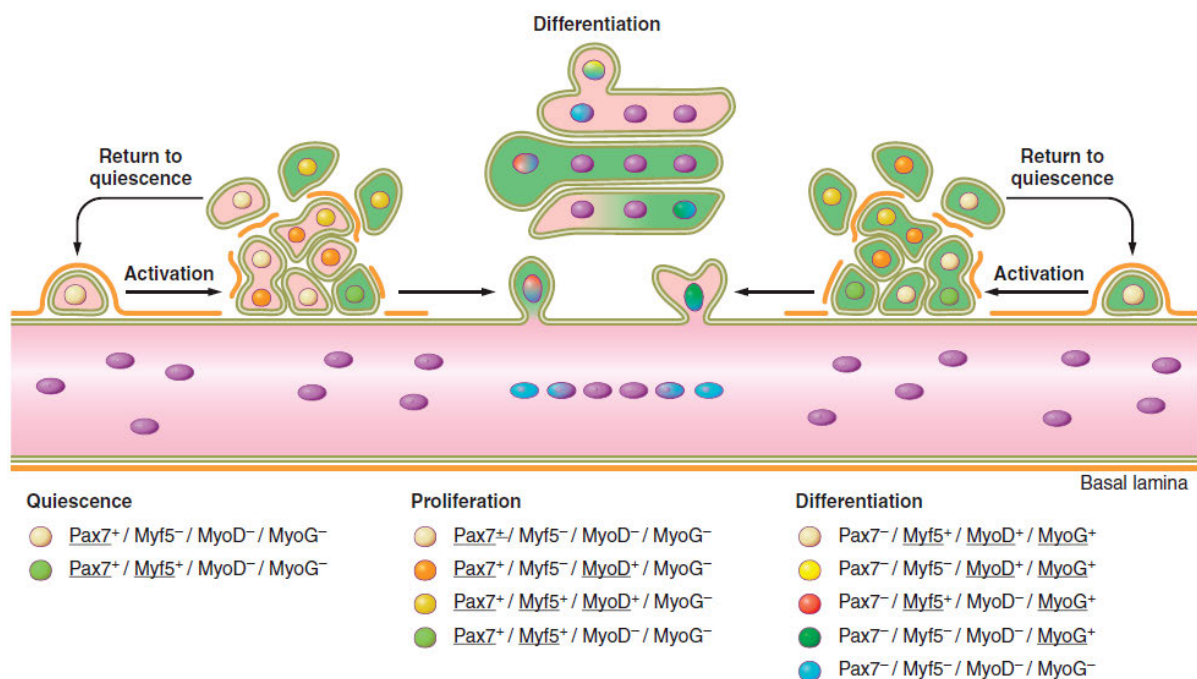


Figure 6: satellite cells express different myogenic markers during activation, proliferation, differentiation and fusion with myofiber. Adapted from (Yin et al., 2013).

After muscle damage, satellite cells exit from the quiescent state, become activated and start to proliferate. Activated satellite cells and their progeny, also referred to as adult myoblasts, express the myogenic transcription factors MyoD and Myf5 (Relaix et al., 2005; Zammit et al., 2004). A proportion of the adult myoblasts begins differentiation by downregulating Pax7 expression, whereas others return to quiescence by downregulating MyoD/Myf5 expression (Kuang et al., 2007; Zammit et al., 2004).

At the final step of differentiation process, cells start the expression of the myogenic transcription factor myogenin. Together, the myogenic transcription factors will activate muscle specific structural and contractile genes (Cornelison et al., 2000; Cornelison and Wold, 1997).

During proliferation, precursor cells undergo either symmetric or asymmetric division (Figure 7). The decision to undergo asymmetric division was analyzed in an assay that is called 'floating fiber assay'. In this assay, a fiber is incubated in medium containing serum, and the attached satellite cells become activated under these conditions. They then undergo symmetric or asymmetric division, which appear to be determined by the relative position of the daughter cells (Kuang et al., 2007). 90% of all divisions are symmetric and are characterized by a parallel orientation of the mitotic spindle and the muscle fiber axis. These symmetric divisions generate either two self-renewing cells that are Pax7⁺/Myf5⁻ or two committed cells that are Pax7⁺/Myf5⁺. The identical daughter cells have both contact to the basal lamina and the plasmalemma. When the mitotic spindle is oriented perpendicular to the fiber axis, asymmetric division occur that generate one self-renewing cell (Pax7⁺/Myf5⁻) that remains in contact with the basal lamina, and one committed cell (Pax7⁺/Myf5⁺) that is adjacent to the plasmalemma but has lost contact with the basal lamina. The molecule implicated in the process is Wnt7a, a member of the large family of secreted signaling molecules that act throughout the stabilization of beta-catenin in cytoplasm, which then translocates to the nucleus and activates transcription of the target genes (Dierick and Bejsovec, 1999). The Wnt7a binds to receptor Frizzled7 and stimulates symmetric satellite cell division, for instance from 30% to 67% in cultured myofibers, and thus allows the increase of the satellite cell pool (Le Grand et al., 2009).

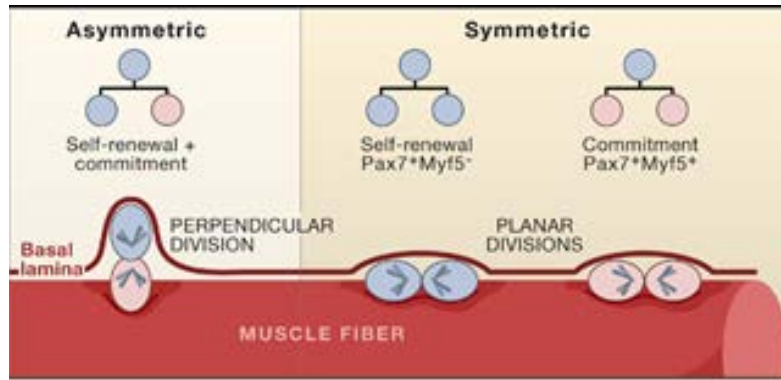


Figure 7: Symmetric and asymmetric division of muscle satellite cells. Adapted from (Cossu and Tajbakhsh, 2007)

1.3.5. The satellite cell niche

Satellite cells interact with each other and with their microenvironment, the so-called satellite cell niche (Ohlstein et al., 2004). These interactions regulate satellite cell quiescence, self-renewal, proliferation, and differentiation. At close range, known signaling pathways that regulate such interactions are Wnt, Notch and sphingolipids. In particular, non-canonical Wnt signals such as Wnt7a directly regulate MyoD expression and thus initiate differentiation (Kim et al., 2008). Notch signaling is required for satellite cell quiescence, maintains the undifferentiated state and controls colonization of the satellite cell niche (Brohl et al., 2012; Conboy and Rando, 2002; Yoshida et al., 2013). Sphingolipids have been described as signaling molecules regulating satellite cell proliferation and muscle regeneration (Nagata et al., 2006). Wnt7a, the Notch ligand Delta-like-1 and sphingolipid signaling molecules are provided by the myofiber and thus are present in the niche.

Various stimuli can trigger satellite cell activation, which are thought to be released by damaged myofibers, macrophages, and fibroblasts (Charge and Rudnicki, 2004). Hepatocyte growth factor is released by crushed muscle and stimulates activation and proliferation of adult satellite cells in culture (Tatsumi et al., 1998). Insulin like growth factor (IGF) that is present in the plasma, induces mitogenic and myogenic responses (Clemmons, 2009; Miura et al., 1992). Similarly, fibroblast growth factors such as FGF-2, which are secreted at low levels in many tissues, promote myoblast proliferation during muscle regeneration (Abraham et al., 1986; Cornelison et al., 2001). Matrix metalloproteinases are secreted by regenerating myofibers and are implicated in satellite cell activation, migration, and differentiation (Carmeli et al., 2004; Fukushima et al., 2007). Further, fibrocyte/adipocyte progenitors, immune cells, fibroblasts and vascular endothelial cells secrete IL-6, a factor regulating satellite cell-mediated muscle hypertrophy and regeneration (Serrano et al., 2008). The activity of the nervous system acts as an anti-apoptotic stimulus, and after denervation satellite cells lose their capability to enter mitosis when cultured (Kuschel et al., 1999). Finally, nitric oxide regulates migration of satellite cell and can reverse the age-related reduction of their migratory ability (Collins-Hooper et al., 2012).

1.4. Regulation of muscle mass growth

The postnatal growth of skeletal muscle fibers depends on their fusion with muscle precursors and on protein synthesis (Sartorelli and Fulco, 2004; White et al., 2010). Satellite cell-dependent mechanism that is involved in hyperplastic muscle growth, contributes to muscle mass increase during early postnatal development. Muscle hyperplasia is the increase in the size of a muscle mass due to an increase in the number of myofibers.

In adulthood, the muscle growth ceases and muscle stem cells become mostly quiescent. However, the regeneration of muscle tissue upon damage is dependent on satellite cells. The studies of Rosenblatt et al., based on irradiation of muscle stem cells, demonstrated that satellite cells are indispensable for muscle maintenance and regeneration in adults (Rosenblatt and Parry, 1992; Rosenblatt et al., 1994). In analogy, the study of Sambasivan et al. used the conditional ablation of satellite cells and came to similar conclusions (Sambasivan et al., 2011).

In adults, skeletal muscle can increase its size and elevates its functions in response to contractile activity and signaling factors. This process can be independent of satellite cells, i.e. is not accompanied by an increase in the number of myonuclei per fiber. This process is also called hypertrophy and accompanied by an increase in muscle protein. A few major signaling pathways are known to control protein synthesis and the size of the muscle:

IGF1–Akt–mTOR pathway acts as a positive regulator of muscle mass. Insulin-like growth factor 1 (IGF1) activates AKT. AKT signaling regulates protein synthesis by activating mTOR that stimulates phosphorylation of eukaryotic translation initiation factor 4E-binding protein 1 (EIF4BP1). This protein interacts with eukaryotic translation initiation factor 4E (eIF4E), which is a component of the multisubunit complex that recruits 40S ribosomal subunits during mRNA translation (Eguchi et al., 2006).

The family of mitogen-activated protein kinases (MAPK) is implicated in muscle growth and hypertrophy in response to exercise. These proteins are considered to couple cellular stress with an adaptive transcriptional response by activation of mitogen-activated protein kinase kinase (MAP2K) (Keren et al., 2006; Williamson et al., 2003). MAP2K phosphorylates the target protein MAPK that stimulates protein

synthesis by activation of eIF4E and regulates myogenic differentiation (Bennett and Tonks, 1997; Kramer and Goodyear, 2007; Lluís et al., 2005; Smith et al., 2013). The growth factors such as HGF and FGF2 signal also via MAPK pathway (Yablonka-Reuveni et al., 2008).

Calcium signaling also regulates muscle hypertrophy. In this pathway, a rise in intracellular Ca^{2+} leads to the activation of calcineurin. Calcineurin, a serine/threonine phosphatase, dephosphorylates the transcription of nuclear factors of activated T cells (NFAT). This results in the translocation of these factors from the cytoplasm to the nucleus and subsequent activation of target genes. One of the target genes is peroxisome proliferator-activated receptor gamma coactivator-1 α (PGC-1 α) that inhibits the atrogen-1 transcription, a protein that controls muscle loss (see also below; cf. (Krawiec et al., 2007; Sandri et al., 2006; Schiaffino, 2010) (Crabtree, 1999; Hudson and Price, 2013).

1.5. Regulation of muscle mass loss

One of the characteristics of adult muscle tissue is its plasticity. Skeletal muscle adapts its size and functions to different physiological conditions and can undergo atrophy that is characterized by a reduction of the cytoplasmatic volume and by the loss of organelles and proteins (Bonaldo and Sandri, 2013). During atrophy of skeletal muscle, protein degradation rates exceed synthesis rates. The following molecular mechanisms are known to cause muscle atrophy: protein degradation through the ubiquitin-proteasomal and autophagic-lysosomal pathways, and apoptosis due to activation of caspases and calpains. Proteolytic mechanisms are particularly important in the loss of muscle mass (Wing et al., 2011).

1.5.1. Ubiquitin-proteasomal system

Ubiquitin-proteasomal degradation is based on proteolysis of proteins, which are labeled with poly-ubiquitin chains. Cell proteins destined for degradation are labeled with a covalent bond of multiple ubiquitin monomers. Ubiquitin is a small protein composed of 76 amino acids. Ubiquitin can be conjugated with specific protein substrates, a process that requires three enzymes (Figure 8): E1, an ubiquitin-activating enzyme; E2, an ubiquitin-conjugating enzyme; and E3, an ubiquitin ligase. Initially, E1 is activated and, in an energy-dependent reaction, transfers ubiquitin through E2 to E3. The E3 ubiquitin ligases identify and poly-ubiquitylate protein substrates, which are destined for degradation (Teixeira Vde et al., 2012; Yamao, 1999). The ubiquitylated proteins are recognized and delivered by ZNF216 to the proteasomes. Proteasomes are large complexes that contain enzymes that degrade ubiquitylated proteins, for instance proteins that are unnecessary or damaged (Hishiya et al., 2006).

The human genome encodes more than 650 ubiquitin ligases (Lee and Goldberg, 2011), which together recognize many different proteins. However, each E3 ligase recognizes a specific set of proteins and selectively regulate the degradation of these. The amount of different E3 ligases varies between tissues types and under different physiological conditions (Bonaldo and Sandri, 2013). Genes that regulate muscle atrophy are called atrophy-related genes or 'atrogenes' and many of these encode E3 ligases (Sacheck et al., 2007). For instance, during muscle atrophy, two muscle-specific ubiquitin ligases, MAFbx and MuRF1, are strongly up-regulated

(Bodine et al., 2001a; Gomes et al., 2001; Sandri et al., 2004). The identified targets of MAFbx (also known as atrogin-1) are MyoD and eukaryotic translation initiation factor 3F (eIF3F) (Csibi et al., 2010; Tintignac et al., 2005). MuRF1 substrates are structural sarcomeric proteins like MHC (Clarke et al., 2007; Cohen et al., 2009; Polge et al., 2011). The studies of Bodine et al. demonstrated that mice deficient in either MAFbx or MuRF1 develop normally, but are resistant to denervation-induced atrophy (Bodine et al., 2001a). MuRF2 encodes another ubiquitin ligase that is also expressed in muscle. However, mice that lack MuRF1 and MuRF2 developed extreme cardiac and milder skeletal muscle hypertrophy (Witt et al., 2008).

Another ligase that plays an important role in atrophy is the E3 ubiquitin ligase TRAF6 (Paul et al., 2010). TRAF6 knockout mice are resistant to muscle loss induced by denervation, cancer or starvation (Kumar et al., 2012; Paul et al., 2012; Paul et al., 2010). The studies of Paul et al. suggested that the inhibition of TRAF6 reduces the maximal induction of other autophagy-related genes by a still unknown mechanism. This then allows the preservation of muscle mass under conditions which favor catabolism.

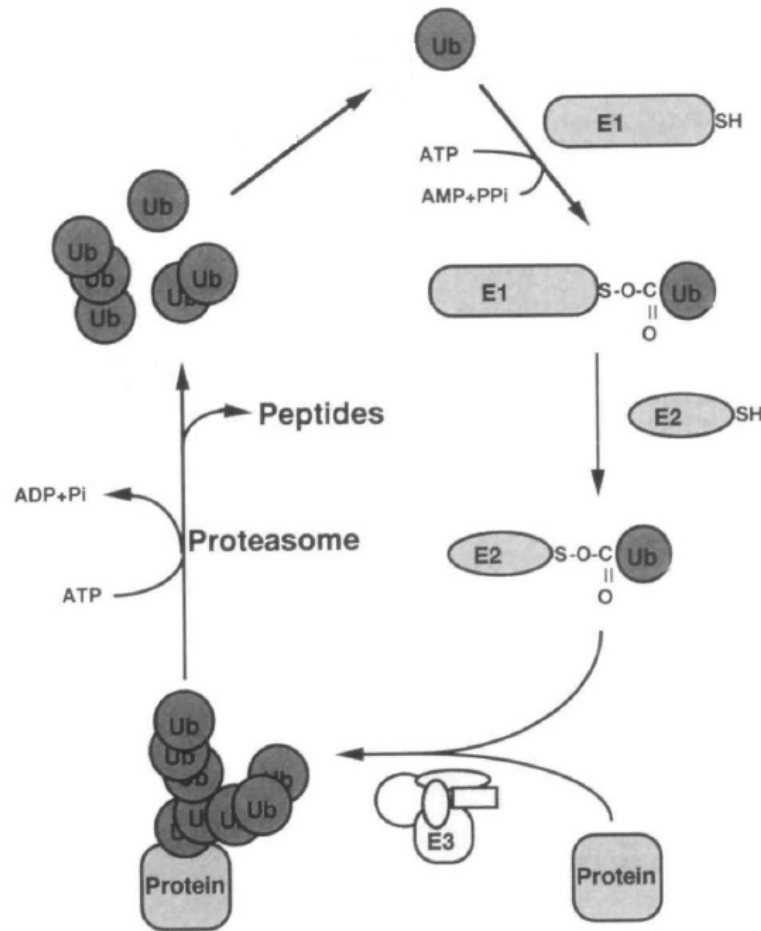


Figure 8: Ubiquitin-proteasome system of muscle degradation. This pathway requires the participation of three distinct proteins (E1, E2 and E3). The E3 ligases provide specificity to the protein substrates and label them with ubiquitin monomers (Ub). Adapted from (Yamao, 1999).

In skeletal muscle, E3 ligases can also have important regulatory functions on signaling pathways. For example, Shi et al. demonstrated that the Fbxo40 ligase regulates anabolic signals (Shi et al., 2011). Fbxo40 ubiquitylates and affects the degradation of IRS1, a downstream effector of insulin-receptor-mediated signaling. The RNAi-mediated inhibition of Fbxo40 results myotubes hypertrophy *in vitro*. *In vivo*, the Fbxo40 deficient mice display muscle hypertrophy (Shi et al., 2011). All of these identified ubiquitin ligases promote degradation of fibrillar and soluble muscle proteins and/or reduce signals that stimulate protein synthesis.

There are few pathways that regulate atrogene expression in muscles. As it was indicated previously, the IGF1-AKT pathway controls protein synthesis via expression of eIF2B and eIF4E factors. Suppression of IGF1-AKT signaling causes loss of

muscle mass. Known IGF1 downstream targets are FoxO molecules that are members of the family of forkhead transcription factors (Figure 9) (Murphy et al., 2003; Sandri et al., 2004). AKT phosphorylates and suppresses the activity of FoxO factors that control protein degradation by regulating the expression of the ubiquitinylation factors atrogin1/MAFbx and MuRF1. FoxO3 induces the transcription of numerous genes involved with the autophagy-lysosome pathway, such as LC3, in response to starvation and denervation (Mammucari et al., 2007; Zhao et al., 2007). The mitochondrial E3 ubiquitin protein ligase 1 (Mul1) responds to muscle-wasting stimuli through involving FoxO1/3-mediated mechanism (Lokireddy et al., 2012). The study of Lokireddy et al. demonstrated that this ligase controls mitophagy and regulates remodeling of mitochondrial network during muscle atrophy.

Various signaling pathways can interact with the IGF1/AKT pathway in order to regulate FoxO activity. For instance, Peroxisome proliferator-activated receptor gamma coactivator-1 α (PGC-1 α) and AMP activated kinase (AMPK) inhibit FoxO-dependent transcription of the atrogin-1 genes (Krawiec et al., 2007; Sandri et al., 2006).

In addition, **myostatin**, a negative regulator of skeletal muscle growth, activates FoxO transcription factors and promotes muscle wasting. This pathway is discussed in detail below.

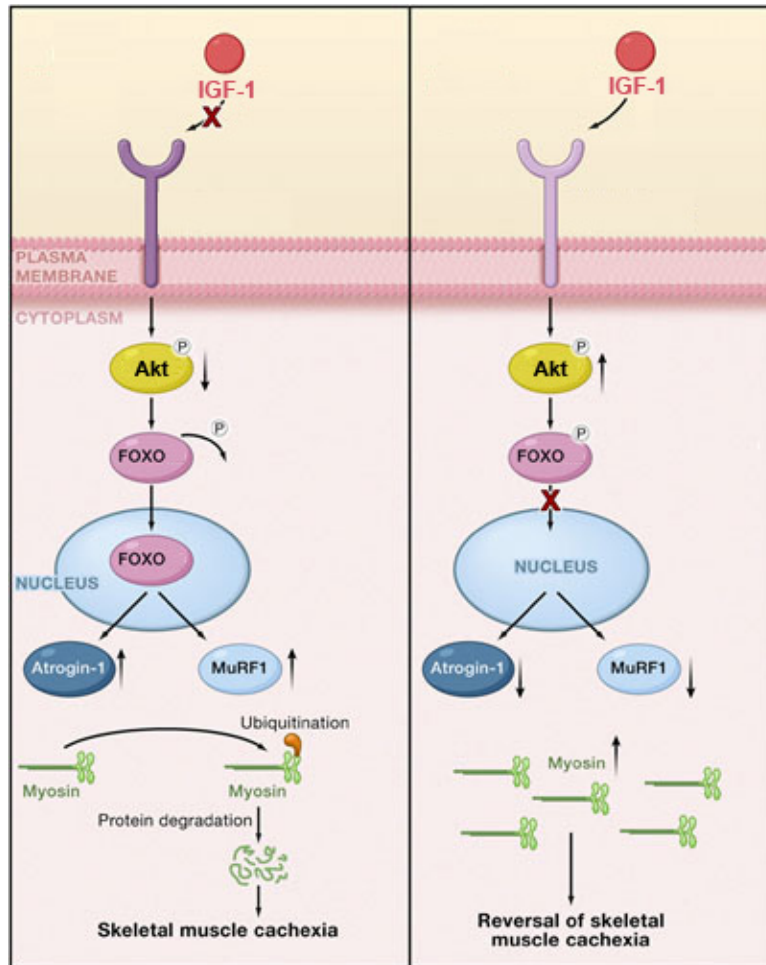


Figure 9: Intracellular signaling pathway that is activated by IGF-1 and the role of FoxO factors in muscle atrophy (**left panel**) and hypertrophy (**right panel**). Adapted from (Sandri et al., 2004) and (Tisdale, 2010).

1.5.2. Autophagy–lysosomal degradation system

There are three mechanisms to deliver intracellular protein substrates to lysosomes: microautophagy, chaperone-mediated autophagy or macroautophagy (Figure 10). To date, there is no evidence found that microautophagy is active in skeletal muscle cells, and therefore its importance in muscle proteolysis remains to be established (Bechet et al., 2005).

