# The role of GSK-3 in myogenesis and recovery of atrophied muscle

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# The role of GSK-3 in myogenesis and recovery of atrophied muscle

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# Chapter 1

## General introduction

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# GENERAL INTRODUCTION

The Dutch population of 16.7 million people comprises 17% <15yrs, 60% 15-60yrs and 23% >60yrs with <15yrs decreasing 0.8% annually and >60yrs increasing with 2% annually, clearly indicating a progressive aging of the population (WHO data 2002-2012 on the Netherlands). Average life expectancy, which is still rising, in the Netherlands is 81 years and the mortality rate of people aged 15-60 years is approximately 6% (WHO data 2011). This shows that a large proportion of deaths occur at an older age. In 2008 WHO investigated the general composition of the annual deaths occurring in the Netherlands. These were divided grossly into injuries (22), communicable (28) and non-communicable (377) disease deaths per 100.000 people. This indicates that 88% of those deaths, >62.000 people/year, are attributable to non-communicable diseases such as chronic heart failure (CHF), obstructive pulmonary disease (COPD), chronic renal failure (CRF), rheumatoid arthritis (RA) and cancer. Chronic diseases often coexist with aging [1, 2] and reduce patient quality of life [3-5]. Chronic diseases, in particular in advanced stages are also a significant economic burden for our society [6-9]. Since 1995 health costs in dollars spent per person in the Netherlands has risen over $200 per year to $5122 in 2011. With a population of 16.7 million this adds up to an 85.4 billion dollar expense, which is approximately 14% of our GDP (607billion 2012). On average, health expenditure per capita increases 6.8% per year. With average inflation rate of 2%, the economic burden would double every 15-16years. This is likely unsustainable and therefore underscores the need for a shift in health care towards prevention and improvement of current medical intervention strategies.

Chronic diseases [10-13] and cancer [14] are often associated with wasting and weakness of skeletal muscle, leading to decreased physical performance, reduced efficacy of medical intervention and increased mortality [15]. Muscle wasting was recently identified by the World Health Organization as an unmet-medical-need [16]. Developing successful intervention strategies requires better understanding of the mechanisms involved contributing to impaired muscle maintenance. This not only refers to reducing or preventing muscle wasting, but also improving muscle mass recovery.

The focus of this thesis is on better understanding muscle mass recovery and more specifically the role of Glycogen Synthase Kinase (GSK)-3, a negative regulator of muscle mass, therein. As a prelude, the regulation of muscle mass maintenance and processes that govern muscle mass are briefly reviewed.

## Regulation of muscle maintenance

Muscle mass is determined by the balance between muscle protein synthesis (anabolism) and muscle protein degradation (catabolism) and by the balance between apoptosis and myonuclear accretion, which are responsible for the continuous low level turnover of muscle tissue [17].

### *Protein turnover*

During muscle hypertrophy or regeneration there is a shift in the protein balance leading to a net increase in total protein by either increasing protein synthesis, decreasing protein degradation or both. mRNA translation is a rate-limiting step in this process, and can be regulated by IGF-I/Akt mediated signalling pathways [18, 19]. Muscle protein synthesis is stimulated by insulin-like growth factor-I (IGF-I), activating PI-3K

signalling resulting in Akt phosphorylation [20-22]. Akt activation subsequently results in phosphorylation of mammalian target of rapamycin (mTOR)- of mTOR-raptor complex 1 (mTORC1) [23, 24]. mTORC1 then hyper-phosphorylates eIF4E binding protein 1 (4E-BP1), effectively removing the brake on translation initiation. mTORC1 also phosphorylates p70-S6K1 and thereby stimulates translation capacity [24-27]. In parallel, activated Akt also phosphorylates GSK-3, thereby inhibiting GSK-3 activity [28, 29]. This inactivation alleviates GSK-3-mediated inhibitory phosphorylation of eIF2Bε [30, 31] allowing eIF2B interaction with the eIF2-complex permitting mRNA translation initiation [32, 33].