The substrates of **chaperone-mediated autophagy** are glycolytic enzymes, such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH), diverse transcription factors and the proteasomes (Cuervo and Dice, 1998). The substrate degradation involves three major steps: 1) The heat-shock cognate protein of 73 kDa (hsc73) recognizes the specific peptide sequence on the protein substrate and builds a complex. 2) This

complex translocates to the lysosomal membrane and binds to its receptor, the lysosome associated membrane protein (LAMP2a) (Cuervo and Dice, 1996). 3) Finally, the protein-substrate is translocated across lysosomal membranes (Agarraberes and Dice, 2001).

Macroautophagy is a conserved homeostatic process carrying out degradation of cytoplasmic components including damaged organelles, toxic protein aggregates and intracellular pathogens (Masiero et al., 2009). These cytoplasmic components are localized in double membrane-bound vesicles, the autophagosomes (Mortimore, Poso, & Lardeux, 1989). The autophagosome then fuses with a lysosome to become an autolysosome. The outer membrane of the autophagosome fuses with a lysosome, exposing the inner single membrane of the autophagosome to lysosomal hydrolases, where sequestered components are degraded. In yeast, the molecular machinery required for autophagy involves approx.. 30 ATG genes, and many Atg products have related homologous proteins in mammals (Ohsumi, 2001). The important proteins are, for instance, the cargo receptor p62, which is conjugated to microtubule-associated protein light chain 3 (LC3) (Lamark et al., 2009). Other proteins like Bnip3/3L interact directly with LC3 and induce selective removal of endoplasmic reticulum and mitochondria via autophagy (Hanna et al., 2012; Novak et al., 2010). There are also other proteins that are involved in remodeling of mitochondrial network. For instance, parkin protein promotes mitophagy through ubiquitylation of outer mitochondrial membrane proteins. The autophagosome recognizes these proteins via p62 protein and proceeds with organelle breakdown (Narendra et al., 2010a; Narendra et al., 2010b; Youle and Narendra, 2011).

The autophagy-lysosome pathway is critical for muscle homeostasis. The inhibition of autophagy results in myofiber degeneration and weakness in muscle disorders such as Bethlem myopathy and Ullrich congenital muscular dystrophy (Grumati et al., 2010). This process is characterized by accumulation of abnormal mitochondria (Masiero et al., 2009). Importantly, inhibition of autophagy also promotes the accumulation of nuclear abnormalities, reduces cell viability, and disrupts the function of mitochondria (O'Leary and Hood, 2009) (Bechet et al., 2005; Park et al., 2009).

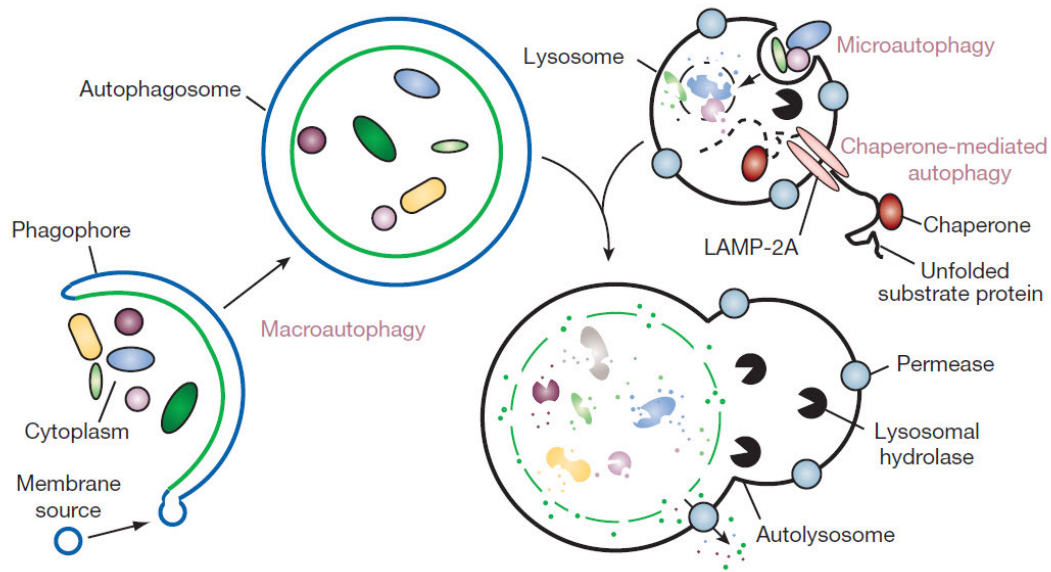


Figure 10: Different types of autophagy. Adapted from (Mizushima et al., 2008).

1.5.3. Calpains

Cell membrane damage, osmotic imbalance or abnormal ion fluxes are mechanisms potentially leading to the fiber necrosis. These destructive changes are associated with membrane wounds during muscle contraction and can result to muscular pathologies such as seen in dystrophinopathies or sarcoglycanopathies. These processes are accompanied by an increased calcium influx resulting in an elevation of intracellular calcium levels (Turner et al., 1991). This flux activates calpains, the family of cysteine-proteases (Alderton and Steinhardt, 2000). Their enzymatic activity depends on calcium concentration (Khorid 2002). Additionally, activation of the calpain system was demonstrated during muscle atrophy, such as consequences of immobilization and ageing (Goll et al., 2003). To date, the calpain family comprises 15 different members: Calcium-dependent proteases, called μ -calpains and m-calpains, and a polypeptide calpastatin, whose function is to inhibit calpains (Goll et al., 2008; Goll et al., 2003). Muscle tissue expresses mainly three distinct calpains: the calpains 1, 2 and 3 (Bartoli and Richard, 2005). Calpains are involved in the cleavage of myofilament proteins such as alpha-actinin that are major constituents of Z-discs (Ohtsuka et al., 1997). When Z-discs are disrupted, actin, myosin and other proteins from the sarcomere are released in the cytoplasm. This facilitates further

protein degradation by another cell proteolysis system (Bullard et al., 1990; Williams et al., 1999).

1.5.4. Caspases

Caspases are non-calcium-dependent cysteine-proteases that cleave their substrate proteins after an aspartic acid residue. Internal and external signals can activate inactive pro-caspases. In particular, the pro-apoptotic regulatory proteins of the B-cell lymphoma 2 (Bcl-2) family trigger caspase mediated protein degradation (Fischer et al., 2003). Caspase were found to be involved in protein degradation during muscle weakness in response to endotoxin (Supinski and Callahan, 2006). In addition, the denervation-induced atrophy was associated with an increase in caspase-3 and -8 activity (Alway et al., 2003; Siu and Alway, 2005).

To date, more than 280 caspase targets are identified (Fischer et al., 2003). For instance, under catabolic conditions, caspases fragmentize long filamentous actin proteins. These cleaved actin fragments are degraded further by the ubiquitin-proteasome system (Du et al., 2004). The study of Du et al. demonstrated that the blocking of caspase activity in atrophic muscle following diabetes or chronic uremia reduced both the accumulation of actin fragments and deprived protein degradation rate by other proteolytic systems.

The importance of caspase-mediated proteolysis is not fully elucidated. Generally, the mode by which atrophy occurs determines the involvement of different pathways and this is also valid for caspase system. Various studies suggested that activation of caspases may serve a different role in distinct models of muscle atrophy (Alway et al., 2003; Leeuwenburgh et al., 2005; Siu and Alway, 2005; Siu et al., 2005).

1.5.5. Cytokines

Role of cytokines during local and systemic inflammation is one of the major factors initiating muscle loss. Elevated levels of various cytokines and chemokines are present in patients with sepsis and the systemic inflammatory response syndrome (Callahan and Supinski, 2009). Human skeletal cell cultures *in vitro* constitutively

express low levels of proinflammatory cytokines such as interleukin-1 (IL-1) and interleukin-6 (IL-6). Moreover, exposure of these cells to exogenous cytokines triggers a positive feed-back loop and induces the release of other cytokines (Bartoccioni et al., 1994; Nagaraju et al., 1998). The exposure of isolated myofibers and muscle cell lines to the cytokines *in vitro*, in particularly to tumor necrosis factor alpha (TNF- α), reduces protein content and decreases contractile force of myofibers (Li and Reid, 2001, 2000; Li et al., 1998; Reid et al., 2002).

Various studies confirmed the same effects *in vivo*. The study of Shindoh et al. reported the increase of TNF- α expression in the diaphragm muscle after administration of endotoxin (Shindoh et al., 1995). Other studies demonstrated that elevated levels of TNF- α in serum induce atrophy of the limb musculature (Buck and Chojkier, 1996). Acharyya and colleagues overexpressed IL-6 and TNF- α in mice and observed the loss of muscle mass and a selective down-regulation of specific myofibrillar proteins (Acharyya et al., 2004). TNF- α induces apoptosis directly through its interaction with the death domain receptors that leads to the activation of procaspase 8 (Greiwe et al., 2001). Another molecular mechanism describes the indirect participation of TNF- α in activation of its downstream effectors. Both TNF- α and IL-1 β can activate I κ B kinase (IKK) that phosphorylates the inhibitory protein I κ B. The phosphorylated I κ B releases NF- κ B, a protein that controls among others the expression of ubiquitin ligase MuRF1. The study of Cai et al. reported that the overexpression of IKK leads to severe muscle atrophy in particular by ubiquitin-proteasome pathway (Cai et al., 2004).

1.6. Muscle pathologies

Damage to muscle tissue can occur due to exercise and contraction, acute physical and chemical insult, abnormalities in the immune system, or genetic defect of muscle components. The pathological consequence of acute physical damage is the rapid myofiber necrosis. Acute myonecrosis can be induced through the intoxication of venoms, such as myo- and cardiotoxins (Duchen et al., 1974; Habermann, 1972; Ownby et al., 1976; Ownby et al., 1997). These toxins cause depolarization and contraction of myofibers. The low-molecular-mass myotoxins act via voltage-sensitive sodium channels in the sarcolemma and induce uncontrolled sodium influx and oedema. Other toxins lead to myonecrosis through rapid lysis of the sarcolemma. The resulting calcium influx leads to the hyper-contraction of sarcomeres.

Insufficient blood supply, so-called ischemia, also causes acute muscle damage. Oxygen insufficiency during ischemia converts cellular metabolism to anaerobic pathways, initiating a cascade of reactions that generate large quantities of superoxide and other free radicals and increase intracellular calcium concentrations (Ratych et al., 1987). This leads to the activation of apoptosis, mediated, in particular, by calpains (Riedemann and Ward, 2003).

Another pathological damage to myofibers can be induced by contractions. Especially, eccentric contractions cause severe damage to sarcomeres, tearing T-tubules apart, disorganizing myofilaments and disrupting membrane integrity. The damage of sarcoplasmic reticulum results in intracellular calcium influx that activates calpains (Belcastro et al., 1998).

Immune response represents another pathological treat that causes muscle damage. Inflammatory myopathies are a heterogeneous group of subacute, chronic, or sometimes acute muscle diseases (Dalakas, 2011). Inflammatory myopathies can be grouped in four different types according to histological, immunopathological, and clinical manifestations: dermatomyositis, polymyositis, sporadic inclusion body myositis, and necrotizing autoimmune myositis (Dalakas, 2010). They all are characterized by inflammation in myofibers initiated by cellular response of innate and adaptive immune system. The immune cells such as cytotoxic T cells, B cells, macrophages, and plasmacytoid dendritic cells, attack endothelial cells or myofibers in muscle tissue and cause cell lysis and degradation.

In addition, muscle degenerative diseases caused by deficiencies in stability, repair, or gene-expression regulation in muscle fibers represent more than 100 human disorders (Tabebordbar et al., 2013). Age-related progressive muscle loss and Duchenne muscular dystrophy are introduced in more detail below.

1.6.1. Age-related muscle wasting

The loss in muscle mass and muscle strength associated with age is called sarcopenia. In contrast to cachexia, a pathological muscle mass loss secondary to other diseases, sarcopenia is a chronic process associated with the age and has a low grade of systemic inflammation (Figure 11) (Kwan, 2013). Sarcopenia is accompanied by elevated muscle fragility and decreased exercise performance. Sarcopenia affects approx. 25% of individuals older than 70 years of age and 40% of individuals older than 80. Individuals suffering from sarcopenia experience decreased independence and reduced life quality.

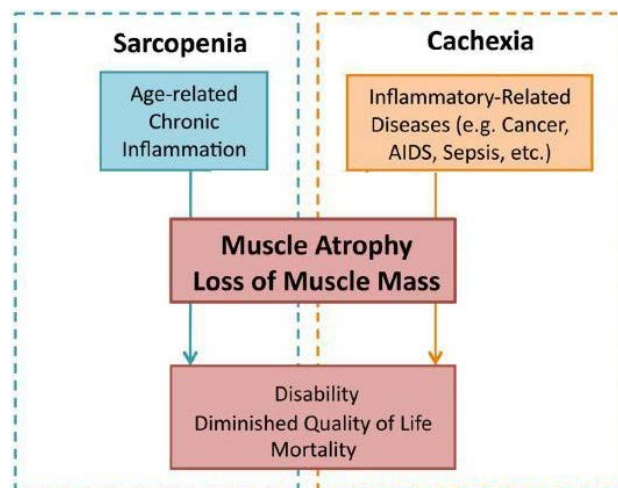


Figure 11: Sarcopenia and cachexia represent two distinct diseased states. Whereas sarcopenia (blue) results from chronic inflammation associated with age, cachexia (orange) results from inflammation associated with a primary disease, for instance the cancer. Adapted from (Hall et al., 2011).

Sarcopenia is caused by multiple factors ranging from external conditions, such as physical activities, to internal parameters, such as genetic risk of an individual. Aging-associated alterations were monitored by many clinical studies. The loss of muscle power and muscle strength is associated with a reduction in the amount of calcium available for the mechanical response in muscles (Delbono et al., 1995). In elderly patients, the loss of muscle strength occurs even more rapidly than the loss of muscle mass (Goodpaster et al., 2006). Sarcopenia is characterized by a reduction in muscle fiber number as well as fiber size, with the preferential reduction of type 2 myofibers (Garatachea and Lucia, 2013; Lexell et al., 1983). Aging is associated with a reduction in cell density of the satellite cell population in type 2 fibers as well as a diminished satellite cell proliferation capacity (Renault et al., 2002a; Renault et al., 2002b; Verdijk et al., 2007). In addition, age-related neurological factors, such as alterations in motor axon conduction velocity and the number of myelinated axons, can contribute to the loss in muscle strength. Aging is also associated with the changes in microcirculation, for instance in the reduction of vasodilatory capacity and capillarization (Degens, 1998).

External conditions like fasting and declined physical activity play an essential role in the development of sarcopenia. Other age-related factors can contribute to muscle loss. Mitochondrial dysfunction may arise from the age-related decline in mitochondriogenesis and accumulation of defective mitochondria (Derbre et al., 2012). Mitochondrial dysfunction impairs mitochondrial oxidative function and energy production and thus affects cell viability of myocytes (Martin, 2011). In addition, the elevated oxidative stress in muscles leads to the downregulation of myogenic transcription factors such as MyoD, and the inhibition protein translation synthesis (Frost et al., 2009; Guttridge et al., 2000). Another known cause of sarcopenia is the age-related increase in proinflammatory cytokines, a process known as “inflamm-aging” (Franceschi et al., 2007). The elevated baseline levels of cytokines, such as TNF- α and IL-6 affect the metabolic balance in muscle fibers, leading to muscle atrophy and apoptosis (Hall et al., 2011).

There are also other molecular and cellular manifestations associated with sarcopenia, such as the role of elevated myogenic inhibitor of differentiation (Id) factors. However, the molecular mechanism is still not well understood (Alway et al., 2003; Arthur and Cooley, 2012).

1.6.2. Duchenne muscular dystrophy

One frequent genetically caused muscle disorder is Duchenne muscular dystrophy (DMD). It was characterized in 1861 by Guillaume Benjamin Armand Duchenne (1806-1875). This disorder is characterized by progressive degeneration of muscle tissue. One of the clinical striking feature, apparent in many cases, is an enlargement of severely weakened muscles ("pseudo-hypertrophy") in the early steps of the disease. Initially, Duchenne considered this disease as a type of brain disorder and named the disease "hypertrophic paraplegia childhood" (Duchenne, 1868). He proposed diagnostic criteria based on anatomical observations, responses to electrical stimulation, and findings on the muscle biopsy as characterized by the replacement of muscles with fibrous connective tissue (Figure 7).

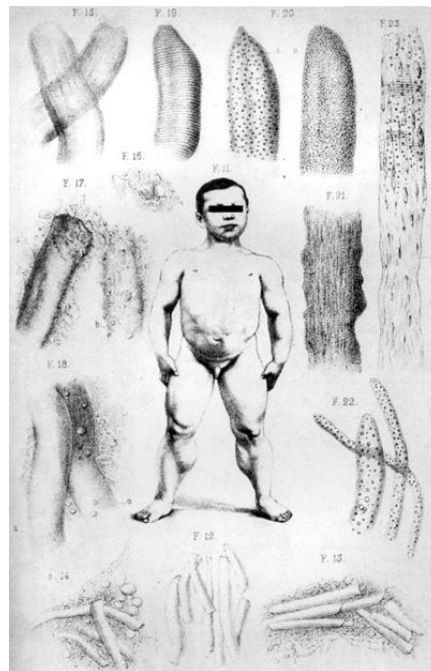


Figure 12: Duchenne's Case XII is in the center (F. 11), "showing atrophy of the pectorals." The surrounding images are muscle specimens "from subjects affected with pseudohypertrophic paralysis of differing severity; showing the considerable quality of connective and fibrous interstitial tissue." According to (Duchenne De Boulogne, 1965). Adapted from (Tyler, 2003).

DMD is the X-linked genetic disorder and affects one in about 3500 males (Emery, 1993). Most boys with DMD manifest symptoms within the first years of life. Women are carriers of mutated gene. The first symptoms are Gowers' sign and waddling gait that usually appear between 3 and 5 years (Figure 13) (Tyler, 2003). The decline in

muscle strength is progressively to all limb and trunk. This muscle weakness leads to the loss of ambulation usually between 7 and 13 years. The disease results in the wide heterogeneity in the fiber size, the presence of atrophic and hypertrophic fibers, signs of myofiber degeneration / regeneration and the infiltration of inflammatory cells. While the cell membrane is damaged, creatine kinase is released from the myofiber into the serum. The necrosis of muscle fibers therefore results in very high levels of creatine kinase in the serum of the DMD patients. The weakening of the respiratory muscles leads to the retention of carbon monoxide and anoxia. The failure of respiratory system often triggers frequent respiratory infections. Nowadays, the progress in respiratory assistance prolongs life expectancy by DMD patients up to 29 years (Emery, 2002). DMD affects also the heart leading to progressive cardiomyopathie that is one of the leading causes of death in the patients.

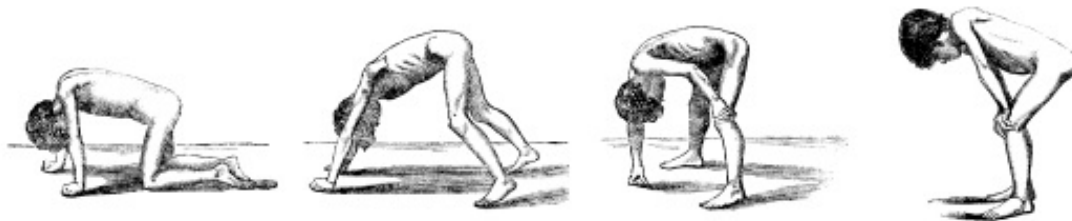


Figure 13: Gowers' sign: Children use the upper limbs in support when rising from a squatting position: "He helps himself up in a very peculiar way—by putting his hands upon his knees, and grasping his thighs higher and higher, and so by climbing up his thighs he pushes his trunk up." According to (Gowers). Adapted from (Tyler, 2003).

The DMD locus was identified on the short arm of the X-chromosome in the Xp21 region (Davies et al., 1983). Becker muscular dystrophy (BMD) was linked to the same locus but the disease progression by BMD is much milder compared to DMD and some patients remain asymptomatic until a very advanced age (Kingston et al., 1984).

The DMD gene is now the largest known human gene and extends over 2.5 mega base pairs (Mb). It represents about 0.1% of the human genome (Lander et al., 2001). However, 99% of the gene comprises introns. The mRNA has of a size of 14 kb and contains 79 exons. In approximately two-thirds of DMD cases, the mutation of DMD gene is a deletion or duplication of one or more of the exons causing shift in

reading frame (White et al., 2002). Other mutations are caused by very small deletions and point mutations, most of which introduce premature stop codons (Koenig et al., 1987; Prior et al., 1995; Roberts et al., 1994). T

he protein dystrophin encoded by the DMD gene is not detectable by DMD patients (Bonilla et al., 1988; Hoffman et al., 1987b; Watkins et al., 1988). In contrast to DMD patients, the expression of truncated form of dystrophin is detectable by BMD patients. Dystrophin is composed of 3685 amino acids and has a molecular weight of 427 kD (Koenig et al., 1988). Dystrophin is a member of the β -spectrin/ α -actinin protein family. This family is characterized by an NH₂-terminal actin-binding domain followed by a variable number of repeating units known as spectrin-like repeats. Dystrophin is linked indirectly to the membrane of the myofiber through the complex of dystrophin associated proteins (DAPC). DAPC consists of several protein groups: the dystroglycan complex, the sarcoglycan-sarcospan complex and cytoplasmic complex, which comprises dystrophin, the syntrophins, the α -dystrobrevin and the neuronal-type nitric oxide synthase (nNOS) (Blake et al., 2002). The cohesion of the complex is essential for the stability of the plasma membrane and depends on the expression and the correct location of each of its parts.

Dystrophin-deficient organisms, such as mice, dogs, and cats and nematodes, provide models of DMD and allow to study the pathophysiological processes (Blake et al., 2002). The murine model of DMD is the *mdx* mouse. It has elevated plasma levels of creatine kinase and displays a muscle pathology (Bulfield et al., 1984b). The dystrophin transcript has a premature stop codon because of a point mutation in exon 23 of the DMD gene (Sicinski et al., 1989). The *mdx* muscles are hypertrophic, however, the muscle function is significantly reduced (Lynch et al., 2001a). At the age of 3-4 weeks, *mdx* mutants develop a massive myofiber necrosis. Thereafter, muscle fiber necrosis decreases in intensity. The necrosis is compensated by a very strong regeneration and the *mdx* mice develop no obvious muscle weakness and wasting in contrast to DMD patients (Coulton et al., 1988; Tanabe et al., 1986). The majority of fibers become centrally nucleated upon regeneration (Figure 14). After the first round of myofiber necrosis, the myofiber degeneration and regeneration continue, however, less intense (DiMario et al., 1991; Pagel and Partridge, 1999).