During muscle atrophy there is a shift in the protein balance leading to a net decrease in total protein either by increasing protein degradation, decreasing protein synthesis or both. This can be activated by e.g. inflammation and glucocorticoid signalling molecules. Proteolysis involves several cellular proteolytic systems including the ubiquitin 26S-proteasome system (UPS) and the autophagy-lysosomal pathway (ALP) [17, 34-37]. UPS primarily targets myofibrillar and short-lived proteins [38-41]. This selective proteolysis is achieved by multi-ubiquitination of marked proteins by rate-limiting E3 ubiquitin ligases [42], which results in degradation of the tagged proteins by the 26S-proteasome [43]. Two well established muscle-specific E3 ligases are atrogin-1/muscle atrophy F-box and muscle-specific RING finger protein 1 (MuRF1) when upregulated [44, 45] target myofibrillar proteins, muscle-specific enzymes and transcription factors for 26S-proteasome mediated degradation [38, 39, 46, 47]. ALP regulates the clearance of long-lived proteins and dysfunctional organelles [48, 49] by encapsulation and fuse with acidic lysosomes for degradation [50]. These fusion event are highly regulated and involve various autophagy-related proteins including LC3 and Gabarapl1 [51]. Although, unlike with UPS, no muscle specific ALP related proteins are known, ALP is strongly upregulated during muscle atrophic conditions [51-54]. Crosstalk at multiple levels exists between the signaling pathways regulating muscle protein synthesis and degradation, which is crucial for proper protein turnover in muscle mass maintenance.

***Myonuclear turnover***

Muscle fibers are large multinucleated cells. During embryogenesis multinucleated muscle fibers are formed by the fusion of mono-nucleated muscle cells stemming from the embryonic myotome [55, 56]. The remaining muscle-related non-fusing single nucleated cells form a specialized cellular niche and are termed “satellite cells” [57] because of their location in between the epimysium, the membrane surrounding the muscle fibers and the muscle fibers themselves [58]. The cytoplasmic volume surrounding a muscle fiber nucleus (myonucleus) is referred to as the myonuclear domain, and its gene expression is thought to be mainly controlled by this myonucleus. The myonuclear domain concept states that during muscle atrophy the cytoplasmic space of the myofiber decreases, possibly progressing beyond a sustainable myonuclear domain size. This results in myonuclear excess and subsequent removal, potentially through apoptosis which has been reported for aging related muscle atrophy [59-61], muscle disuse [62-64], burn injury [65] and cancer cachexia [66].

During either muscle regeneration or hypertrophy, cytoplasmic space increases and when reaching the upper limit of the myonuclear domain, new nuclei need to be provided to keep sufficient gene expression regulation possible [67, 68]. Post-natal

satellite cells proliferate in response to muscle regeneration or hypertrophy [69-72]. These cells differentiate into myoblasts and fuse with existing muscle fibers and start with muscle-specific gene expression regulated by muscle regulatory factors (MRFs), including Myf5, MyoD and Myogenin [73, 74]. The myonuclear domain theory however was recently disputed in studies showing that during muscle hypertrophy nuclei addition did not appear to be required in the process [75-77]. Nonetheless, both myonuclei loss, and conversely activation and proliferation of satellite cells have been correlated to muscle atrophy and growth, respectively [60, 63, 78-80], implying a role for post-natal myogenesis and myonuclear accretion during muscle growth.

## AIMS AND OUTLINE OF THE THESIS

Resistance exercise is a powerful stimulus to gain muscle mass in health and to combat ageing induced loss of muscle mass [81]. However, it is less effective as single intervention in acute or chronic disease induced muscle wasting. This is partly related to diminished feasibility of high intensity exercise in these conditions but also related to decreased efficacy due to disease and -treatment induced catabolic stimuli [13, 82-84]. More targeted pharmacological intervention is therefore indicated. Pharmacological intervention trials have indeed been performed previously to enhance muscle mass gain. Anabolic agents investigated so far included growth hormone, insulin-like growth factor (IGF) and testosterone or analogues thereof [85-88]. These agents elicit anabolic signaling by stimulation of the IGF-I/Akt pathway. This results in abrogation of proteolysis, increased protein synthesis, increased proliferation and muscle-specific gene expression [23, 89-96]. Still, side-effects and variable efficacy [87, 97, 98] indicate that a good therapeutic window of pharmacological anabolic stimulation remains to be determined. Furthermore the potential influence of coinciding pharmacological treatment such as systemic glucocorticoids is currently unclear. To exemplify this we reanalyzed a clinical trial investigating the efficacy of anabolic supplementation in patients with COPD participating in a pulmonary rehabilitation program. Overall there was a positive effect of testosterone analogue nandrolone decanoate (ND) on muscle mass gain; however there was a clearly greater significant increase in muscle mass gain in patients who were concurrently receiving maintenance doses of glucocorticoids (GC). Therefore a potential interaction between GC and anabolic steroid stimulated IGF-I/Akt signalling was investigated. For this we used a translational research approach in **CHAPTER 2** by reanalysing the clinical data, and investigating the interaction between GC and IGF-I stimulation during myogenic differentiation *in vitro*.