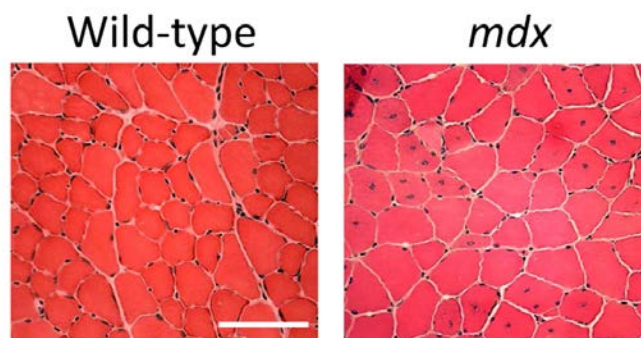


Figure 14: Representative images of TA muscles from wild-type and mdx mice at ages of 2 months with hematoxylin and eosin staining. Scale bar is 100 μ m. According to (Echigoya et al., 2013).

1.7. TGF- β super family of signaling molecules

Members of the TGF- β (transforming growth factor- β) superfamily regulate activation, proliferation, differentiation or cell death. The first member of this family, TGF- β , was discovered by its ability to transform cells (de Larco and Todaro, 1978), and named the superfamily of secreted proteins with similar structure (Vitt et al., 2001). Besides TGF- β 1-4, bone morphogenetic proteins (BMPs), growth and differentiation factors (GDFs), activins and nodal are members of this family (Moustakas and Heldin, 2009). In addition to these ligands, *Xenopus laevis* expresses also unique family members, such as the mesoderm-inducer *Derrière*, and the six nodal-related proteins XNR1-6. Invertebrates, such as *Drosophila melanogaster* have protein homologs of TGF- β family members.

The TGF- β family members share structural features, like the presence of cysteine loops, and displays sequence similarity. The prototypic TGF- β isoforms, inhibin- β ligands and activins, have nine cysteines, eight of which are responsible for protein folding and one that builds intermolecular bridge to form dimers. The inhibin- α polypeptides, BMPs and GDFs have seven cysteines, six of which are responsible for protein folding and one for forming intermolecular bridges. GDF3, GDF9 and BMP15A have six cysteines for protein folding, whereas they do not form covalent dimers.

TGF- β molecules are synthesized as long inactive pre-pro-peptides. After cleavage of the N-terminal signal peptide, further proteolytic maturation steps follow, and the processed molecules form homo- or heterodimers. The mature peptide and an N-terminal precursor remnant (also known as latency associated peptide) remain non-covalently attached and form the small latent complex that is still inactive. This complex can remain in secretory vesicles or be secreted into the extracellular space (ten Dijke and Arthur, 2007). To become active, the latency associated peptide needs to be released, for instance by pH change or proteolytical cleavage through thrombospondin-1 (Lyons et al., 1988; Schultz-Cherry and Murphy-Ullrich, 1993).

Table 2: TGF- β pathway. Receptors are listed as type II (RII) and type I (RI). Dashed lines separate groups of ligands or receptors based on the division into BMP and TGF- β /activin-like pathways. Ligands, type I receptors and R-Smads are color-coded: blue, BMP-like pathways; red, TGF- β /activin-like pathways. N/A, not applicable. According to (Patel and Amthor, 2005).

Pathway	<i>H. sapiens</i>					
	BMP	GDF	Activin	TGF β	AMH	Inhibitors
Ligand	BMP2, 4 BMP5, 6, 7 BMP8A, 8B BMP9, 10	GDF5, 6, 7 GDF9b GDF10, 11 GDF15 (MIC1) ----- GDF1, 3 Myostatin GDF9	Inhibin β A Inhibin β B Nodal	TGF β 1 TGF β 2 TGF β 3	AMH (MIS)	BMP3 Inhibin α Inhibin β C Inhibin β E LEFTYA LEFTYB
RII	BMPRII ActRIIA, ActRIIB	BMPRII ActRIIA, ActRIIB	ActRIIA ActRIIB	T β RII	AMHRII	N/A
RI	BMPRIA (ALK3) BMPRIIB (ALK6) ALK2 ALK1	BMPRIA (ALK3) BMPRIIB (ALK6) ALK2 ----- ActRIB (ALK4) ALK7 T β RI (ALK5)	ActRIB (ALK4) ALK7	T β RI (ALK5) ----- ALK1 ALK2 BMPRIA (ALK3)	BMPRIA (ALK3) BMPRIIB (ALK6) ALK2	N/A
R-Smad	SMAD1, 5, 8	SMAD1, 5, 8 ----- SMAD2, 3	SMAD2, 3	SMAD2, 3 ----- SMAD1, 5, 8	SMAD1, 5, 8	N/A

The activated factors bind and signal via receptors that possess serine/threonine kinase activity (Figure 15). Two receptor molecules, a type I and type II receptor, cooperate to transduce the signal and different combinations of type I and type II receptor bind specifically to particular ligands of the TGF- β family (Groppe et al., 2008; Wrana et al., 1992). Upon ligand binding, the receptors become phosphorylated and their kinase activity is activated, which triggers phosphorylation of intracellular components – most importantly the receptor-activated Smad proteins (R-Smads) (Hoodless et al., 1996; Kretschmar et al., 1997). Smad proteins are classified into three groups: receptor Smads (Smad1, Smad2, Smad3, Smad5, Smad8) that bind to and are phosphorylated by particular receptors, the common-mediator Smad (Co-Smad; Smad4) and the inhibitory Smads (I-Smads; Smad6 and Smad7) (Moustakas and Heldin, 2009). Two phosphorylated R-Smads bind to the Co-Smad, and the trimeric Smad complex is shuttled into the nucleus, where it binds to DNA (Kawabata et al., 1998). Together with other transcription factors, they regulate the expression of specific target genes. Some Smad complexes (those that mediate BMP signals) induce the expression of I-Smads which creates a negative-feedback loop (Ishida et al., 2000). Mutation in Smad proteins may have severe consequences. For instance, mutations in the *SMAD4* gene in humans result in

Myhre syndrome, which is characterized by reduced growth, generalized muscular hypertrophy, facial dysmorphism, joint stiffness, and skeletal anomalies (Caputo et al., 2012). Interestingly, missense mutations in *SMAD4* caused an increase in the levels of phosphorylated R-Smad proteins in patients with Myhre syndrome (Le Goff et al., 2012).

TGF- β members use different combinations of the type II and type I receptors. For instance, a TGF- β member activin signals via activin type IIa (Acvr2A) or type IIb (Acvr2B) receptors in combination with activin receptor type I (Acvr1B), also known as activin receptorlike kinase 4 (ALK4). TGF- β itself binds mainly to TGF- β receptor type II (TgfbR2) and type I (TgfbR1), also known as activin receptor-like kinase 5 (ALK5). Another TGF- β member myostatin, like activin, can bind to AcvR2A, AcvR2B and ALK4, but unlike activin, is also able to signal via ALK5 (Rebbapragada et al., 2003). It was suggested that other ligands BMP9, BMP10 and BMP11 may signal in a similar way as myostatin (Souza et al., 2008).

The activity of co-receptors regulates the affinity towards the type I or II receptor complex and thus modulates ligand-receptor sensitivity, selectivity and function of TGF- β family members. For example, betaglycan (TgfbR3) interacts with TGF- β and enhances its affinity toward TgfbR2 and ALK5, acts as inhibins co-receptor with AcvR2 and antagonizes BMP signaling (Lewis et al., 2000; Lopez-Casillas et al., 1993; Wiater and Vale, 2003).

Complementary to this canonical pathway, non-canonical pathways also exist. For instance, the transcription intermediary factor 1 γ interacts with receptor-activated Smad2/3 and competes for Smad4 binding (He et al., 2006). Several studies indicate that TGF- β signaling impinges onto the activation of various signaling pathways, and for instance NF- κ B, p38 MAPK, Rho-Rock1 and Cdc42/Rac1-PAK2 activity is modulated by TGF- β signaling (Lu et al., 2007, Bhowmick et al., 2001, Suzuki et al., 2007).

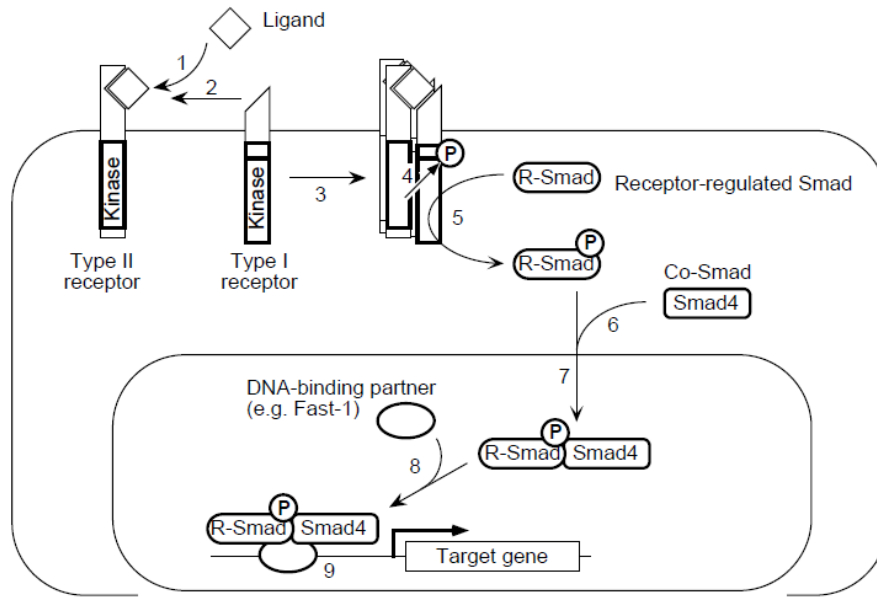


Figure 15: The transforming growth factor β (TGF- β)/Smad pathway. Binding of a TGF- β family member to its type II receptor (1) in concert with a type I receptor (2) leads to formation of a receptor complex (3) and phosphorylation of the type I receptor (4). Thus activated, the type I receptor subsequently phosphorylates a receptor-regulated Smad (R-Smad) (5), allowing this protein to associate with Smad4 (6) and translocate into the nucleus (7). In the nucleus, the Smad complex associates with a DNA-binding partner, such as Fast-1 (8), and this complex binds to specific enhancers in the proximity of target genes (9), activating transcription. Adapted from (Massague, 1998).

1.8. BMP signaling

BMPs are members of the TGF- β superfamily and were originally discovered by Marshall Urist by their ability to induce formation of bone and cartilage (Urist et al., 1977). This phenomenon was attributed to the presence of substance in bone matrix named BMPs (Urist et al., 1977). However, the proteins responsible for bone induction remained unknown until the identification of human BMP-2 and bovine BMP-3 (osteogenin) (Wozney et al., 1988). To date, around 20 BMP family members are identified and characterized (Chen et al., 2004). BMPs are considered to constitute a group of morphogenetic signals, orchestrating tissue architecture throughout the body (Jain et al., 2013). The invertebrate homologue of BMPs, in particular BMP2 and BMP4, is the fly morphogen, decapentaplegic (Dpp), a key molecule involved in *Drosophila* embryonic development (Gelbart, 1989; Padgett et al., 1987).

BMPs are synthesized inside the cell in a precursor form and consist of a signal peptide, propeptide and mature peptide (Figure 16). After the cleavage of the signal peptide, the precursor protein undergoes glycosylation and dimerization. The pro-domain is cleaved and the bioactive BMPs are secreted as either hetero- or homodimers.

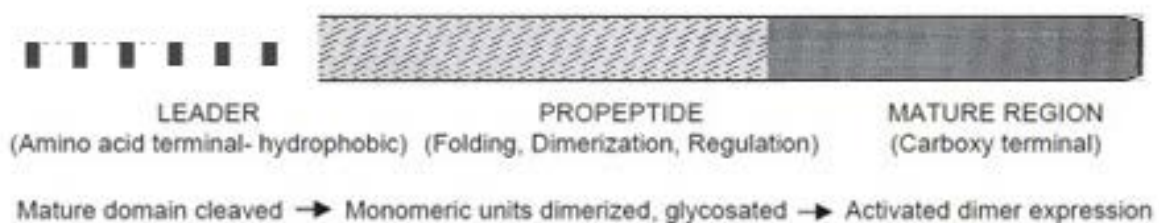


Figure 16: Chemical structure of BMPs. According to (Jain et al., 2013)

BMPs bind to and activate dedicated BMP receptors like activin-receptor-like kinase 3, (ALK3, a type I receptor) and BMP receptor type 2 (BMPR2, a type II receptor) (Figure 17). BMPs are classified into several subgroups, each of which again contains several members: BMP-2/4, BMP-5/6/7/8, growth and differentiation factor (GDF)-5-7 and BMP-9/10 (Miyazono et al., 2010). BMP-1 is not a member of TGF- β superfamily but a protein with homology to pro-collagen C-proteinase, a family of

putative proteases involved in formation of the extracellular matrix (Kessler et al., 1996). BMP-2/4, BMP-5/6/7/8 and BMP-9/10 can induce bone and cartilage formation *in vivo*, hence the name BMP- bone morphogenic protein. BMP-3 and myostatin (also known as GDF-8, which does not belong to the BMP family) fail to induce bone and cartilage formation *in vivo* (Arounleut et al., 2013; Kokabu et al., 2012; Miyazono et al., 2010).

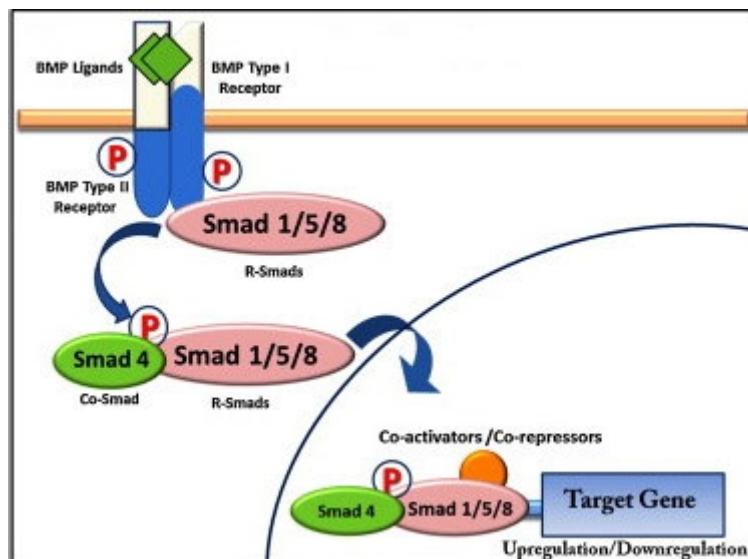


Figure 17: Schematic of BMP signaling pathway. Binding of BMP ligands to the Type II receptor leads to phosphorylation of the Type I receptor, which in turn phosphorylates the BMP pathway specific receptor-regulated SMADs (R-SMADs) i.e. SMAD1 or SMAD5 or SMAD8. Phosphorylated R-SMADs form heterodimers with SMAD4 and translocate into the nucleus According to (Bandyopadhyay et al., 2013).

The R-Smad proteins that are phosphorylated upon BMP signaling are Smad1, Smad5 and Smad8, and all of them bind to Smad4. Upon their translocation into the nucleus, they activate target genes, among them genes of the inhibitor of DNA binding (Id) family. Id proteins (ID1-4) control cell growth and differentiation (Korchynskiy and ten Dijke, 2002; Miyazono and Miyazawa, 2002). Id proteins compete with basic helix-loop-helix (bHLH) factors for E-protein binding, and prevent thus their function. bHLH act as tissue-specific transcription factors, for instance during myogenic differentiation and expression of MyoD (myf3), Myogenin (myf4), Myf5 and MRF4 (myf6) (Benezra et al., 1990; Schafer et al., 1994).

BMP signaling has important roles in development (Winnier et al., 1995), but also in the regulation of many processes in the adult. For instance, dysregulation of BMP signaling in adults triggers the remodeling of bone and cartilage, such as ossification in the hereditary disease fibrodysplasia ossificans progressive (FOP) and in osteoporosis (Wu et al., 2003; Yu et al., 2008). To understand the role of BMP signaling, different animal models were developed that carry constitutive and conditional mutations of BMP genes. Many constitutive mutations are lethal, for instance BMP-2 and BMP-4 that regulate gastrulation (Winnier et al., 1995; Zhang and Bradley, 1996). Other BMPs act in a functional redundant manner (Dudley and Robertson, 1997; King et al., 1994), so only double mutations (e.g. BMP-5/7) are lethal, e.g. due to disrupted cardiac development (Solloway and Robertson, 1999).

GDF-5, known also as BMP-14, is involved in many developmental processes, like chondrogenesis and joint formation. Interestingly, BMP-14 signals via another type I receptor, the activin-receptor-like kinase 6 (ALK6) (Nickel et al., 2005). Spontaneous mutations in GDF-5 trigger alterations in skeletogenesis to mal-formed limbs (Storm et al., 1994). During development of GDF-5 mutant mice, the digit condensations are thin and malformed and the chondrogenesis is delayed. In humans, GDF-5 deficiency leads to diseases, for instance chondrodysplasias like Hunter-Thompson, Grebe and DuPan syndromes and autosomal dominant brachydactyly type C (Polinkovsky et al., 1997).

BMP signaling is antagonized by molecules like noggin, sclerostin, dan, gremlin, inhibin, follistatin and chordin (Abe et al., 2000; Brunkow et al., 2001; Gaddy-Kurten et al., 2002; Gazzo et al., 1998; Hanaoka et al., 2000; Lim et al., 2000; Pereira et al., 2000; Winkler et al., 2003). For instance, the secreted noggin binds with varying affinities to BMP-2, -4, -5, -6, -7, -13, -14 and competes with binding to type I and II BMP receptors (Groppe et al., 2002; Seemann et al., 2009; Song et al., 2010; Zimmerman et al., 1996).

Mutations of BMP antagonists have severe consequences (Devlin et al., 2003; Wu et al., 2003). Multiple synostoses syndrome and proximal symphalangism are caused by mutations in noggin (Gong et al., 1999; Takahashi et al., 2001). Although BMP antagonists have been well characterized due to their role in bone formation, their activity is not restricted to skeletogenesis but extends to other tissues like muscle

(Frank et al., 2006; Ono et al., 2011; Stafford et al., 2011). In chick embryos, noggin is expressed in the dorsomedial dermomyotome, the notochord and the dorsal neural tube (Hirsinger et al., 1997). Studies performed on noggin mutant mouse embryos showed that it is required for the development of skeletal muscle in the myotome (Hirsinger et al., 1997; McMahon et al., 1998; Smith and Harland, 1992). It was observed that noggin is upregulated during myogenic differentiation (Ono et al., 2011). *In vitro*, noggin effectively stimulates differentiation of myoblasts by binding and thus inhibiting BMPs that is present in the culture medium (Ono et al., 2011; Terada et al., 2013). Noggin-mediated blocking of Bmp signaling diminishes the number of fetal muscle progenitors (Wang et al., 2010). However, the function of noggin signaling in adult muscle tissues remains poorly understood.

BMP signaling can also be modulated by mutation of the receptors. Loss of function mutation of the BMPR-IA gene causes embryonic lethality in mice, since BMPR-IA is required for mesoderm formation (Mishina et al., 1995). Although BMPR-IA and BMPR-IB can bind similar ligands, the two receptors are expressed in distinct cells types and take over different functions. In particular, BMPR-IB deficiency alters cartilage formation (Gannon et al., 1997).

Mutation of intracellular components of the Smads that act downstream of BMP can also have severe phenotypes. Smad1 mutant embryos fail to form the umbilical connection to the placenta (Tremblay et al., 2001), and Smad5 mutants have defects in angiogenesis (Yang et al., 1999). Another important mechanism by which the activity of BMP signaling proteins is regulated is mediated via Smad6. Smad6 participates in a negative feedback loop (Ishida et al., 2000), but *in vivo* Smad6 appears to be mainly expressed in the heart, lungs and blood vessels (Alejandre-Alcazar et al., 2008; Galvin et al., 2000).

1.9. Myostatin

Myostatin, a TGF- β superfamily member, is a negative regulator of the muscle mass (Lee et al., 2005). Spontaneous mutations of myostatin are found in cattle, dogs and sheep, or have been induced by gene targeting in mice (Figure 18) (Clop et al., 2006; Kijas et al., 2007; McPherron et al., 1997; McPherron and Lee, 1997; Mosher et al., 2007). Thus, these mutants are also called the double muscle animals. Furthermore, one mutation was also detected in humans (Schuelke et al., 2004). Regardless, of the species, myostatin mutants display excessively sized skeletal muscle. In the myostatin mutant mice developed by McPheron and colleagues, the C-terminal coding sequence was replaced by a neo cassette. The homozygous mutants are viable and fertile (McPherron et al., 1997).



Figure 18: A 3-year-old male of Whippet breed is a medium-sized Greyhound-like dog (right). A heavily muscled female (left) is called double muscle Whippet and associated with myostatin deficiency. According to (Shelton and Engvall, 2007).

Myostatin is expressed during many stages of muscle development, i.e. expression begins in developing somites and extends to adult muscle (Amthor et al., 2004). Myostatin transcript contains a small signal sequence at the N-terminus followed by a large (approximately 28 kDa) pro-peptide region, also called the latency association protein, and a smaller 12 kDa 'mature' region at the C-terminus (Patel and Amthor, 2005). The signal sequence is required for processing and secretion. The propeptide

region regulates the biological activity of myostatin. Biological inactive myostatin is secreted in form of homo-dimers.

Complementary to the inhibitory effect of pro-peptides, there are other myostatin binding proteins that regulate its biological activity. BMP-1/Tolloid (TLD) family of metalloproteinases can cleave the propeptide region of myostatin, which frees the mature fragment of myostatin and thereby permitting receptor activation (Wolfman et al., 2003). Follistatin is a powerful endogenous antagonist of myostatin but also of other members of the TGF- β family (Amthor et al., 2002; Amthor et al., 2004). The homologue of follistatin encoded by follistatin related gene (FLRG) inhibits also the biological activity of myostatin. In addition, circulating myostatin can bind to growth and differentiation factor-associated serum protein-1 (GASP-1) that inhibits myostatin activity (Hill et al., 2003).

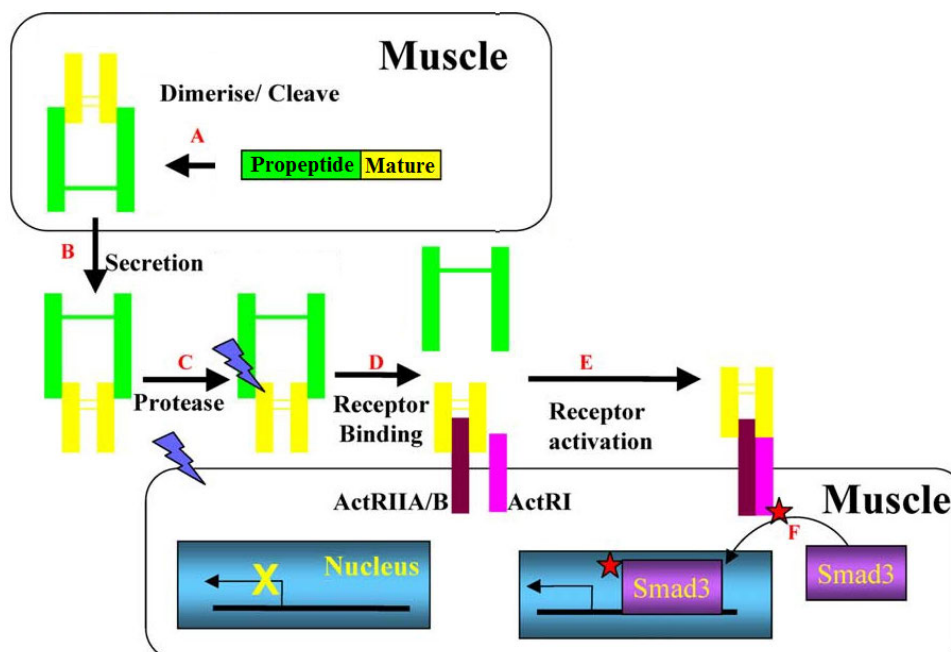


Figure 19: Myostatin processing and receptor activation. (A) Di-sulphide bonds cause dimerisation of myostatin. Mature region cleaved from propeptide region. Propeptide portion forms non-covalent link to mature region. (B) Inactive Myostatin is secreted by muscle cells. (C) Proteases on muscle cells release propeptide from mature region. (D) Mature region binds type II Activin receptor. (E) Transphosphorylation leads to activation of type I receptor. (F) Type I receptor phosphorylates Smad3 which facilitates translocation into nucleus where it initiates gene transcription. According to (Patel and Amthor, 2005).

Myostatin binds to the type II receptor activin receptor type 2b (ActRIIB) and the type I receptor activin-receptor-like kinase (ALK5) (Figure 19) (Rebbapragada et al., 2003; Trendelenburg et al., 2009). Upon activation of the receptors, Smad2 and Smad3 are phosphorylated, and these Smads form a complex with Smad4 (Lagna et al., 1996; Zhang et al., 1996). This complex translocates into the nucleus to activate gene transcription, but little is known about direct target genes. Inhibition of the myostatin-Smad2/3 pathway promotes muscle hypertrophy (Bogdanovich et al., 2002; Whittemore et al., 2003). Myostatin inhibits Akt/mTOR/p70S6K signaling in myoblasts and myotubes (Bodine et al., 2001b; Trendelenburg et al., 2009), which enhances expression of E3 ubiquitin ligases MuRF1 and MAFbx (Bodine et al., 2001a). Inhibition of myostatin signaling was introduced as a therapeutical approach. Treatment with a soluble ActRIIB extracellular domain sequesters myostatin, preventing cachexia and extending survival in tumor bearing mice (Zhou et al., 2010). Furthermore, treatment with follistatin increases muscle mass and strength (Lee, 2007; Lee and McPherron, 2001). However, expression of dominant-negative ActRIIB, or downregulation of Smad2 and Smad3, does not prevent muscle loss in denervated muscle (Sartori et al., 2009). Thus, various reports show that myostatin inhibition increases the muscle mass, but the beneficial effects of myostatin blockade in pathological settings like myopathies are still actively investigated (Dumonceaux et al., 2010; Foster et al., 2009; Kawakami et al., 2013; Rodino-Klapac et al., 2013).

1.10. Muscle physiology in myostatin mutant mice

The mechanisms controlling muscle mass had attracted much attention, especially the gain of muscle mass in myostatin mutant mice (Amthor and Hoogaars, 2012; McPherron et al., 1997; Patel and Amthor, 2005). Larger muscle of myostatin mutant mice results from the increase in muscle fiber number as well as increase in size of individual fibers. It was reported that absolute maximal force of EDL muscle is increased by 34% in male myostatin mutant mice (Mendias et al., 2006). The number of motor axons and the innervation of muscle fibers is also increased in myostatin mutant mice compared to controls, and is accompanied by a reduced capillary density (Gay et al., 2012; Matsakas et al., 2012). The increased absolute maximal force is observed in the mutant mice. The ratio of force to muscle mass (i.e. specific muscle force) is an indication of the efficiency of muscle contraction, and it was also found that this is reduced in myostatin mutant mice (Amthor et al., 2007; Mendias et al., 2011). Furthermore, fatigue resistance of muscle during repetitive contractions is reduced in myostatin mutants, and myofibers appear to be more fragile and susceptible to contraction-induced muscle injury (Mendias et al., 2006; Ploquin et al., 2012). Finally, the absence of myostatin reduces mitochondrial content and leads to an overall faster and more glycolytic muscle phenotype. It results in increased ratio of glycolytic type 2b myofibers at the expense of type 1 and oxidative 2a fibers (Amthor et al., 2007; Girgenrath et al., 2005), and in consequence affects the redox status as well as muscle endurance. In the *tibialis anterior* (TA) muscle of aged mice, the proportions of oxidative type 2a myofibers increase at the expenses of glycolytic type 2b and 2x fibers (Alnaqeeb and Goldspink, 1987; Larsson et al., 1993). In contrast, no fiber type switching was observed in mutant mice with ageing (Siriett et al., 2006). Thus, the consequences of the myostatin mutation are complex, and not all aspects are beneficial for muscle function.

2. Objectives of my thesis

Pre- and postnatal muscle growth relies on muscle precursor dependent hyperplasia and on myofiber hypertrophy. Members of TGF- β superfamily have been implicated in these processes. BMPs and their antagonist Noggin regulate embryonic and fetal myogenesis (Amthor et al., 2002; Amthor et al., 1998; Hirsinger et al., 1997; Ono et al., 2011; Pourquie et al., 1996; Wang et al., 2010). Moreover, a recent study demonstrated that BMP/Noggin signaling also regulates the balance between proliferation, differentiation and self-renewal of adult satellite cells, however, this study was largely performed *ex vivo* and the effect on muscle growth and homeostasis remains unknown (Ono et al., 2011).

Therefore, my first objective was to investigate whether BMP signaling regulates muscle stem cells and stem cell-dependent muscle growth during postnatal development. To answer this question, I focused investigation:

- To study whether BMP signaling is active during postnatal muscle development.
- To establish experimental conditions which allow abrogation of BMP signaling *in vivo*.
- To investigate the consequences of BMP signaling blockade on postnatal satellite cell function and muscle growth.
- To monitor the dynamics of molecular events after manipulation of BMP signaling in cultures of primary myoblasts.

In late postnatal and in adult age, the growth of striated muscle fibers occurs in a satellite cell independent manner. This is associated with an increase of the volume and protein content of myofibers. The role of BMPs during this process was entirely unknown and we were the first to study this function. Another member of the TGF- β family, myostatin, signals via different receptors than BMPs. Myostatin is a known negative regulator of muscle mass. This function became first evident in animals that carry null mutations of myostatin. Such mutants display a pronounced increase in the muscle size (McPherron et al., 1997). However, the exact molecular mechanism

underlying the growth inhibitory effect of myostatin on skeletal muscle remains unknown. Consequently, in the second part of my thesis, my objective was to investigate the role of BMP signaling in precursor cell independent growth and homeostasis of adult skeletal muscle. Moreover, I intended to define the interaction between BMP and myostatin signaling.

Therefore, I set out to investigate the following questions:

- Is the BMP signaling active in adult skeletal muscle? Does BMP signaling regulate differentiated muscle in adults?
- What is the underlying growth stimulus of postnatal hypertrophic muscle growth?
- What is the molecular mechanism that underlies the muscle mass increase in lack of myostatin?
- What is the role of BMP signaling in the context of muscle atrophy?

The mutation in myostatin can arise spontaneously in various domestic animals and are characterized by exaggerated muscle mass and increased muscle force (Boman et al., 2010; McPherron and Lee, 1997; Mosher et al., 2007). The force generation and functional physiology of skeletal muscles in myostatin mutants were extensively studied. However, these studies report conflicting conclusions (Baligand et al., 2010; Giannesini et al., 2013; Matsakas et al., 2012; Mendias et al., 2006). My third objective was to re-investigate the force generation in myostatin mutant mice.

To pursuit this objective, I intended:

- To define the efficiency of force generation *in situ* in mutants.
- To study functional consequence of hypertrophic muscle growth by sex- and age-matched animals.
- To compare our results with reports elsewhere.

Mutations in the dystrophin gene cause Duchenne muscular dystrophy (Duchenne, 1868; Hoffman et al., 1987a). Mdx mice carry mutations in the dystrophin gene and,

therefore, are extensively used as a model for DMD (Bulfield et al., 1984a; Love et al., 1989). The treatment with adeno-associated virus (AAV)-mediated expression of U7 results in skipping of dystrophin-exon-23, and restores dystrophin expression (Goyenvalle et al., 2004). In addition, previous studies showed that soluble activin receptor IIB (sAcvR2B-Fc) binds and inhibits myostatin, and results thus in muscle hypertrophy (Lee and McPherron, 2001). Our group investigated whether the combined treatment with sAcvR2B-Fc and AAV that carries U7 expressing vector (AAV-U7) may act synergistically to improve muscle function in mdx mice.

To answer this question, we aimed:

- To restore dystrophin expression using the AAV-U7 exon skipping approach
- To stimulate muscle growth by treatment with sAcvR2B-Fc
- To study functional quality of muscle upon multi-drug treatment
- To evaluate whether the combined treatment acts synergistically to improve muscle function

3. Results

3.1. Publication no. 1: BMP signaling controls satellite cell dependent postnatal muscle growth

3.1.1. Summary

In this part of my project, I abrogated BMP signaling in postnatal skeletal muscle of mice using an Adeno-associated virus (AAV) serotype 2/1 that carries Noggin expressing vector. Overexpression of Noggin during postnatal muscle growth severely retarded muscle growth and strongly reduced the pool of satellite cells. A decreased satellite cell proliferation caused the formation of smaller myofibers that were depleted of myonuclei. In collaboration with Dr. Fabien Le Grand and colleagues (Paris, France), I used primary myoblast cell culture and I found that BMP signaling is pivotal to maintain muscle precursors in an activated and proliferating state. I conclude from this work that BMP signaling is required for myofiber growth by regulating satellite cell proliferation during postnatal development.

The results of this work are summarized in the presented manuscript (Pages 47 - 82):

Schirwis, E., Amalia Stantzou, A., Mouisel, E., Alonso-Martin, S., Rochat, A., Zammit, P., Relaix, F., Birchmeier, C., Le Grand, F., Amthor, H. BMP signaling controls satellite cell dependent postnatal muscle growth.

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BMP signaling controls satellite cell dependent postnatal muscle growth

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ABSTRACT

Antagonistic gradients of Bone Morphogenetic Proteins (BMPs), a subfamily of diffusible morphogens of the TGF- β family of signaling molecules, and their antagonist Noggin have been implicated in determining the entry of embryonic myogenic precursors into muscle differentiation. Here we demonstrate that the signaling system BMP/Noggin regulates the balance between the generation and differentiation of the resident stem cells of postnatal skeletal muscle, so-called satellite cells, thereby defining postnatal muscle growth. Satellite cells from postnatal muscle expressed BMPs, the transmembrane receptor BMPR1A as well as the BMP antagonists Noggin and up-regulated BMP dependent intracellular signaling components. Treatment of postnatal mice with the BMP antagonist Noggin decreased satellite cell proliferation, diminished the myonuclear recruitment during myofiber growth which severely retarded muscle growth. Moreover, in lack of BMP signaling muscle depleted the satellite cell pool. Abrogation of BMP signaling in primary myoblast cultures decreased expression of Pax7, MyoD and markers of cell proliferation, whereas expression of p21 was strongly stimulated, suggesting that cells gained quiescence. Importantly, presence of BMP4 was sufficient to activate the intracellular BMP pathway and MyoD expression. In conclusion, these results show that BMP signaling during postnatal muscle development is required for satellite cell dependent myofiber growth and the generation of adult satellite cell pool.

INTRODUCTION

The basic cellular units of skeletal muscle are myofibers, which are multinuclear syncytia capable of contraction. Myofibers are continuously formed during embryonic, fetal and postnatal/juvenile development from a source of mononuclear muscle progenitors. Multiple molecular events commit these precursors towards a muscle fate, in which the expression of the paired box genes Pax3/Pax7 followed by MyoD and other members of the myogenic regulatory factor family play a central role to gain myogenic identity (1). Terminal differentiation starts with expression of muscle specific proteins and this process coincides with multiple fusions of muscle precursors into myotubes and subsequent maturation in myofibers. However, differentiated muscle cells become mitotically quiescent (2). Therefore, continuous muscle growth requires a sufficient large pool of muscle precursors that from early embryonic development intermingle in between differentiated muscle cells (3). By the end of fetal development, muscle precursors typically locate between basal lamina and sarcolemma of muscle fibers and are henceforth called satellite cells (4). In mice, the number of muscle fibers that form a specific muscle is laid down during prenatal development. During the first three weeks of postnatal mouse development, muscle fibers grow by recruiting satellite cells thereby enlarging myonuclear number as well as by expending cytoplasmic domain (5). Thereafter, muscle fibers grow mainly by expending the cytoplasmic volume without further addition of myonuclei. Whereas postnatal/juvenile satellite cells cycle to generate progenitors to fuse with muscle fibers, they become quiescent in adult muscle and are only reactivated to regenerate damaged muscle fibers (6). The postnatal/juvenile growth period is accompanied by a steady decline in number of muscle satellite cells until 21 days and remains stable thereafter throughout adulthood (5).

Little is known about which intercellular signaling systems guide postnatal/juvenile muscle growth and the setup of adult satellite cell number. We and others previously determined the crucial role of bone morphogenetic proteins (BMPs) during embryonic, fetal and adult muscle growth (7-13). Reminiscent to other members of the TGF- β signalling molecules, BMPs act on target cells via transmembrane serine/threonine kinases receptors. BMPs bind to type II and type I receptors, thereby forming a ligand-receptor complex and permitting the phosphorylation of the type I receptor via the constitutively active type II receptor (14, 15). The type I receptor in

turn phosphorylates the BMP responsive proteins Smad 1/5/8. Phosphorylated Smad1/5/8 proteins subsequently form complexes with co-Smad4 and translocate to the nucleus to regulate transcriptional activity of target genes such as Ids (16). Inhibitors of differentiation/DNA-binding proteins (Id1-4) block E-proteins from binding with the myogenic regulatory transcription factor MyoD and inhibit terminal differentiation (17).

A number of secreted proteins, amongst Noggin, can non-covalently bind BMPs and inhibit receptor binding (8, 18). Previous work demonstrated that during embryonic development, concentration gradients of BMP maintain the Pax3 muscle precursor population and counter gradients of Noggin specify the onset of myogenesis by down-regulating Pax3 expression and up-regulating MyoD expression (12, 13). Noggin induced precocious loss of Pax-3 expression resulted in a growth deficit of embryonic muscle anlagen, whereas BMP stimulated embryonic muscle growth. However, BMP was suggested to act concentration dependent on embryonic muscle, with high concentrations inducing apoptosis, sub-apoptotic concentrations stimulating Pax3 dependent muscle development, and BMP withdrawal inducing myogenic differentiation (7). Interestingly, myotube formation is delayed in Noggin null mice, pointing to a muscle differentiation defect in lack of Noggin (19).

The signaling system BMP/Noggin also regulates satellite cell (SC) function. We previously showed that BMP signaling stimulated SC division and inhibited myogenic differentiation, whereas abrogation of BMP signaling induced precocious differentiation (10). Importantly, we could demonstrate that the receptor BMPRI1A transmits BMP signaling on muscle precursors. Importantly, injection of Noggin into regenerating muscle inhibits BMP signaling and reduces P-Smad1/5/8, Id1 and Id3 proteins levels (20). Moreover, in the Id-mutant mice, the number of proliferating Pax7+ cells is reduced following muscle injury. These data suggest that BMP signaling regulates Id1 and Id3 in muscle satellite cells thereby maintaining their proliferation before terminal myogenic differentiation (20). Furthermore, MyoD binding elements were found in the promoter region of BMPRI1A and MyoD enhances BMPRI1A expression, suggesting that BMP signaling may have an important role for myogenic progression (21).

We here investigated the contribution of BMP signaling during satellite cell dependent postnatal muscle growth. We employed Noggin as a strategy to interfere with BMP signaling and show that myonuclear recruitment as well as the generation of the adult satellite cell pool is severely inhibited.

MATERIALS AND METHODS

Animals

C57Bl6 mice were purchased (Charles River) and kept according to institutional guidelines in the animal facility of the Medical Faculty of the Paris VI University. Animal studies have been approved and were carried out under the laboratory and animal facility licenses A75-13-11 and A91-228-107.

AAV-production

The Noggin construct, prepared by PCR amplification of chick cDNA (Suppl. 1), was subcloned into the pCR2.1-TOPO plasmid vector (TOPO Cloning, Invitrogen) and thereafter introduced into an AAV-2-based vector between the 2 inverted terminal repeat and under the control of the cytomegaly virus promoter using the XhoI and EcoRI sites. The AAV2/1-Noggin (thereafter called AAV-Noggin) was produced in human embryonic kidney 293 cells by the triple-transfection method using the calcium phosphate precipitation technique with both the pAAV2 propeptide plasmid, the pXX6 plasmid coding for the adenoviral sequences essential for AAV production, and the pRepCAp plasmid coding for AAV1. The virus was then purified by 2 cycles of cesium chloride gradient centrifugation and concentrated by dialysis. The final viral preparations were kept in PBS solution at -80°C. The particle titer (number of viral genomes) was determined by a quantitative PCR.

AAV-injection

AAV quantity for intramuscular delivery was calculated dependent on total body weight ($x \text{ } [\mu\text{l}] = 1.5 \times \text{body weight [g]}$), which was 5-30 μl . AAV was injected into the muscles of the anterior compartment of the lower leg of three days old C57Bl6 mice. We here used PBS and AAV-U7-scramble for control experiments as in previous work we never observed any effect on muscle morphology or histology in control injected animals when injecting PBS or AAV-U7-scramble intramuscularly except of some regenerating fibers along the injection trajectory (22-24). AAV-Noggin was used at concentration of 1×10^{13} viral genome (vg)/ml, AAV-BMP4 at 2×10^{13}

vg/ml, and AAV-control at 5×10^{12} vg/ml. At four weeks of age, muscle were isolated and prepared for cryosections/histology, western blotting, qPCR or isolation of single muscle fibers.

Immunocytochemistry/Immunohistochemistry

Immunocytochemistry/histological analyses were performed using primary antibodies against Pax7 (1:2, mouse IgG1, HBSS) and (1:100, guinea pig, produced by (25)), P-Smad1/5/8 (1:100, rabbit, Cell Signaling), Dystrophin (1:50, mouse IgG2a, NCL-dys1, Novocastra), Laminin (1:400, rabbit, Dako), anti-BrdU (1:100, rat, Abcam), MyoD (1:100, mouse IgG1, Sigma), P-HH3 (1:150, rabbit, Millipore) followed by secondary antibodies with various fluorophores (1:400, AlexaFluor®, Invitrogen) and DAPI (1:5000, Sigma). Fluorescence was visualized either by Zeiss Axio Imager with an Orkan camera (Hamatsu) and AxioVision software or by LSM 700 laser scanning microscope (Carl Zeiss) with ZEN-2009 software. Immunoblotting was performed using primary antibodies against P-Erk (1/1000, mouse, Santa Cruz), Erk (1/1000, rabbit, Cell Signaling), P-Smad1/5/8 (1:1000, rabbit, Cell Signaling), Actin (1/10000, Sigma), Smad4 (1/200, mouse, Santa Cruz). Western blots were analysed with Amersham ECL Prime detection reagents and Bio-Rad Image Lab system.

BrdU analysis

Mice were treated with AAV-Noggin or AAV-control which were intramuscularly injected into TA muscle at postnatal day (P) 3. At P14, animals were labeled with BrdU (Invitrogen) by subcutaneous injection with 10 μ l per 1 g body weight for 3 consecutive days. Following sacrifice, histological sections of TA muscle (0.8 μ m) were fixed with 4% paraformaldehyde for 10 min, and permeabilized with methanol (-20°C) for 6 min. Antigens were retrieved by boiling for 30 min at 70-80°C in 0.01 M sodium nitrate (pH 6). This was followed by incubation with HCl (1N) for 20 min at 37°C. Samples were washed in PBS and incubated for 1h in blocking buffer (2% BSA + 2% swine serum). Samples were incubated overnight at 4°C with anti-BrdU (rat, Abcam) in combination with other primary antibodies (see above). Following the

incubation, samples were treated with secondary antibodies Alexa A488 (1:400, Invitrogen) to visualize the anti-BrdU labeled cells.

RNA isolation and real-time PCR

Total RNA was isolated from cells or muscle tissue using Trizol-Chloroform extraction. cDNA synthesis was performed using Superscript II reverse transcription (Invitrogen) and random hexamer primers. PCR was carried on LightCycler 480 Real-Time PCR Systems (Roche) using SSoFast EvaGreen Mic (Bio-Rad) with specific primers. The thermocycling conditions used applied as in manufacture protocol (Bio-Rad). Transcript levels were normalized to GAPDH or Actin-B transcript levels. Statistical analysis was performed using Δ Ct method. The list with 5'-Primers is provided in supplementary data.

Single fiber preparation

Age-matched male and female C57BL6 mice (n=4) at postnatal age P3 and P28 were sacrificed whereby *tibialis anterior* (TA) and *extensor digitorum longus* (EDL) muscles were surgically isolated. Muscles were thereafter digested in 0.2% collagenase Type 1 dissolved in DMEM (Sigma). Individual, viable, non-damaged myofibers were isolated by gently passing through Pasteur pipettes with different sized apertures and abundantly washed in PBS, as described in detail elsewhere (Rosenblatt JD, 1995). Then the myofibers were fixed in 4% paraformaldehyde dissolved in PBS (Sigma) for 10 min, washed, stained with DAPI and mounted on slides.