Contemporary knowledge on governance of muscle mass plasticity regulation consists of complex interactions between different cellular signaling pathways regulating protein and myonuclear turnover. Certain signaling molecules can act as 'molecular switchboards' channeling cellular responses upon stimulation; such 'master regulators or nodal points' in skeletal muscle mass plasticity are preferred targets for pharmacological modulation for combating skeletal muscle wasting and improving muscle mass recovery.

GSK-3 is a signaling molecule which is suppressed by activation of IGF-I/Akt pathway, suggesting it may serve as a target for pharmacological inhibition to modulate muscle mass. GSK-3 consists of two isoforms from two separate genes namely GSK-3α and GSK-3β, which can operate in a redundant manner in various cellular processes.

McManus *et al.* reported that in human and mouse muscle GSK-3β protein content is three to four times higher than GSK-3α, indicating GSK-3β as the predominant GSK-3 isoform in muscle [99]. Therefore many studies have focussed on modulation of GSK-3β. Inhibition of GSK-3(β) has been shown to abrogate proteolysis and improve myogenic differentiation [100, 101]. We therefore reviewed the literature on GSK-3 as a potential master regulator of muscle mass plasticity in **CHAPTER 3**.

Targeting GSK-3(β) for treating skeletal muscle wasting requires better understanding of the effects of modulation of GSK-3(β) activity on the molecular mechanisms governing muscle mass plasticity. The **MAIN HYPOTHESIS** of this thesis is that GSK-3(β) inactivation is required and sufficient to stimulate muscle regeneration. This hypothesis was tested in **CHAPTERs 4-6** using a combination of *in vitro* and *in vivo* experimental models of muscle (-cell) differentiation and regeneration.

Previous work by our group has shown that inactivation of GSK-3(β) through stimulation of the IGF-I/Akt pathway or by pharmacological inhibition with Lithium Chloride (LiCl) salt promotes *in vitro* myogenic differentiation [90]. In that work alleviation of GSK-3(β) mediated inactivation of Nuclear factor of activated T-cells (NFAT) c3 was postulated as the basis for increased myogenic gene expression in response to GSK-3 inhibition [101]. Another phospho-substrate of GSK-3, β-catenin, has also been implicated in muscle regeneration and hypertrophy [102, 103]. β-catenin regulation via GSK-3 has been well described in response to the Wnt ligand rather than IGF-I mediated signaling. We therefore assessed the role of GSK-3(β) and NFAT/β-catenin signaling in relation to myogenic differentiation following Wnt, or IGF-I stimulation and pharmacological GSK-3 inactivation in **CHAPTER 4**.

A vast majority of the studies implying a role for GSK-3 in muscle mass plasticity and specifically muscle regeneration was performed using *in vitro* models of myogenesis. A major part of the experimental work conducted for this thesis was to address the role of GSK-3 in muscle regeneration *in vivo*. To do this, a mouse model of reversible disuse-induced muscle atrophy was employed, consisting of two weeks hindlimb suspension (HS) resulting in atrophy of the unloading-sensitive calf muscles. The calf consists of the soleus muscle, plantaris and gastrocnemius muscles. As control for non-unloading muscle mass changes, the unloading-insensitive shin muscle Extensor Digitorum Lungus (EDL) was used. After two weeks the mice are released from hindlimb suspension, and the subsequent resumed loading and use of the muscles was deployed as a model to study muscle mass recovery.

Previous work by our group has shown that in this mouse model of disuse-induced muscle atrophy, the soleus muscle during hind limb reloading-induced muscle mass recovery was associated with significantly increased GSK-3β phosphorylation indicating GSK-3 inactivation [104]. To investigate if inactivation of GSK-3 is indeed required during the reloading-induced muscle mass growth, a mouse strain carrying a serine to alanine mutation point mutation for both GSK-3α (S21A) and GSK-3β (S9A) was deployed [99]. This serine to alanine mutation prevents IGF/Akt signaling mediated inactivation of GSK-3 [28, 105]. These mice expressing whole-body constitutively active (CA) GSK-3, and their control counterparts were subjected to the hindlimb suspension and muscle reloading model. The obtained results are described in **CHAPTER 5**.