Primary myoblast cultures

Cell cultures were performed using primary myoblasts extracted from juvenile mice (4 weeks of age). Cells were obtained from the minced muscle tissue following digestion in 0.2% collagenase Type1 (Sigma). Cells were thereafter re-suspended in growth medium consisting of Ham's F10 (Invitrogen) supplemented with 20% FBS and 2.5

ng/ μ l of basic FGF (R&D systems) and pre-plated onto a non-coated culture plates (fibroblasts will adhere to the plate whereas myoblasts remain in suspension) for 2 h. At the end of the pre-plate procedure, the media containing enriched myoblasts was transferred onto collagen-coated Petri dishes. Cultures were maintained in growth medium until cells reached 80% confluence. The myoblast population was enriched by differential adhesion compared with fibroblasts (either by shaking off the myoblasts, by tapping the culture dishes, or by serial 20 min pre-plate procedures following trypsinization), and usually cultures were 95% pure after the fourth passage. Cells were grown on collagen coated plates in presence of 20% calf serum (Gibco). BMP4 (100 ng/mL, Invitrogen) and LDN193189 (10nM, Sigma) was added to the media. Partly, cells were grown in serum-depleted medium for 5 h before exposure to BMP and LDN193189.

Statistical analysis

The wet muscle weights, the mean of myofiber length and diameter and myonuclei number per myofiber were analyzed from at least three mouse legs assumed as independent. The results were expressed as the mean \pm S.E.M. / S.D. as stated in the figure legends. The probability of statistical differences between experimental groups was determined by the Student's t-test. The significance is annotated as (*) for $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$.

RESULTS

BMP signaling is active in postnatal and adult muscle satellite cells

We recently demonstrated that BMP signaling regulates adult skeletal muscle homeostasis (Sartori et al. 2013). We here asked whether the BMP pathway is also active during the postnatal/juvenile growth phase of skeletal muscle. We found the presence of transcripts of exemplary genes involved in the BMP signaling cascade BMP-2 and -4, BMP receptor ALK3 and the ID1 target gene in skeletal muscle from 2, 4 and 8 week old mice (Fig. 1A). Interestingly, ID1, a target gene of BMP signaling pathway (16), tended to decrease its transcription level with increasing age. In order to differentiate which cells respond to BMP signaling, we monitored the presence of P-Smad1/5/8 using immunohistochemistry. Surprisingly, we found expression of P-Smad1/5/8 in myonuclei as well as satellite cells of juvenile and adult muscle (from 2, 4 and 8 weeks old mice respectively) (Fig. 1B; Suppl. Fig. 1). Adult satellite cells, however, expressed only rarely P-Smad1/5/8 at high levels (Fig 1B; Suppl. Fig. 1). We next studied the expression of BMP pathway components on a pure subset of satellite cells which were FACS sorted by using a *gfp* reporter gene targeted into the Pax3 locus as an identifier (26). Real-time PCR analysis revealed the expression of BMP4, the BMP receptors ALK3 and BMP inhibitor Noggin in satellite cells from adult muscles (Fig 1C). These results suggest that BMP signaling plays a role during satellite cell dependent postnatal muscle growth.

BMP signalling is required for postnatal/juvenile muscle growth

We next challenged the role of BMP signaling on postnatal/juvenile muscle growth *in vivo* and abrogated BMP signaling using an AAV vector to overexpress Noggin, a BMP antagonist (Suppl. Fig. 2). Transfection of skeletal muscle with AAV-Noggin at postnatal day 3 (P3) resulted in a severe impairment of muscle growth compared to saline injected controls as evidenced by the anatomical analysis of skeletal muscle at 4 weeks of age (Fig. 2A). The muscle weight of Noggin treated muscles was considerably smaller compared to control limbs (Fig. 2B). Morphometrical analysis of single fibers from *tibialis anterior* (TA) muscles revealed a strong shift of myofiber diameters towards smaller sizes (Fig. 2C), whereas fiber length remained of the

same size compared to controls (Fig. 2D). We next aimed to understand the cellular mechanism behind this growth retardation. Myonuclear number can be considered as a cumulative history of previous satellite cell recruitment during myofiber growth. We therefore determined the total myonuclear number on isolated muscle fibers from TA muscles prior and after Noggin overexpression (Fig. 2E, Suppl. Fig. 3). Prior Noggin transfection at day P3, muscle fibers contained 74 ± 2 myonuclei, which increased in control treated muscles to 419 ± 11 myonuclei, whereas Noggin treated myofibers increased myonuclear number only to 244 ± 5 (mean \pm S.E.M.). Thus, whereas myonuclear number doubled 2.4 fold between days P3 and P28 in controls, presence of Noggin reduced the doubling to 1.6 fold, clear evidence for decreased satellite cell recruitment during postnatal/juvenile myofiber growth.

We next studied the effect of Noggin on satellite cell proliferation during the postnatal growth phase. We treated mice at P3 with AAV-Noggin and administered BrdU at P14 for 3 consecutive days. The population of Pax7+/BrdU+ represents proliferating satellite cells, Pax7+/BrdU- quiescent satellite cells, whereas the Pax7-/BrdU+ population at sublaminar position represent former satellite cells that became differentiated and hence mitotically inactive once recruited into the myofiber (Fig. 3A). Interestingly, Pax7+/BrdU+, Pax7+/BrdU- and the Pax7-/BrdU+ cell population were reduced following Noggin mediated abrogation of BMP signaling (Fig. 3B, 3C). Decreased satellite cell proliferation was therefore the main cellular mechanism causing delayed myonuclear recruitment and reduced postnatal muscle growth, whereas we found no evidence for satellite cell exhaust by precocious differentiation. Interestingly, the reduced Pax7+/BrdU- population suggests a decreased satellite cell self-renewal in lack of BMP signaling.

At about 21 days of age, final satellite cell number is established and does not further increase towards adulthood (5). We therefore asked for the consequence of decreased satellite cell proliferation during the postnatal/juvenile stages for the generation of the adult muscle stem cell pool. Remarkable, treatment with Noggin at day P3 decreased the reservoir of young adult satellite cells at 4 weeks of age to about half size compared to controls, when counting satellite cells using markers Pax7 and m-cadherin (Fig. 4A, 4B). Thus, adult muscle stem cells are generated during the postnatal/juvenile growth phase under the control of BMP signaling.

To test whether AAV-Noggin induced myofiber hypotrophy can alter number of satellite cells per myofiber, we quantified satellite cells following denervation induced muscle atrophy. We found no evidence that denervation induced muscle atrophy reduced the number of satellite cells. Instead, satellite cell number was moderately increased (Suppl. Fig. 4).

BMP signaling is required for myoblast activation and proliferation

We next used primary myoblast cultures to further explore the role of BMP signaling on muscle precursor cells. The *in vitro* system has for advantage to permit monitoring of the rapid dynamics of BMP signaling on cell behavior. Control myoblasts, when cultured with serum conditioned medium, robustly expressed nuclear P-Smad1/5/8 as clear evidence for active BMP signaling (Fig. 5A, 5C). Treatment of myoblasts with the BMP type 1 receptor antagonist LDN193189 strongly decreased nuclear P-Smad1/5/8 expression, whereas treatment with recombinant BMP4 increased P-Smad1/5/8 (Fig. 5A, 5C). P-ERK levels were not altered and thus exclude the involvement of non-canonical pathway in BMP signal transduction (Fig. 5B). LDN193189 induced abrogation of BMP signaling resulted in a strong down-regulation of Pax7 and a complete loss of MyoD expression that was associated with a decreased cell proliferation as evidenced by the lack of pHH3 marked myoblasts (Fig. 5C, 5D). Treatment with recombinant BMP4 had no effect on myogenic markers in serum conditioned medium, likely because the BMP mediated activation of myoblasts cannot be further increased in the presence of serum. Real-time PCR analysis revealed a down-regulation of *Pax7* and *MyoD* already after 6 hours of LDN193189 treatment, whereas the expression of cyclin-dependent protein kinase (CDK) inhibitor *p21* but not *p57* was strongly up-regulated (Fig. 5E). G2/mitotic-specific *cyclin-B2* tended towards a lower transcription level compared to control cultures (Fig. 5E). Interestingly, the BMP signaling pathway interacted with the Delta/Notch pathway as well as with the TGF- β pathway as evidenced by the up-regulation of *Jagged-1* and *ALK5* transcripts (Fig. 5E).

We next explored the effect of BMP signaling on serum starved myoblasts in order to circumvent the non-controlled effect of serum on BMP pathway activation. Serum depletion during 5 hours caused complete loss of nuclear P-Smad1/5/8 accumulation

and a strong down-regulation of MyoD (Fig. 6A). Both, expression of P-Smad1/5/8 and MyoD, was re-established after only 4 hours treatment with recombinant BMP4, evidence that BMP signaling on its own can activate muscle precursors. The effect of BMP4 was blunted in presence of LDN193189, showing that BMP4 truly acts on myoblasts via BMP type 1 receptors. Real-time PCR analysis further supported immunocytochemical findings: *ID1* and *MyoD* were upregulated following BMP4 treatment, whereas LDN193189 had the inverse effect and blunted the effect of BMP4 (Fig. 6B). Other genes such as *Pax7*, *Myogenin*, *p21* and *CycB2* did not change their expression at this early time point following BMP exposure and are therefore less likely direct target of BMP signaling.

DISCUSSION

The present study has provided several important new insights concerning the BMP signaling and its contribution to the regulation of satellite cell dependent muscle growth in postnatal/juvenile skeletal muscle.

We here showed *in vitro* as well as *in vivo* that postnatal/juvenile satellite cells express components of the BMP signaling pathway and respond to BMP signaling, which is reminiscent to previous observations on the role of BMP during embryonic and fetal chick myoblast development as well as adult murine satellite cell regulation (7-11). Therefore, BMP signaling is an important regulator of muscle stem cell activity during all stages of muscle development.

We deciphered for the first time the function of BMP signaling on satellite cell function during postnatal/juvenile muscle growth *in vivo*. We used Noggin, an efficient BMP antagonist (18), as a mean to abrogate BMP signaling during the postnatal/juvenile growth phase. We show that Noggin mediated BMP blockade decreased satellite cell proliferation, which supports previous observations on the role of BMP on fetal and adult muscle precursor regulation (10, 11). This decreased proliferation caused a diminished recruitment of satellite cells during postnatal/juvenile myofiber growth resulting in a largely diminished myonuclear number. However, our *in vivo* experiments neither gave ultimate evidence whether BMP signaling was indeed abrogated at cell autonomous level, nor can we exclude secondary effects on satellite cells, such as signaling via differentiated myofibers. We therefore turned towards cultures of primary myoblast. Treatment with the BMPRI receptor antagonist LDN193189 abrogated BMP signaling, evidence for efficient silencing of the BMP pathway at cell autonomous level. Similar as BMP blockade using Noggin *in vivo*, treatment with LDN193189 decreased cell proliferation of cultured myoblasts. Moreover, we show that MyoD was completely lost in lack of BMP signaling, whereas BMP4 on its own was sufficient to activate myogenic cells and drive MyoD in serum deprived culture condition. In addition to loss of MyoD and failure of proliferation, expression of *Pax7* decreased, whereas expression of *p21* increased, suggesting that absent BMP signaling caused quiescence of muscle stem cells. Interestingly, this is paralleled *in vivo* by a continuous drop in the expression of the BMP target gene *ID1* in skeletal muscle from juvenile development towards adulthood. Furthermore,

whereas postnatal/juvenile satellite cells highly expressed P-Smad1/5/8, adult satellite cells do so rarely. This supports previous findings showing that satellite cells from freshly isolated myofibers did not express BMPRIA and Smad proteins (10). We suggest that a decrease in BMP responsiveness during satellite cell maturation causes quiescence of satellite cells, however, we still lack experimental proof for this hypothesis. Differently to BMP signaling, Notch-signaling is required for maintaining satellite cell quiescence, and interestingly, the Notch pathway becomes increasingly down-regulated during satellite cell maturation (27). Both signaling pathways could be interconnected. In fact, we show here, that BMP blockade up-regulated the Notch ligand *Jagged-1*. Thus BMPs may control satellite cell quiescence via regulation of Notch-signaling.

Neither *in vivo* nor *in vitro* we found evidence of precocious differentiation of myogenic precursors following BMP pathway inhibition, contrasting previous findings in cultures of satellite cells (10). It is important to note that we used far shorter time windows to monitor the effect of BMP signaling *in vitro* as compared to Ono *et al.* (10), who observed signs of precocious differentiation following BMP blockade not before two days culture period. We cannot exclude that satellite cells gain quiescence and thereafter differentiate without passing through S-phase, similarly as reported following suppression of Notch signaling in adult satellite cells (27).

It is now clear that adult muscle satellite cells are of somite origin (3), however, it is less studied how expansion of the satellite cell pool is controlled to give rise to final cell number at adult stages. Here we show that BMP signaling controls the generation of adult satellite cells during the postnatal/juvenile growth phase. We suggest that BMPs maintain precursor proliferation to generate a sufficient precursor cell pool and cells become quiescent once BMP signaling is tapered during muscle maturation. The exact underlying cellular mechanism remains to be elucidated, such as whether BMP regulates the destination of myogenic progenitors by symmetric or asymmetric cell division.

The exact source and identity of BMPs that control postnatal/juvenile satellite cell activity remains obscure. We recently showed that adult skeletal muscle expresses a variety of different BMPs (22). We here show the presence of BMP transcripts in whole muscles as well as in satellite cells at different time points of postnatal/juvenile

development. However, the respective role of BMPs synthesized by satellite cells, by mature muscle or derived from extrinsic sources such as growing bone remains to be determined.

We recently showed that increase in BMP signaling in adult muscle caused fiber hypertrophy, whereas its abrogation resulted in fiber atrophy, both processes independent of satellite cells (22). We here observed a strongly delayed fiber growth following BMP blockade, however, we did not determine whether deregulation of non-satellite cell dependent myofiber hypertrophy contributed to this growth defect. As P-Smad1/5/8 was also expressed in myonuclei of growing postnatal/juvenile fibers, we believe that BMPs have similar signaling activity in growing and adult myofibers.

Abnormal levels of BMP signaling in skeletal muscle can trigger ectopic bone formation such as in patients suffering from fibrodysplasia ossificans progressive (28). Interestingly, myogenic precursors contribute only minimally to BMP-mediated heterotopic ossification (<5%) *in vivo* (29). Using AAV-mediated overexpression of BMP4, we observed an immense ossification of soft tissues in hind limbs of adult mice upon 4 weeks of treatment (Suppl. Fig. 5) similarly as previously reported in the literature (29). Therefore non physiological levels of BMPs can induce an osteogenic program in skeletal muscle, whereas physiological BMP signaling is required for muscle growth and homeostasis.

In conclusion, BMP signaling is an important regulatory system during postnatal/juvenile muscle growth and determines satellite cell dependent myofiber growth and the generation of the adult muscle satellite cell pool.

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Figures

Fig.1: BMP signaling is active in satellite cells in postnatal, juvenile and adult muscle.

(A) Components of BMP signaling are detectable at mRNA level in *tibialis anterior* (TA) muscle from 2, 4 and 8 weeks old wild type mice. (n=3). **(B)** Immunohistochemistry to monitor pSmad1/5/8 expression (green) and Pax7 expression (red) in satellite cells from 2, 4 and 8 weeks old cross sectioned wild type TA muscles. DAPI marks nuclei in blue. Each panel represents 9 exemplary images from different regions of the muscle section. Satellite cells express heterogeneous levels of P-Smad1/5/8 at different ages. Scale bar is 10 μ m. Exemplary images from n=3 muscles for each time point. **(C)** Expression profiling of BMP pathway components transcripts in adult satellite cells. Real-time PCR illustrating expression levels of BMP4, Alk3 and Noggin in Pax3^{GFP/+} satellite cells from adult mice. Gene expressions were normalized against acidic ribosomal phosphoprotein P0. n=3. Data are shown as mean \pm S.E.M.

Figure 1

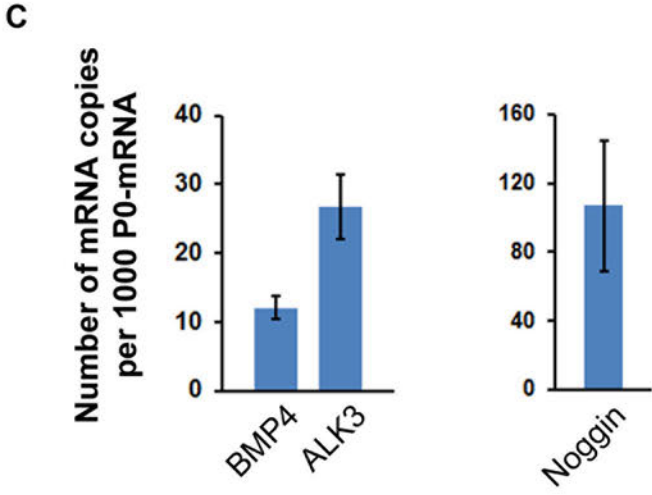
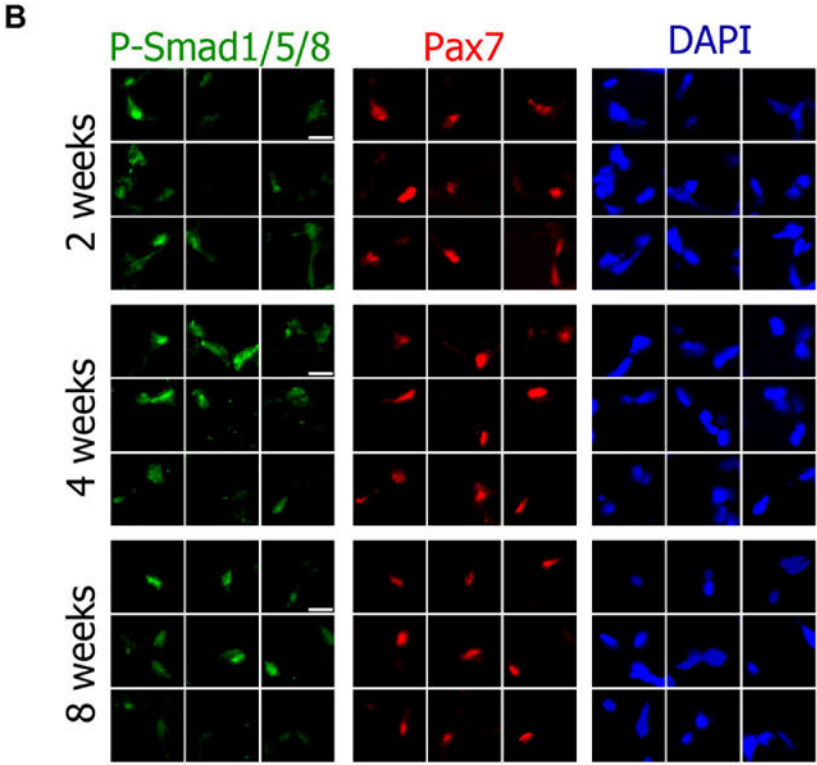
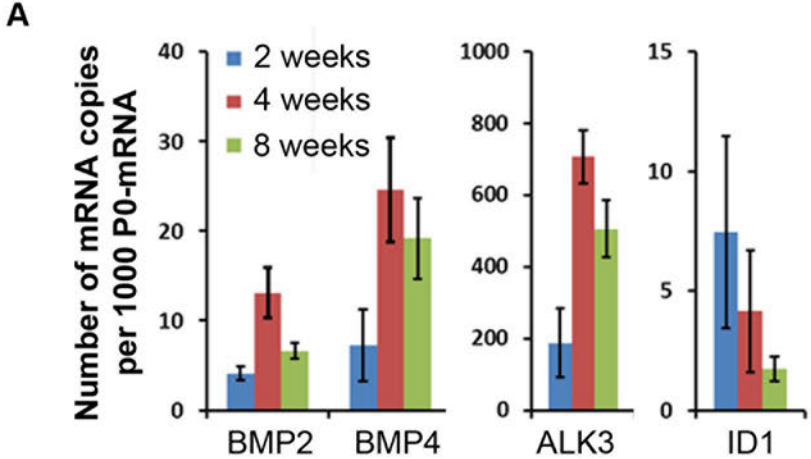


Fig.2: Noggin mediated abrogation of BMP signaling severely retards postnatal muscle growth.

Triceps brachii (TB) muscle and the anterior compartment of the lower hindlimb were transfected with AAV-Noggin at postnatal day 3 (P3). Muscles were analyzed at four weeks of age. **(A)** Dorsal view on the forelimb shows massive muscle hypotrophy of the AAV-Noggin treated TB muscle (left images) compared to the saline injected contralateral side (right image). Exemplary images of n=4 injected mice. **(B)** Diagram depicts muscle wet weights of TB, TA and *extensor digitorum longus* (EDL) muscles. *p<0.05, **p<0.01 and ***p<0.001, n=5 for each condition. Data are shown as mean± S.E.M. **(C, D)** Histogram **(C)** presents the distribution of myofiber diameter of single myofibers from 4 weeks old TA muscles and diagram **(D)** shows myofiber length. For fiber diameter measurement: n=107 and n=128 myofibers were analysed from 3 non-injected control and 5 AAV-treated muscles, respectively. For fiber length measurement: n=65 and n=90 myofibers from 3 non-injected control and 5 AAV-treated muscles, respectively. **(E)** Diagram depicts average myonuclear number per single fiber from TA muscles from 3 days old mice prior AAV-Noggin transfection (control 1) and 4 weeks old TA muscles from AAV-Noggin injected and saline injected muscles (control 3) and non-injected control muscle (control 2). Number of myofibers n=51, n=51, n=47 and n=91 from 4 animals in control 1, 3 animals in control 2, 2 animals in control 3 and 3 AAV-treated animals, respectively. #p<0.05 for control 2 vs. control 3, ***p<0.001 for AAV-treated vs. control 2/3, data are shown as mean± S.D. (D and E).