Ample evidence has been provided to show that inhibition of GSK-3(β) can suppress muscle proteolysis [100, 106, 107]. Furthermore *in vitro* inhibition of GSK-3(β)

is sufficient to induce muscle hypertrophy [108] and enhance myogenic differentiation [90, 101, 109]. However, *in vivo*, the effects of increased GSK-3(β) inactivation on muscle mass recovery have not been investigated. To explore if additional inactivation of GSK-3β during hindlimb reloading could improve muscle mass recovery, a muscle-specific GSK-3β knock-out mouse was used [110]. These mice lacking muscle GSK-3β, and their control littermates were subjected to hindlimb suspension and reloading and the obtained results are described in **CHAPTER 6**.

Finally the insights and implications resulting from the data obtained in this dissertation are critically evaluated and integratively discussed with respect to the most recent literature in **CHAPTER 7**. In addition, future research directions are presented with regard to muscle mass plasticity modulation and the role of GSK-3 as a potential pharmacological target therein.

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# Chapter 2

## Synergistic stimulation of myogenesis by glucocorticoid and IGF-I signaling

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## ABSTRACT

Muscle wasting is associated with poor prognosis in chronic obstructive pulmonary disease COPD. Exercise stimulates muscle recovery but its efficacy is variable depending on the clinical condition and medical treatment. Systemic glucocorticoids, commonly administered in high-doses during acute disease exacerbations or as maintenance treatment in end-stage disease are known to contribute to muscle wasting. As muscle mass recovery involves IGF-I signaling, which can be stimulated by anabolic steroids, the impact of glucocorticoids and the effect of simultaneous IGF-I stimulation by anabolic steroids on muscle recovery and growth were investigated. The effects of and interactions between glucocorticoid and IGF-I signaling on skeletal muscle growth were assessed in differentiating C2C12 myocytes. As proof-of-principle, we performed a *post-hoc* analysis stratifying patients by glucocorticoid use, of a clinical trial investigating the efficacy of anabolic steroid supplementation on muscle recovery in muscle wasted patients with COPD. Glucocorticoids strongly impaired protein synthesis signaling, myotube formation and muscle-specific protein expression. In contrast, in presence of glucocorticoids, IGF-I synergistically stimulated myotube fusion and myofibrillar protein expression, which corresponded with restored protein synthesis signaling by IGF-I and increased transcriptional activation of muscle-specific genes by glucocorticoids. In COPD patients on maintenance glucocorticoid treatment, the clinical trial also revealed an enhanced effect of anabolic steroids on muscle mass and respiratory muscle strength. In conclusion, synergistic effects of anabolic steroids and glucocorticoids on muscle recovery may be caused by relief of the glucocorticoid-imposed blockade on protein synthesis signaling allowing effective translation of glucocorticoid-induced accumulation of muscle-specific gene transcripts.

## INTRODUCTION

COPD is a chronic disabling disease characterized by progressive, irreversible airflow obstruction, skeletal muscle wasting and weakness. It is well established that muscle wasting negatively impacts physical performance and mortality independent of the severity of airflow obstruction [1]. Therefore muscle mass maintenance or recovery after exacerbation-induced catabolic stress, is considered an important therapeutic target. Effective and feasible single or multimodal therapeutic approaches focusing on muscle recovery are currently limited and further development requires elucidation of the mechanisms governing muscle-plasticity during muscle atrophy and recovery.

COPD related factors that may contribute to muscle atrophy include disuse, inflammation, and systemic glucocorticoids (GC) [2-5]. GCs are frequently applied during acute exacerbations and sometimes also as maintenance treatment during end-stage disease. The efficacy of GC as maintenance medication is controversial [6] and Schols *et al.* even reported a dose-dependent increased mortality risk in severe disease [7]. In addition, proposed catabolic effects of GC could impair beneficial effects of pulmonary rehabilitation [8]. Nevertheless ill-manageable, frequently exacerbating patients often receive long-term low-dose GC.

Besides increased proteolysis, GC-induced muscle atrophy involves decreased protein synthesis signaling. This relies on reduced activity of mammalian target of rapamycin complex-1 (mTORC1), and decreased phosphorylation of its downstream targets p70-S6K1 and 4E-BP1, which stimulate translation capacity and suppress translation initiation, respectively [9-15]. Conversely anabolic steroids, like nandrolone decanoate (ND), prevent muscle atrophy and stimulate muscle growth and hypertrophy, partly by increasing muscle IGF-I production and signaling in a para-/autocrine fashion [16-20]. In contrast to GC, IGF-I signaling reduces proteolysis and increases protein synthesis, including mTORC1 signaling [10, 21].

Muscle regeneration and myogenesis involve activation, proliferation and subsequent differentiation of satellite cells into myoblasts that fuse with existing or form new myofibers [22-24]. This also involves increased muscle-specific gene expression, including contractile/sarcomeric proteins e.g. myosin heavy chain (MyHC), myosin light chain (MLC), and enzymes involved in muscle energy metabolism e.g. muscle creatine kinase (MCK), through increased transcriptional activity of the muscle regulatory factors (MRFs) [25]. Myogenesis can be stimulated by anabolic steroids [23], and their potential to restore muscle function during pulmonary rehabilitation has been studied in COPD patients [26-28].

The effects of GC on myogenesis and muscle recovery have received limited attention. Therefore, we investigated the effects of GC and the interaction with anabolic signaling on muscle re-growth using a translational research approach. First, the effect of GC on and interaction with IGF-I signaling on myogenesis was evaluated in differentiating myocytes. As proof-of-principle, a Randomized Controlled Trial (RCT) assessing the effect of ND supplementation on muscle recovery in wasted male COPD patients, of which a sub-population was on chronic GC medication, [26] was reanalyzed.

## MATERIALS AND METHODS

### Cell culture

The murine skeletal muscle cell line C2C12 (ATCC # CRL1772) was cultured in growth medium (GM). This was composed of low glucose Dulbecco's Modified Eagle Medium (DMEM) containing antibiotics (50U/ml Penicillin and 50µg/ml Streptomycin) and 9% (v/v) Fetal Bovine Serum (FBS) (all from Gibco, Rockville, MD). C2C12 Cells were plated at $10^4/cm^2$ on Matrigel (BD Biosciences, Bedford, MA) coated (1:50 in DMEM low glucose) dishes as described previously [29] over night in GM. To induce spontaneous differentiation by growth factor withdrawal [30], GM was replaced with differentiation medium (DM), which contained low glucose DMEM with 1.0% heat-inactivated FBS and antibiotics. As indicated additionally differentiation was induced in the presence murine IGF-I (Calbiochem, La Jolla, CA) with or without the glucocorticoids dexamethasone (DEX) or prednisolone (PRED) added directly with induction of differentiation and again 24h later when the cells were provided with fresh DM and every 48hrs thereafter. IGF-I was used at 5nM, which is sufficient to stimulate myogenic differentiation [31]. DEX was used at a relatively low dose of 10µM compared to other studies [21, 32, 33]. Furthermore PRED, the prescribed oral glucocorticoid in the *post-hoc* stratification analyzed RCT [26], was used in equimolar and equipotent (5-fold compared to DEX [34-36]) doses. To obtain mature myotubes, myoblasts were cultured in DM to differentiate, with medium changes at 24 and 72 hrs for 5 days. At day 5 medium was changed with or without the presence of murine IGF-I and with or without the glucocorticoids dexamethasone (DEX)

### Transfections and plasmids

Transient transfections were performed using Nanofectin (PAA, Pasching, Austria) and in all cases included co-transfection with pSV-β-gal to correct for differences in transfection efficiency (Promega, Madison, WI). According to manufacturers' instructions 1.0µg plasmid per 3.2µl Nanofectin was used. Per transfection 1.0-2.5µg DNA of expression plasmids or empty vector controls were used in 35mm-dishes. The transfection mix was added 6h prior to differentiation induction. GRE- luciferase reporter plasmid was used to measure glucocorticoid induced transcriptional activity, the 4RTK MRF-sensitive luciferase plasmid [37, 38] was used to determine MRF transcriptional activity. To determine luciferase and β-galactosidase activity, cells were lysed in luciferase lysis buffer and stored at –80°C. Luciferase (Promega, Madison, WI) and β-galactosidase (Tropix, Bedford, MA) were measured according to the manufacturers' instructions.

### May-Grunwald Giemsa Staining

C2C12 cells were grown on Matrigel coated 60mm dishes and differentiation was induced in the presence or absence of IGF-I, Dexamethasone or Prednisolone, which were added again 24h later with media renewal. After 72h of differentiation cells were washed twice in PBS (RT), fixed in methanol and stained in May-Grunwald Giemsa (Sigma, Saint Louis, MO) according to the manufacturer's instructions. Pictures were taken at 40x and 100x magnifications using a microscope connected to a digital camera (DXM 1200F), both from Nikon (Nikon, Kanagawa, Japan). The 100x magnified images were taken in series of four with fixed overlap.