Figure 2

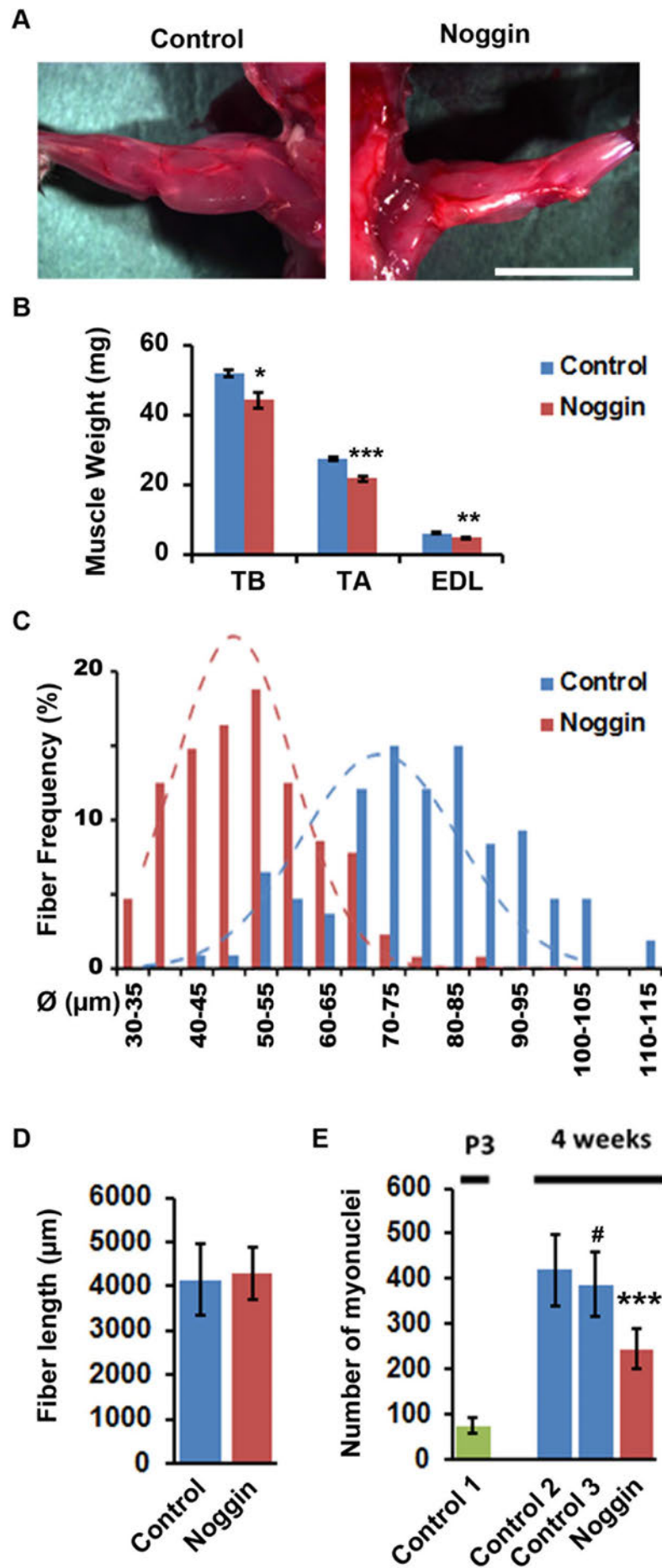


Fig.3: Abrogation of BMP signaling decreases proliferation rate of satellite cells.

(A) Immunohistochemistry to visualize BrdU treated satellite cells. Anterior compartment of the lower hindlimb were transfected with AAV-Noggin at P3. Following two weeks, mice were treated daily with subcutaneous injection of BrdU for 3 days. TA muscle from P17 old mice were stained against Pax7, BrdU and Laminin. Pax7+/BrdU+ cells are indicated with yellow and white arrows (high and low BrdU incorporation, respectively). Pax7+/BrdU- satellite cells and Pax7-/BrdU+ myonuclei are indexed with purple and green arrows, respectively. Scale bar is 20µm. **(B)** Diagram presents number of Pax7+ satellite cells normalized per 100 myofibers (left panel). Lower fraction depicts Pax7+/BrdU- fraction and upper fraction depicts the number of Pax7+/BrdU+ satellite cells that is additionally demonstrated in a separate diagram (right panel). n=4 for each condition. *p<0.05 and ***p<0.001, data are shown as mean± S.E.M. **(C)** Diagram presents number of myonuclei (sublamina situated Pax7-/BrdU+ nuclei) normalized per 100 myofibers. n=4 for each condition. **p<0.01, data are shown as mean± S.E.M.

Figure 3

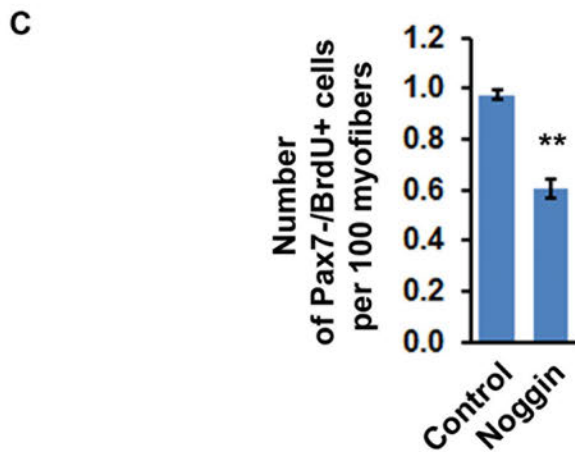
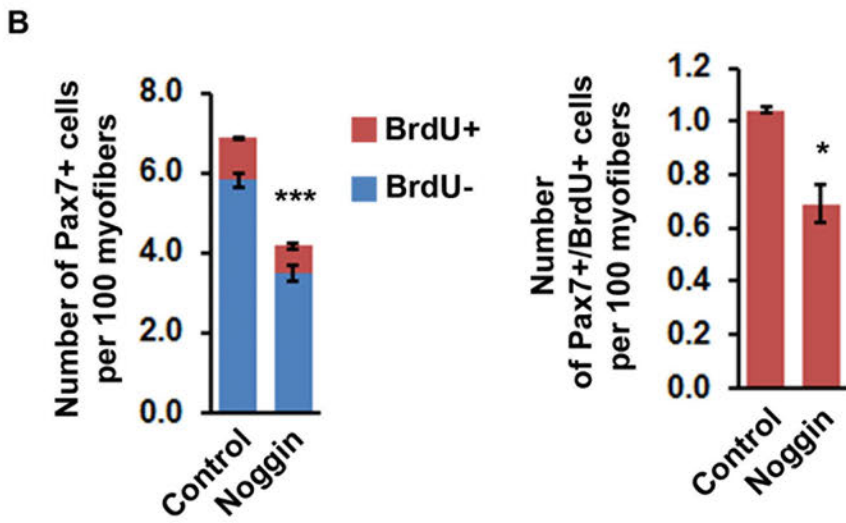
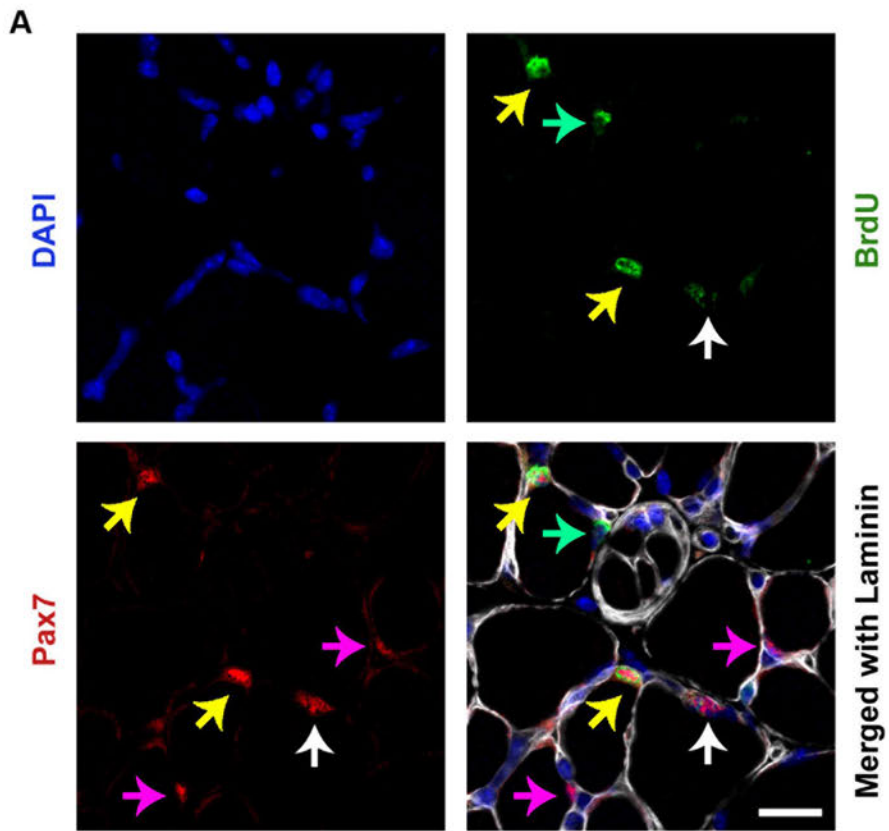


Fig.4: Postnatal BMP signaling determines the satellite cell pool.

TA muscles were injected with AAV-Noggin or saline as control at P3 and muscles were analyzed at 4 weeks of age. **(A)** Exemplary images of immunohistochemistry to illustrate Pax7 positive satellite cells (purple). Laminin (green) illustrates the size of myofibers and DAPI was used as nuclear stain. Scale bar is 20 μ m. **(B)** Diagram depicts number of satellite cells normalized per 100 myofibers. Satellite cells were stained against Pax7 and m-Cadherin and they were quantified from whole TA muscle sections. n=4 for each condition. *p<0.05, data are shown as mean \pm S.E.M.

Figure 4

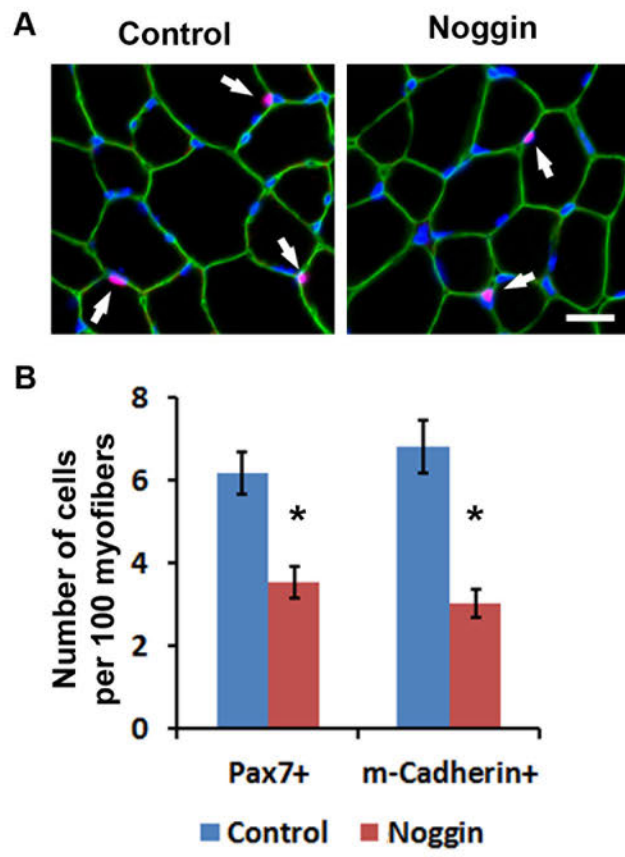


Fig.5: Primary myoblasts respond to BMP signaling.

(A-D) Primary myoblasts were treated for 6 (A, E) and 24 (B-D) hours with LDN193189 (10 μ M) or BMP4 (100 ng/ml). **(A)** Western blot showing P-Smad1/5/8 levels (upper panel). P-Smad1/5/8 was quantified following normalization against Smad4 (lower panel). n=3 for each condition. *p<0.05, data are shown as mean \pm S.E.M **(B)** Western blot showing P-Erk1/2 and total Erk levels (upper panel). P-Erk1/2 was quantified following normalization against total Erk (lower panel). n=3 for each condition. **(C)** Immunocytochemistry to depict Pax7 and P-Smad1/5/8. Merged image is showing Pax7, P-Smad1/5/8 and DAPI. n=2 cell culture replicates. **(D)** Immunocytochemistry to depict MyoD and pHH3. Merged image is showing MyoD, pHH3 and DAPI. n=2 cell culture replicates. **(E)** mRNA expression profile in myoblast cell cultures following 6h treatment with LDN193189 (10nM). Genes involved in myogenic program (*Pax7*, *MyoD*, *Alk5* and *jag1*) and cell cycle (*p21* and *p57*) were normalized glyceraldehyde 3-phosphate dehydrogenase *GAPDH*. n=3 for each condition. *p<0.05, data are shown as mean \pm S.E.M.

Figure 5

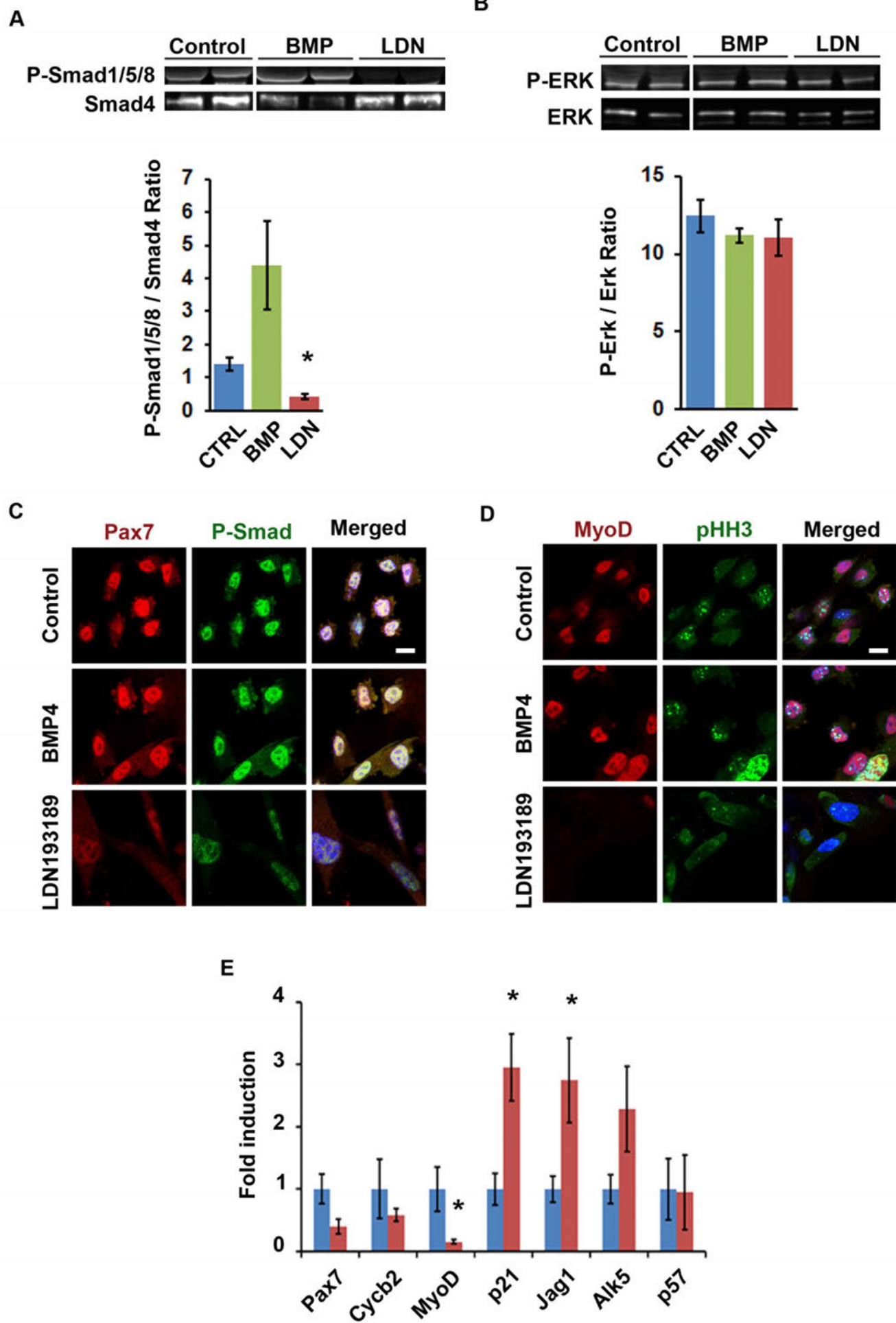
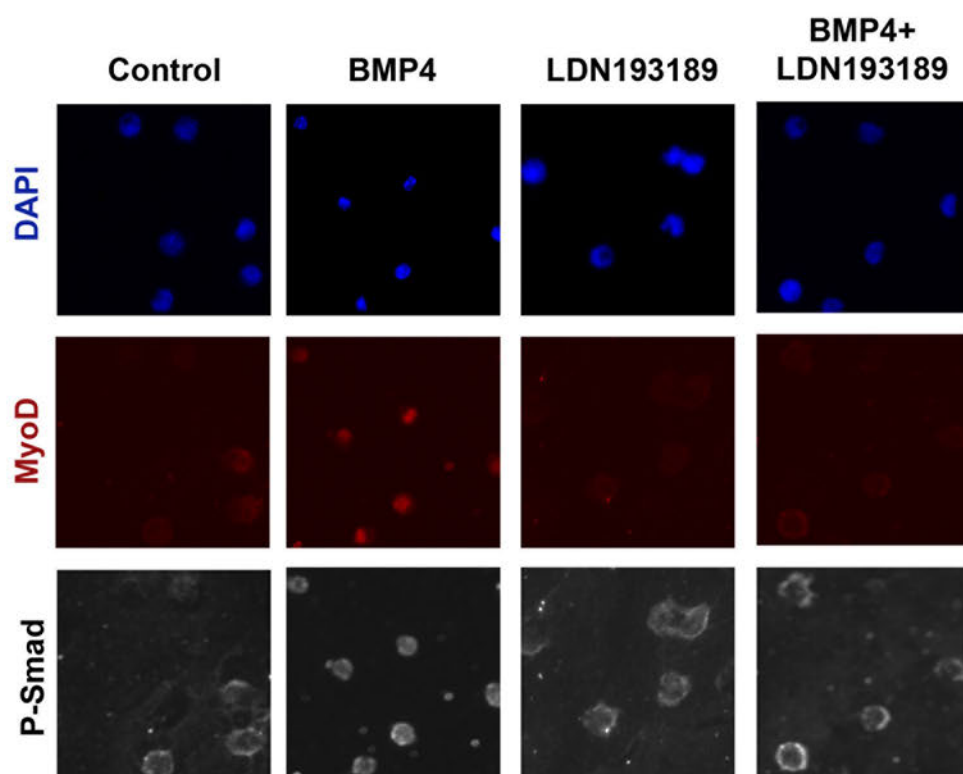


Fig.6: BMP4 is sufficient to activate myoblasts.

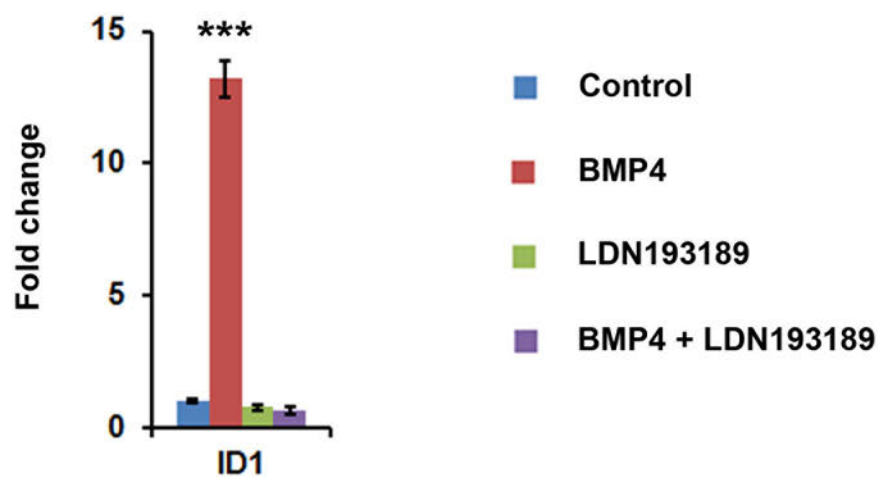
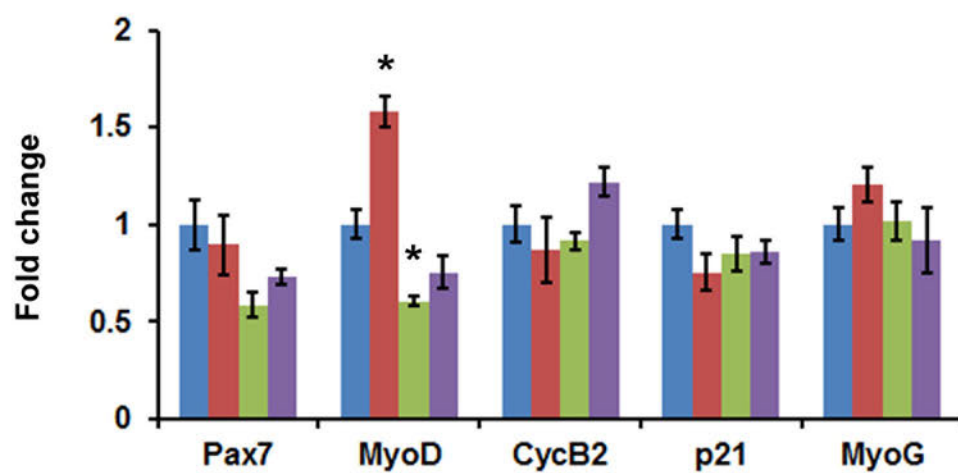
Myoblast cultures were serum depleted for 6 hours and thereafter treated for 4h with LDN193189 (10nM), BMP4 (100ng/ml) and the combination of both. **(A)** Cells were stained against MyoD, P-Smad1/5/8 and DAPI. n=3 culture replicates for each condition. **(B)** Gene expression was analyzed by qRT-PCR. n=3 cultures for each condition and each PCR. *p<0.05 and ***p<0.001, data are shown as mean+/- S.E.M.

Figure 6

A



B

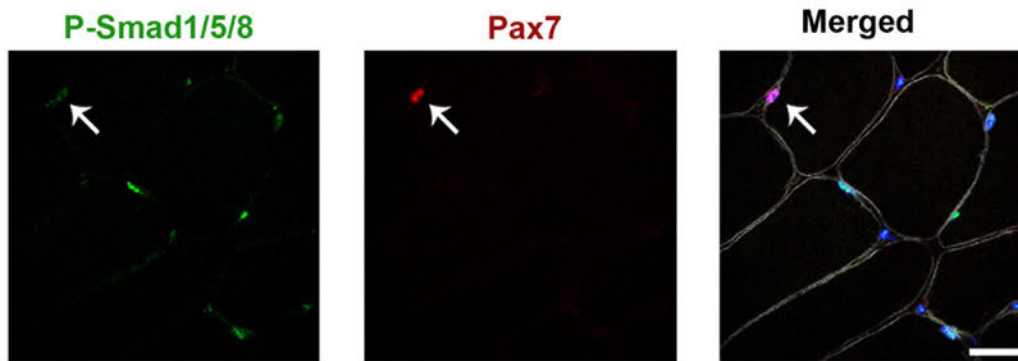


Supplementary Data

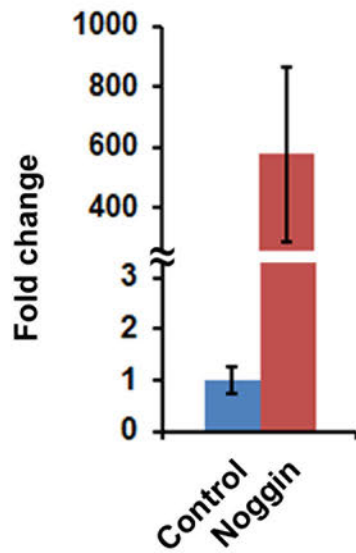
Suppl. Fig. 1: BMPs signal in satellite cells and myonuclei of 4 weeks old mice. Cryosections of TA muscle were stained against Pax7 (red), P-Smad1/5/8 (green) and Dystrophin (white). The Pax7 positive nucleus shows low P-Smad1/5/8 level (white arrow), whereas some but not all myonuclei show strong P-Smad1/5/8 levels. Scale bar is 20 μm .

Suppl. Fig. 2: Validation of AAV mediated Noggin expression in murine TA muscle. To abrogate BMP signaling, adult TA was transfected with AAV-Noggin and examined 2 weeks later. The Noggin vector has a coding sequence of chicken origin. qPCR expression of chicken Noggin in AAV-control treated muscle is considered as unspecific PCR product and set as 1. n=4 for each condition. Data are shown as mean \pm S.E.M.

Supplementary figure 1



Supplementary figure 2

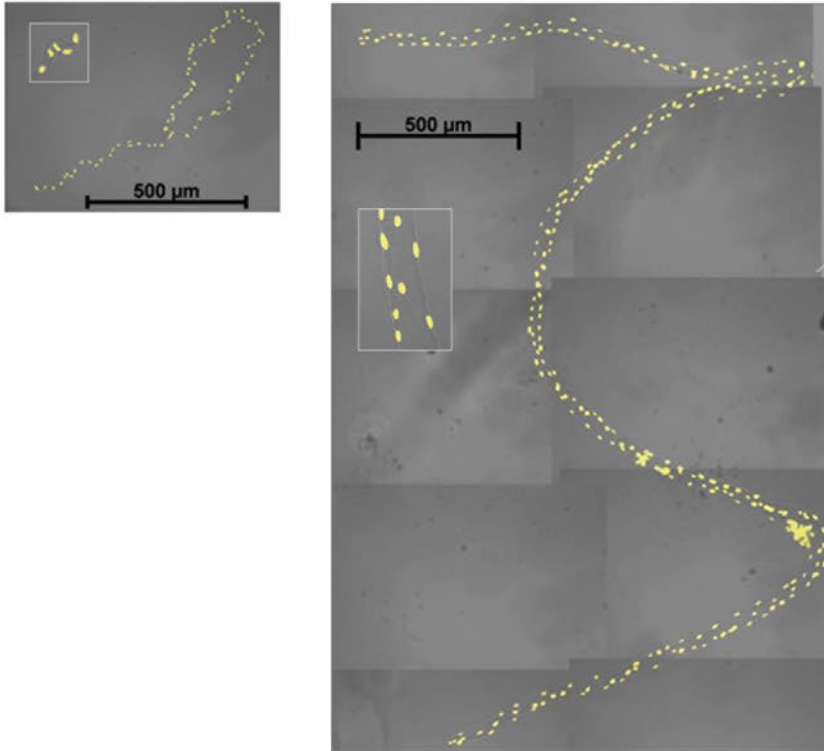


Suppl. Fig. 3: Exemplary images of isolated single fibers of TA muscle from day P3 old mice (left panel) and 4 week old mice (right panel). The inlays show a magnified section of the fibers. Fibers were stained with DAPI and mosaic images were acquired using an automated microscope. Fluorescent images were superimposed over transmission light images. Scale bar is 500 μm

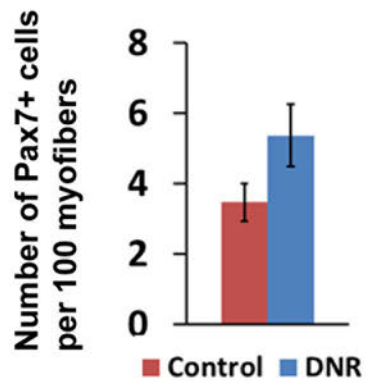
Suppl. Fig. 4: Satellite cell number increases non-significantly in denervated atrophic muscle. Hind limbs of 2 weeks old mice were denervated (ablation of nervus femoralis) and were analyzed after 2 weeks. Crysections of TA muscles were stained against Pax7 and Laminin. n=4 for each condition. Data are shown as mean \pm S.E.M.

Suppl. Fig. 5: AAV mediated overexpression of BMP4 in TA muscles results in massive hindlimb ossification following 4 weeks long treatment of adult C57Bl6 mice. Hindlimbs were stained with alizarin red (bone tissue) and alician blue (collagen rich tissue). Exemplary images are shown of n=5 for each condition. Scale bar is 1 cm.

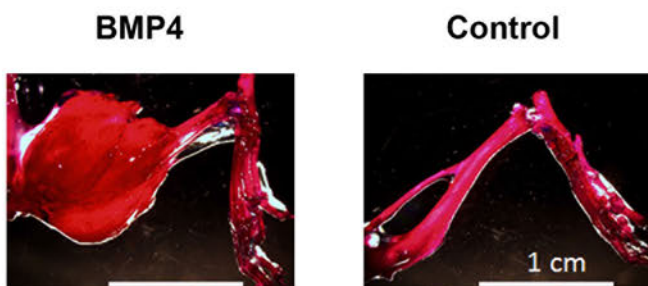
Supplementary figure 3



Supplementary Figure 4



Supplementary figure 5



Supplementary Table

Primers used for quantitative PCR analyses

	Forward primer	Reverse primer
ALK3	CTGGGAGTGGATCTGGATTG	CTGTTTGGCAATAGTTCGCTG
ALK5	TTATGAGAGAATGCTGGTATGCC	AAGAGAGCAGAGTTCCCACGG
BMP2	AGATCTGTACCGCAGGCACT	CCGTTTTCCCACTCATCTCT
BMP4	TGAGGAGTTTCCATCACGAA	CACCTCATTCTCTGGGATGC
chicken Noggin (Plasmid)	CCGCTCGAGATGATGCA AAAAC TGCAAATG	CCGGGATCCCTATTAGTCT CTCCGGGACCTCTT
chicken Noggin	ATGGATCATTCCCAGTGCCTTG	AGCTGCCCACTTTGACGTACCG
GAPDH	GTGAAGGTCGGTGTGAACGG	AATCTCCACTTTGCCACTGC
Jag1	CCCCCTGAGTCTTCTGCTC	GTGACGCGGGACTGATACTC
murine Noggin	TACGTGAAGGTGGGCAGC	ATGGGGTACTGGATGGGAAT
MyoD	CGACTGCTTTCTTCACCACTCC	GGCTGTCCTCTACCCCTCCTC
Myogenin	GCCACAAGCCAGACTCCC	CGCTGGGCTGGGTGTTAG
p21	CCTGCCTGGTTCCTTGC	GAGCAATGTCAAGAGTCGGG
p57	GAGCAAGCGAACAGGCAGG	GGCTGATTGGTGATGGACGG
Pax7	TGGCGTAAGCAGGCAGGAG	GGCAGAAGGTGGTTGAAGG

3.1.3. Additional data not included in the publication no. 1

Apart from my work on postnatal muscle growth, I initiated work to decipher the role of BMP on satellite cell regulation in adult healthy muscle and during muscle regeneration. To determine the role of BMP on the maintenance of the satellite cell pool in the adult muscle, I designed an experiment in which BMP signaling was abrogated by transfecting the anterior compartment of the lower leg of adult C57Bl6 mice with AAV-Noggin. Muscle tissue was analyzed 1.5 month later. I observed a significant decline in the number of Pax7 positive satellite cells in AAV-Noggin treated adult muscle (Figure 20). This finding permitted to conclude that BMP signaling is required for the maintenance of quiescent satellite cells.

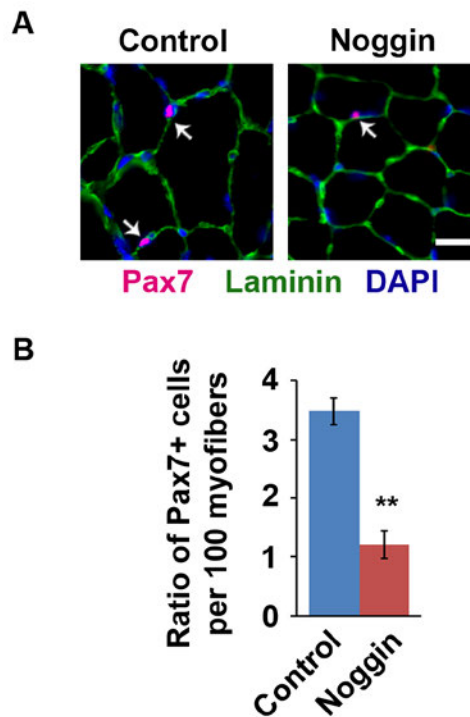


Figure 20: Abrogation of BMP-signaling in adult mice results in loss of Pax7+ satellite cells. AAV-Noggin and AAV-control injection in murine TA muscles at 2 ½ months of age. Muscle tissue was analyzed 1.5 month later. **(A)** Exemplary images of immunohistochemistry to illustrate Pax7 positive satellite cells (purple). Laminin (green) illustrates the size of myofibers and DAPI was used as nuclear stain. Scale bar is 20µm. **(B)** Diagram depicts number of satellite cells normalized per 100 myofibers. Satellite cells were stained against Pax7 and they were quantified from whole TA muscle sections. n=3 for each condition. **p<0.01, data are shown as mean± s.e.m.

I also determined the function of BMP during regeneration of skeletal muscle of adult mice. I designed an experiment in which AAV-Noggin was intramuscularly injected in adult C57Bl6 adult mice one month prior partial muscular cryodamage. Mice were sacrificed one week after cryodamage, a timepoint at which regeneration is well on course (Figure 21A). I counted satellite cells using the marker Pax7 in regenerating muscles and found less muscle precursors in noggin treated muscles (Figure 21B, 21C). Moreover, I observed a strong retardation in the regeneration process of the cryodamaged area after AAV-Noggin treatment (Figure 21D). The myofiber diameter was significantly smaller compared to the controls (Figure 21E). These results suggest that BMP signaling is required for muscle regeneration by permitting sufficient precursor generation and/or by delaying their myogenic differentiation. However, further experiments are required to determine the fate of adult satellite cells and to determine the exact cellular dynamics of muscle regeneration after BMP blockade.

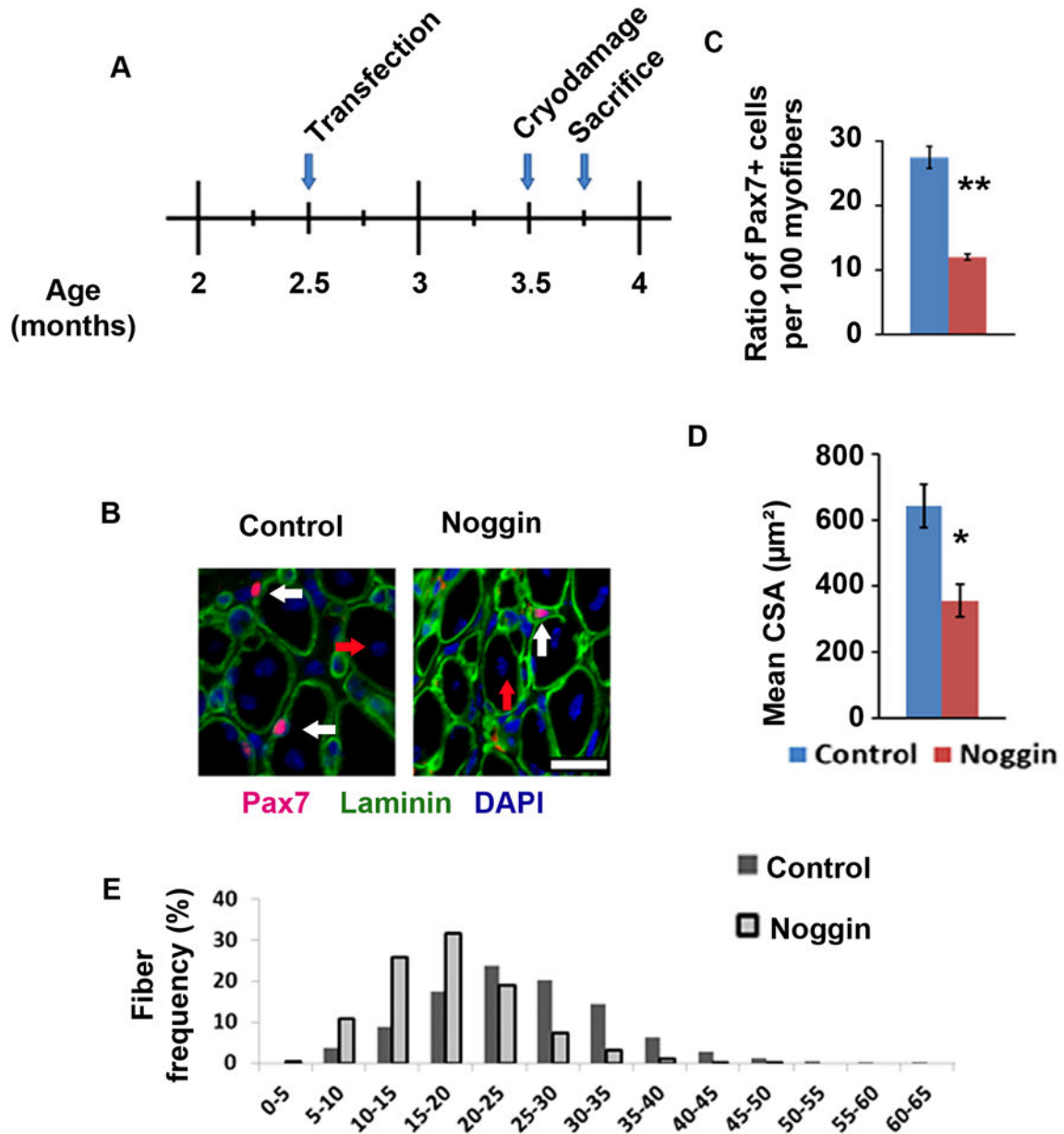


Figure 21: Abrogation of BMP signaling depleted number of satellite cell in regenerating muscle. **(A)** Outline of the experimental protocol. AAV-Noggin was injected into TA muscles of 2.5 month old mice. One month following AAV injection, the cryodamage was applied. After a further week, mice were sacrificed and muscle dissected for analysis. **(B)** Exemplary images of immunohistochemistry to illustrate Pax7 positive satellite cells (purple) that are indicated with white arrows. Laminin (green) illustrates the size of myofibers and DAPI was used as nuclear stain. Centronucleation is indicated with red arrows. Scale bar is 20 μm . **(C)** Diagram depicts number of satellite cells normalized per 100 myofibers. Satellite cells were stained against Pax7 and they were quantified from whole TA muscle sections. $n=3$ for each condition. $**p<0.01$, data are shown as mean \pm s.e.m. **(D, E)** Diagram **(D)** shows the mean of cross section area in new regenerated fibers from 3.5 months old TA muscles and histogram **(E)** presents the distribution of myofiber diameter in cryodamaged area. $*p<0.05$, $n=3$ for each condition.

3.2. Publication no. 2: BMP signaling controls muscle mass

3.2.1. Summary

This part of my thesis was done in collaboration with the group of Prof. Dr. Marco Sandri (Padova, Italy). My contribution to the project consisted in studying the effect of modulation of BMP signaling following intramuscular application of AAV that expressed the BMP inhibitor Noggin or a constitutively activated BMP receptor Alk3. I investigated the impact of BMP signaling following denervation induced atrophy in GDF5 and myostatin mutant mice. I performed on these muscle samples histology, immunohistology, morphometrics, as well as mRNA and protein expression analysis. A number of experiments were performed together with Roberta Sartori, Padua, another first author on our publication. I provided many tissues to our Italian collaborators for further molecular analysis. I participated in the organization of experiments, data analysis and the preparation of the manuscript. In addition, I maintained the mutant mouse colonies (GDF5, myostatin mutants and control littermates).

The results of this project show that BMP activity causes hypertrophic muscle growth in adults. Overexpression of activated BMP receptor induces hypertrophy and rescues denervation-induced muscle atrophy. In contrast, inhibition of BMP signaling by Noggin expression, the use of pharmacological Alk3 inhibition (LDN192189) and the downregulation of Smad expression using RNA interference resulted in muscle atrophy. Abrogation of both, the myostatin and the BMP signaling pathways, by muscle-specific mutation of Smad4 using the cre/loxP technology caused muscle atrophy and aggravated the muscle atrophy caused by denervation or fasting. Similarly, Noggin-induced inhibition of BMP signaling reduced the hypertrophy and aggravated denervation-induced muscle loss in myostatin mutant mice. We observed that canonical BMP signaling negatively regulates the expression of the Fbxo30 ubiquitin ligase, a novel regulator of catabolic processes in skeletal muscle.

The results of this chapter have been published (Pages 87 - 146):

Sartori, R.*, **Schirwis, E.***, Blaauw, B., Bortolanza, S., Zhao, J., Enzo, E., Stantzou, A., Mouisel, E., Toniolo, L., Ferry, A., Stricker, S., Goldberg, A.L., Dupont, S., Piccolo, S. §, Amthor, H. §, Sandri, M[§]. BMP signaling controls muscle mass. **Nat Genet.** 2013 Sep 29.

*Equally contributed; §Co-corresponding authors

<https://dx.doi.org/10.1038/ng.2772>

3.3. Publication no. 3: The beneficial effect of myostatin deficiency on maximal muscle force and power is attenuated with age

3.3.1. Summary

This study was performed in collaboration with Prof. Dr. Arnaud Ferry and colleagues (Paris, France). My contribution to the project was to assist Prof. Ferry in the measurements of muscle force *in situ*. In addition, I maintained the myostatin mutants strain and genotyped the mutants and control littermates used in this study.

We found that absolute differences in maximal force and power are greater in young than old mice when myostatin mutants and controls were compared. Further, we observed a sex-specific effect, i.e. the increase in maximal force and power was more pronounced in female than in male myostatin mutants. Interestingly, young myostatin mutant females were stronger than the mutant or control males. Whereas absolute power was increased, fatigue resistance was decreased in the young myostatin mutants of both sexes.

The results of this chapter have been published (Pages 149 - 157):

Schirwis, E., Agbulut, O., Vadrot, N., Mouisel, E., Hourdé, C., Bonnieu, A., Butler-Browne, G., Amthor, H., Ferry, A. The beneficial effect of myostatin deficiency on maximal muscle force and power is attenuated with age. **Exp Gerontol.** 2013 Feb;48(2):183-90.

<https://dx.doi.org/10.1016/j.exger.2012.11.008>

Supplemental Data

Effect		Groups	Post-hoc (Tukey)/contrast analysis (Fisher PLSD)	Dependant variable
Effect of age in female KO, effect of genotype in old female, effect of sex in old KO	2 way- anova (groups x contractions)	Female KO 6m, Female KO 2y, Female WT 2y, Male KO 2y	Contrast (to examine interaction)	Force drop following lengthening contractions
Effect of gender, effect of age (only in KO)	2 way-anova (gender x age)	Female KO 6m, female KO 2y, male KO 6m, male KO 2y	contrast	P0, sP0, weight, Pmax, sPmax
Effect of genotype, Effect of age (only in KO)	2 way-anova (genotype x age)	Female KO 6m, female KO 2y, female WT 6m, female WT 2y	contrast	P0, sP0...
Effect of genotype in male 6m, effect of age in male KO	1 way-anova	Male KO 6m, male WT 6m, male KO 2y)	Post hoc	P0, sP0...

3.4. Publication no. 4: Combined effect of AAV-U7-induced dystrophin exon skipping and soluble activin type 2b receptor in mdx mice

3.4.1. Summary

This study was not a large part of my thesis, and I contributed to histological and morphological analyses. In collaboration with Dr. Wilhelm Hoogsars (Leiden, Holland), we found that the treatment with AAV-U7 mediates exon-skipping in the dystrophin gene and restores dystrophin expression. The treatment with soluble activin receptor type 2b increases tetanic force in mdx mice that carry a dystrophin mutation. We observed that the combined treatment with AAV-U7 and activin receptor 2b brought an improvement of muscle functions in mutant mice.

The results of this chapter have been published (Pages 159 - 170):

Hoogaars, W. M., Mouisel, E., Pasternack, A., Hulmi, J. J., Relizani, K., Schuelke, M., **Schirwis, E.**, Garcia, L., Ritvos, O., Ferry, A., t Hoen, P. A. and Amthor, H. Combined Effect of AAV-U7-Induced Dystrophin Exon Skipping and Soluble Activin Type IIB Receptor in mdx Mice. **Hum Gene Ther.** 2012 Dec; 23: 1269-1279.

<https://dx.doi.org/10.1089/hum.2012.056>

4. Discussion and perspectives

4.1. BMP signaling in satellite cell-dependent muscle growth

During early postnatal development, muscle growth depends on satellite cells and on their capacity to proliferate. Various factors modulate satellite cell proliferation and differentiation *in vitro* (Collins-Hooper et al., 2012; Conboy and Rando, 2002; Ono et al., 2011; Taylor et al., 2001; Wang et al., 2010), but little is known about their contribution to postnatal and juvenile muscle growth *in vivo*. We observed that components of the BMP signaling pathway are present in satellite cells. We showed that satellite cell proliferation *in vitro* was decreased by block of BMP signaling, and that this reduced satellite cell numbers in juvenile/young adult muscle *in vivo*. The reduced numbers of satellite cells correlated with impaired postnatal myofiber growth. This parallels previous studies that investigated BMP functions during embryonic and fetal chick and mouse development *in vivo*, or mouse satellite cells *in vitro* (Amthor et al., 2002; Ono et al., 2011; Wang et al., 2010). The fact that BMPs are expressed by satellite cells indicates that BMPs act in a cell autonomous manner. However, our *in vivo* experiments cannot distinguish between cell autonomous or non-autonomous mechanisms. To resolve this question, further experiments are required.

We further investigated BMP signaling in cultured myoblasts and used the BMPRI receptor antagonist LDN193189. LDN193189 treatment resulted in decreased cell proliferation of cultured myoblasts as evidenced by a decline of phospho-Histone 3 in myonuclei. LDN193189 also decreased Pax7 and increased p21 expression. Interestingly, P-Smad1/5/8 levels were higher in juvenile than adult satellite cells, suggesting that BMP signaling decreased when satellite cells became quiescent towards adult stages. This supports previous findings that non-activated satellite cells from freshly isolated myofibers do not contain Alk3 and P-Smad proteins (Ono et al., 2011). It is thus possible that decreased or even abrogated BMP signaling during satellite cell maturation results in their quiescence. Notch-signaling inhibits myogenesis by activating expression of the transcription factor Hes1 that in turn suppresses MyoD (Jarriault et al., 1995). I found that Jagged-1 expression is up-regulated by LDN193189 indicating that Notch signaling is activated. Notch signaling was implicated in quiescence, and for instance mutations of the RBP-J, the primary

transcriptional mediator of Notch signals, activate adult satellite cells (Bjornson et al., 2012; Mourikis et al., 2012; Vasyutina et al., 2007). Therefore, both signaling pathways could interact and BMPs may control satellite cell quiescence by regulating Notch-signaling. Such interaction between signaling systems were observed previously in the muscle, for instance the balance between proliferation and differentiation of satellite cells is regulated by a cross-talk between Notch and Wnt/ β -catenin signaling (Brack et al., 2008).

We conclude from our study that BMP is an important regulator during postnatal/juvenile muscle growth. In particular, I found that inhibition of BMP signaling changed the size of the satellite cell pool during the first weeks of postnatal life by controlling their proliferation, which subsequently affected muscle fiber growth. We suggest that BMP signaling controls the proliferation activity of precursor cells to generate a sufficient satellite cell pool. Once BMP signaling is tapered during muscle maturation, satellite cells become quiescent. Likely, this process is coupled to Notch signaling. It is also possible that BMPs play an inverse role than angiopoietin 1 (Ang1) with its receptor Tie-2 in the regulation of myogenic precursor cell fate. Abou-Khalil and colleagues demonstrated that Ang1 overexpression increased the number of quiescent satellite cells *in vivo*, similar to the effect of BMP inhibition (Abou-Khalil et al., 2009). The exact underlying cellular mechanism remains to be elucidated, such as whether BMP regulates the destination of myogenic progenitors by symmetric or asymmetric cell division.

In the second part of my thesis, I showed that increase in BMP signaling in adult muscle caused fiber hypertrophy, whereas its abrogation resulted in fiber atrophy, both processes independent of satellite cells (Sartori et al., 2013). By analyzing postnatal muscle growth, we observed a delayed myofiber growth upon abrogation of BMP signaling. As discussed above, the lack in satellite cells is one factor for this delay in growth. However, we did not determine whether this growth defect was related to the deregulation of satellite cell independent myofiber hypertrophy. As P-Smad1/5/8 was also expressed in myonuclei of growing postnatal/juvenile fibers, we believe that BMPs have similar signaling activity in growing juvenile and adult myofibers.

Despite our extensive study, some questions remain open. As it indicated previously, Noggin does not bind to all BMPs and therefore does not completely abrogate BMP signaling. In our work, we cannot define which ligand(s) of the BMP family signal to satellite cells *in vivo*. It remains also unclear whether Noggin induced a complete block of BMP signaling. In fact, I was only able to demonstrate *in vitro* that LDN193189 induced a downregulation of intracellular components of the BMP signaling cascade in myoblasts, whereas *in-vivo* this experiment was technically very challenging and so far I did not succeed.

Further, the exact source and identity of BMPs that control postnatal/juvenile satellite cell activity remains obscure. I showed that different BMPs are expressed in adult skeletal muscle and satellite cells. Moreover, bone and cartilage also produce BMPs (Chen et al., 1997; Erlacher et al., 1998). The function of these BMPs which are produced by different cell types remains to be determined.

In our study, we used as control either injection of PBS or AAV-control vector that expresses a randomized sequence. Previous work in the laboratory of Luis Garcia did not reveal AAV-induced immune response in muscle, although some regenerating fibers can be observed along the injection trajectory after intramuscular application (Hoogaars et al., 2012; Pietri-Rouxel et al., 2010). In my study, I did not however systematically compare whether injection of PBS or AAV-control vector affected muscle morphology and satellite cell numbers in a different manner.

Previous results indicated that BMPs inhibit differentiation and the expression of transcription factors associated with differentiation; conversely, abrogation of BMP triggered differentiation (Ono et al., 2011). In our work, we did not find any evidence that inhibition of BMP signaling, neither *in vivo* nor *in vitro*, triggered differentiation of myogenic progenitor cells. Instead, our data indicate that ablation of BMP signaling inhibits proliferation and promotes quiescence. Our results indicate that BMPs also participate in regulating the transition from quiescent to proliferation and the self-renewal of satellite cells. However, further investigations are required provide formal proof of these cellular mechanisms. Although my work illustrates the important role of BMP signaling during satellite cell dependent muscle growth, the exact molecular mechanism remains unclear. Further work is required to identify

target genes of BMP signaling and possible interaction with other signaling systems that regulate satellite cells.

We found that BMP4 activates MyoD and also Id1 expression in primary myoblast cell culture. However, Id1 is a negative regulator of MyoD activity. Ono et al. reported that proliferating satellite cell-derived myoblasts express both MyoD and Id1. Id1 levels are then reduced as satellite cells undergo differentiation (Ono et al., 2011; Ono et al., 2009). Further experiments are required to study whether MyoD upregulation not a simple secondary effect to curtail Id1 induction in order to prevent excessive proliferation.

In addition, I initiated to investigate the role of BMP signaling in the process of muscle regeneration in adults. Further experiments are required to determine the fate of adult satellite cells and to determine the exact cellular dynamics of muscle regeneration after BMP blockade.

4.2. BMP and myostatin signaling in muscle hypertrophy and atrophy

In my thesis, I provide important new insights on role of BMP signaling in skeletal muscle. A major part of the thesis is the investigation of BMP function in satellite cell-dependent muscle growth during juvenile life. Beside the study on satellite cells, I investigated the cellular and molecular mechanism of BMP and myostatin signaling in satellite cell-independent growth during muscle hypertrophy and maintenance of adult muscle. Our data revealed a previously unknown role of BMP signaling to cause hypertrophic muscle growth, whereas its abrogation resulted in fiber atrophy. The result was surprising, since a related molecule, myostatin, serves as a negative regulator of muscle growth. It should, however, be noted that myostatin receptors use Smad2/3 whereas BMPs signal via Smad1/5/8 (Taylor et al., 2001; Trendelenburg et al., 2009). The different downstream signaling molecules used by BMP and myostatin receptors could thus account for the different biological effects. The growth promoting activity of BMPs and growth inhibiting activity of myostatin led us to hypothesize that an interaction between both pathways might exist by competing for Smad4, a common Smad used by both signaling systems. For instance, a decrease in myostatin is expected to result in decreased P-Smad2/3, which might release Smad4 that would then be free to bind to P-Smad1/5/8. Vice versa, when the BMP pathway activity is low, more Smad4 would be available for binding P-Smad2/3 (Figure 22). In support of this, we found together with the group of Marco Sandri that recruitment of Smad4 and P-Smad1/5/8 to the ID1 promoter was enhanced in myostatin mutant mice, and this promoter binding was reverted by Noggin expression.

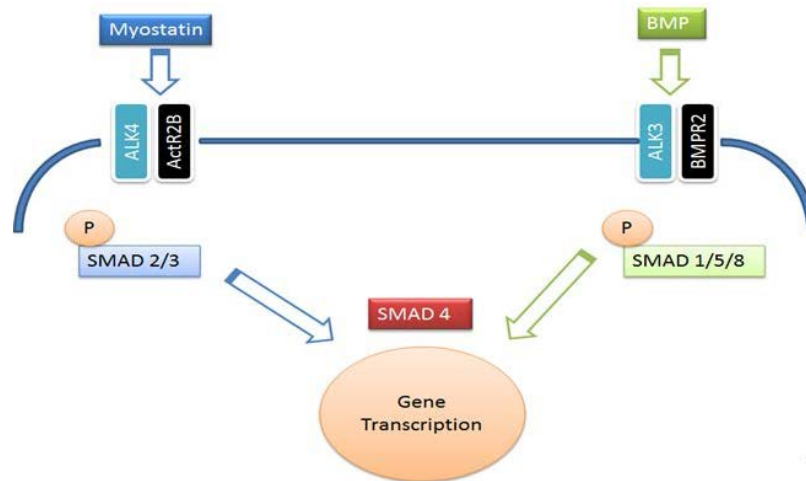


Figure 22: Scheme illustrates the hypothesis of muscle mass regulation in adults that depends on BMP and myostatin signaling.

When both signaling systems are blocked (myostatin mutation and noggin treatment), muscle became atrophic and the effect of the hypertrophic effect of the myostatin mutation was entirely lost. Thus, BMP signaling is the underlying signaling pathway, which is required for the hypertrophic muscle growth in absence of myostatin. In response to denervation-induced atrophy, the muscle compensates by upregulating BMP signaling to prevent excessive muscle loss under pathological conditions. We hypothesized that under normal circumstances, however, BMPs and myostatin compete with each other and the balance between these two signaling pathways maintains muscle mass at its correct size. The disturbance of this balance shifts the muscle mass towards either hypertrophy or atrophy.

We investigated the molecular mechanism underlying the protective action of the BMPs in muscle that undergoes atrophy. Our data indicate that BMP signaling increases muscle mass by repressing protein degradation. We identified a novel E3 ubiquitin ligase, Fbxo30, which is responsible for the protein degradation in in atrophying muscle. We found that inhibition of Fbxo30 using siRNA has no effect on fiber size in mice under normal condition, similar to previous findings on atrogen 1 and MuRF1 (Bodine et al., 2001a). In addition, Fbxo30 undergoes auto-ubiquitination, similar to other E3 ligases in the absence of their primary substrates (Chen et al., 2012). We suggest that activation of Fbxo30 activity during denervation would amplify its degradation in a self-catalyzed manner. Thus, the loss of Fbxo30 protein that

occurs in denervated muscles has to be replenished and this process is tightly controlled by transcriptional regulation. As the blockade of BMP pathway suppresses ligase activity, we concluded that BMP signaling prevents activation of the Fbxo30 gene and protects thus adult muscle from excessive protein degradation.

Several BMPs are expressed during development and close to developing muscle (Amthor et al., 2002; Chang and Hemmati-Brivanlou, 1999; Gamer et al., 2005; Hirsinger et al., 1997; Kim et al., 2001; Seemann et al., 2009). However, the developmental expression profiles of BMPs are highly dynamic. In the adult muscle, BMP-9-14 are not detectable, but upon denervation BMP-13 and BMP-14 are strongly induced. Both of these induced BMPs bind Noggin (Chang and Hemmati-Brivanlou, 1999; Seemann et al., 2009; Song et al., 2010). The denervation induced atrophy after Noggin over-expression is as pronounced as the one observed in BMP14 (GDF5) mutant mice, suggesting that BMP14 is the important BMP during this process. In contrast to other members of the TGF- β superfamily, BMP14 has a high affinity to and exclusively binding to BMP receptor IB (BMPR-IB), known also as ALK6. Interestingly, Winbanks and colleagues reported that BMPR-IB expression was increased after denervation (Winbanks et al., 2013).

Despite our extensive analysis of BMP signaling in the muscle, there are points that remained open. Noggin is a well-known negative regulator of BMP signaling that binds BMPs directly and thus prevents the interaction with BMP receptors. However, Noggin may have additional effects that have not been described. Gremlin, another well-known inhibitor of BMP signaling, also binds BMPs and competes thus for receptor interaction (Hsu et al., 1998). In addition, Gremlin was recently found to bind and activate the VEGF2 receptor (Mitola et al., 2010). In analogy, Noggin may interact with still unidentified receptors and thus regulate other biological processes that were not taken into account here and that directly or indirectly affect muscle size. Arguing against such a scenario is the fact that abrogation of BMP signaling by pharmacological Alk3 inhibitors, RNA interference with Smad1/5 expression and Noggin treatment has similar results on muscle size.

Our analysis of molecular mechanisms defines how muscle hypertrophy can be stimulated by signaling via Alk3 receptor. However, during our experiments we also noted that in mice transfection with AAV encoding constitutive active Alk3 receptor

caused inflammation as assessed by the presence of numerous mononuclear infiltrates, likely being macrophages. Furthermore, we observed many myofibers containing central nuclei four weeks after AVV-mediated Alk3 overexpression, these regenerating fibers indicating that fiber degeneration must have occurred. Although we attributed this effect as a consequence of the massive and fast hypertrophy of myofibers that may have caused a metabolic breakdown followed by fiber degeneration, other scenarios cannot be excluded. For instance, Winbanks and colleagues applied AAV-mediated Alk3 overexpression in muscle tissue and did not observe any signs of fiber regeneration (Winbanks et al., 2013). It should be noted that an activated human Alk3 gene was expressed in our experiments, raising the possibility that the inflammation and degeneration are consequences of an auto-immunoreaction. Furthermore, the overexpression of activated Alk3 receptor may result in activation of non-canonical pathways, for instance in phosphorylation of inappropriate intracellular substrates. Further work might use the target gene that we identified, Fbxo30, and assess the role of direct inactivation of this gene. As yet, mice that carry mutation in this gene have not been generated.

Muscle atrophy induced by denervation is a good model to study the molecular mechanism of muscle atrophy. However, denervation does not perfectly model motoneuron diseases such as amyotrophic lateral sclerosis or spinal muscular atrophy or other pathological processes leading to muscle atrophy such as cachexia or immobility. It therefore remains open whether manipulation of BMP signaling could lead to therapeutic strategies to compensate cachexia or muscle loss caused by immobilization, ischemia or by hereditary muscle diseases.

4.3. The muscle physiology in myostatin deficient mice

Myostatin deficiency enhances absolute maximal force and power production, which accompanies the increase in the muscle mass. We investigated the difference in physiological parameters of myostatin deficient mice taking into account sex and age differences. We compared various groups of mutant and control mice, such as young females and males (6 month of age). In addition, we analyzed the groups of 2 years old mutants of both genders, and compared them to 2 years old female control mice. We were missing the data from 2 years old control males. It was difficult to maintain mice for two years due to natural limitation of the murine life span. This lack of mice prevented us to complete the data set for a better statistical analysis.

We found that the muscle hypertrophy resulted in increased absolute maximal force in 6-month- and 2-year-old myostatin mutant mice. However, absolute tetanic force and power are greater in young than old myostatin mutants. Similarly, the fatigue resistance was decreased in both genders in young animals. We suggest that myostatin deficiency accelerates normal aging and can affect the proteasome ubiquitin system and/or on the transcription of sarcomeric genes. However, our data showed that at least the amount of myofibrillar proteins in mutants was not affected with aging.

Previous studies had already reported that tetanic force is increased after myostatin blockade in adult mice (Lee et al., 2005). However, in relation to the muscle mass, maximal force and maximal power are reduced, and endurance is also impaired in our study. These findings are consistent with previous reports (Amthor et al., 2007; Mendias et al., 2011). The enlarged myonuclear domain might be the responsible mechanism underlying the impaired muscle physiology in myostatin mutants. Previous report suggested that myonuclei may not be able to synthesize sarcomeric proteins (Qaisar et al., 2012). This hypothesis requires however further investigations.

We found that the 6-month-old control mice display sexual dimorphism in tetanic force, power and muscle size that was absent by mutants of the same age. This could be explained by the reduced expression of myostatin in control males compared to females, as it was reported by McMahon and colleagues (McMahon et al., 2003). Myostatin deficiency differently affected muscle physiology in female and

male mice, i.e. increased fatigue resistance was more pronounced in females. Further, the generation of specific force was impaired by 2-years-old mutant males compared to females of the same age. We suggest that myostatin interferes with androgen pathways that are known to modulate force generation (Chambon et al., 2010). The protective effect of the female related factor in this context remains to be determined.

Our results on muscle physiology of mutants were not coherent with some reports from other groups. For example, Matsakas and colleagues (Matsakas et al., 2012) reported an approx. 50% drop in absolute tetanic force of myostatin mutants compared to controls. How the discrepancies between our studies arise is currently unclear. Both groups used mice of the same genetic background (C57BL6) from the same original *Mstn*^{-/-} strain (McPherron 1997). We observed the 32-77% increased absolute muscle force in myostatin mutants which is supported by findings of Mendias and others (Mendias et al., 2006). A likely reason for the diverse results reported by different groups could be the different voluntary motor behavior of mice during housing. It has been published that voluntary and forced exercise dramatically increase muscle function in myostatin deficient mice (Matsakas et al., 2012). Alternatively, secondary mutations may have accumulated in the different colonies of myostatin mutants when using homozygous mating systems.

4.4. Myostatin blockade in therapy of muscle dystrophy

Modulation of myostatin signaling can be used as a possible therapeutic strategy for treatment of progressive pathologies like Duchenne muscular dystrophy. Myostatin can be blocked by various agents, for instance soluble activin receptor 2b (sAcvR2B-Fc) (Lee et al., 2005). The treatment with AAV-U7 mediates exon-skipping in the dystrophin gene and restores dystrophin expression, improving thus muscle function in mdx mice that carry a dystrophin mutation. Administration of soluble activin receptor 2b, combined with AAV-U7 treatment, stimulates muscle growth in control and mdx mice. This effect was maintained in combination with AAV-U7-mediated exon skipping. In addition, treatment with sAcvR2B-Fc increased total body weight as soon as 7 days following the first injection and remained stable thereafter. The combined treatment brings together both positives effects and increases the efficacy of the single treatments with respect to a gain of muscle mass and function, i.e. improved dystrophin expression and muscle mass increase, supporting the idea that multidrug therapies might be useful.

Our data showed that the treatment of mdx mice with sAcvR2B-Fc reduced specific maximal force. Other groups reported that the specific force was not compromised upon inhibition of myostatin signaling (Morine et al., 2010; Pistilli et al., 2011). We can only speculate about the nature for these different physiological responses on myostatin blockade. One possible reason for the failure of force increase in our study could be the shorter treatment period during which skeletal muscle has not yet fully adapted to its larger size. In contrast to the effect of sAcvRIIB-Fc, we showed that AAV-U7 treatment improved absolute maximal force.

To test the efficacy of our therapeutic approach, we analyzed the resistance to eccentric contractions upon AAV-U7 mediated exon-skipping and observed an improvement, which is in line with previous reports (Goyenvalle et al., 2004). In contrast, myostatin blockade with sAcvR2B-Fc has no differences in the eccentric contraction-induced force drop that is similar to previous reports (Pistilli et al., 2011).

We conclude from our study that a combination of dystrophin exon skipping and myostatin depletion in mdx mice is beneficial but had no clear synergetic effect on muscle physiology compared to each treatment alone. It is also important to consider that we tested the effect of combined approach only on a single muscle and we lack

knowledge about the effect on whole body function when both strategies are systemically applied.

Another approach to block myostatin signaling was used by Dumonceaux and colleagues (Dumonceaux et al., 2010). They inhibited the myostatin pathway using the technique of AAV-mediated RNA interference directed against AcvR2B receptor (sh-AcvR2B). The combined strategy of shRNA-mediated downregulation of AcvR2B together with U7-mediated exon skipping presented improved tetanic and specific muscle forces. Differently to our study, this combined approach has a synergistic effect on muscle function.

The complexity in the interactions between the activin receptors and their ligands could explain the discrepancy in these studies. Myostatin is not the only protein that can bind to activin receptor AcvR2A and AcvR2B but also activins and some BMPs, e.g. BMP-11. All these ligands are blocked with soluble AcvR2B (Souza et al., 2008). These AcvR2B ligands regulate muscle growth via other receptors, as suggested by experiments on mutant mice deficient in activin receptor II. Lee and colleagues observed an increase in muscle mass in these mutants upon treatment with sAcvR2B-Fc (Lee et al., 2005).

Another important point is the role of the satellite cells in this mechanism of muscle mass gain in adults. Several groups demonstrated that satellite cells play little or no role in myostatin signaling *in vivo* and that muscle hypertrophy triggered by myostatin blockade was satellite-cell independent (Amthor et al., 2009; Lee et al., 2012).

It remains difficult to conclude from these studies whether a combination of both therapeutic strategies could be beneficial for DMD patients. In our study, we used AAV-U7 to restore quasi-dystrophin expression. However, the application of synthetic antisense oligonucleotides (AONs) is more advanced in clinical trials but less potent than AAV-U7 mediated exon-skipping (Goyenvallé et al., 2004). For instance, AONs have low efficacy in specific tissues and poor cellular uptake but allow repeated systemic administrations (Benchaouir and Goyenvallé, 2012). It remains to be elucidated whether AONs could be applied in multi-drug therapy in combination with other strategies that block myostatin pathway.

The animal model used in this study, mdx mice, mimics the human pathology only incompletely. Although the same gene, dystrophin, is mutated in patients and in mdx mice, there are a number of important pathological differences (Hanft et al., 2007; Lefaucheur et al., 1995). In particular, the muscles of DMD patients undergo chronically degeneration and regeneration, and muscle function is strongly impaired. In mdx mice, only the diaphragm muscle in mice mimics these processes (Lynch et al., 2001b). However, in our laboratory we lack expertise and technics to measure functional parameters of diaphragm muscle, although this has been done in other groups (Lynch et al., 1997). In addition, canine models of DMD, for instance a breed of golden retriever, appear to provide a better model for muscular dystrophy in humans, and might be used in such future studies (Kornegay et al., 2012).

4.5. Final word

Signaling of TGF- β family members like BMP and myostatin are important in satellite cell-dependent and -independent muscle growth. Modulation of this signaling cascade might provide new ways for therapeutic intervention in muscle disorders. In particular, identification of muscle-specific targets of BMP and myostatin signaling will be very important. Future work using transgenic mouse lines for stage specific conditional mutation of BMP pathway components might yield new insights in satellite cell biology. Further experiments can help to resolve function of BMPs in muscle aging, regeneration or pathology. Especially, Fbxo30 blockade may prevent muscle loss induced by various triggers, for instance fasting, muscle immobilization or inflammation, ageing or diseases like cancer. Complementary, targeting the myostatin pathway might be useful for muscle rehabilitation after injury or as a part of palliative care in neuro-muscular diseases.

6. References

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