### Nuclei count and myogenic index

The total number of nuclei of 4 or more fields (100x magnification) were counted to determine the number of nuclei present after 72h of differentiation. The myonuclear distribution was assessed by counting all nuclei within four linked images obtained at 100x magnification. Counted nuclei were assigned to one of three classes: single nucleated myoblasts, dividing or fusing bi-nucleated myoblasts or multi-nucleated (>2) myotubes. Per condition >1900 nuclei were counted and assigned. Of the nuclei contained in myotubes a subdivision was made in four groups with 3-9, 10-19, 20-29 or >30 nuclei per myotube.

### Muscle Creatine Kinase activity

Myogenic differentiation was assessed biochemically via determination of muscle creatine kinase (MCK) activity. Cells were grown on Matrigel coated dishes. After induction of differentiation, cells were washed twice in cold PBS, lysed in 0.5% Triton X-100, and scraped from the dish with a rubber policeman. Lysates were centrifuged for 2 min at 16,000g and 4°C, and the supernatant was stored in two aliquots at −80°C for determination of protein content or MCK activity in presence of 1.25%BSA. MCK-activity was measured by using a spectophotometric–based [39] kit (Stanbio, Boerne, TX). Specific MCK activity was calculated after correction for total protein concentration [40], $R^2$ values were >0.98 for the standard curve.

### Western blotting

The C2C12 cells were washed twice with ice-cold PBS after which they were scraped and lysed in a whole cell lysate (WCL) buffer (20mM Tris, pH 7.4; 150mM NaCl; 1% Nonidet P-40; 1mM DTT; 1mM $Na_3VO_4$; 1mM PMSF; 10µg/ml Leupeptin and 1% aprotenin) or luciferase lysis buffer using rubber policemen. Next, crude lysates were frozen at −80°C, WCL lysates were thawed followed by 30 minutes centrifugation at 16.000g and 4°C. A portion of the supernatant was saved for protein determination, prior to the addition with 4x Laemmli sample buffer (0.25M Tris-HCl pH 6.8; 8% (w/v) SDS; 40% (v/v) glycerol; 0.4M DTT and 0.04% (w/v) Bromophenol Blue). The samples were boiled for 5 minutes at 95°C and stored at −20°C. Total protein was assessed by a BCA Protein Assay kit (Pierce Biotechnology, IL, USA) according to the manufacturers' instructions. For SDS-PAGE 0.5µg -15µg of protein was loaded per lane and separated on a Criterion™ XT Precast 4-12% Bis-Tris gel (Bio-Rad, #3450124), followed by transfer to a 0.45µm Whatman® Protran® Nitrocellulose Transfer membrane (Whatman GmbH, #7324007) by electro blotting using a Bio-Rad Criterion Blotter (Bio-Rad, Hercules, CA, USA). The membrane was blocked for 1-2h at room temperature in 5% (w/v) non-fat dried milk (NFDM) (ELK, Campina, the Netherlands) diluted in TBS-Tween-20 (0.05%). Nitrocellulose blots were washed in TBS-Tween-20 (0.05%) on a rotating platform, followed by overnight (o/n) incubation at 4°C with specific antibodies directed against: p-Akt ($Ser^{473}$) (#9271), Akt (#9272), GAPDH (#2118), p-mTOR(#2971), mTOR (#2972), p-p70-S6K1 (#2906), p70-S6K1 (#2902), total 4E-BP1 (#9452) (all from Cell Signaling Technology, Inc., Danvers, MA,) Myosin Heavy Chain fast (MyHC-f) (#M4276, Sigma-Aldrich, Saint-Louis, MO USA), Myosin Light Chain (MLC)-1 and 3 (#F310) (Developmental Studies Hybridoma Bank, Iowa City, IA, USA) all diluted in TBS/0.05% Tween-20 with or without 5%BSA/NFDM. After 3 washing steps of 10 minutes each, the blots were probed

with a peroxidase conjugated secondary antibody (Vector Laboratories, #PI-1000), and visualized by chemiluminesence using Supersignal® WestPico Chemiluminescent Substrate (Pierce Biotechnology, Inc.) according to the manufacturers' instructions and exposed to film (Biomax light film, KODAK) or live imaged (Bio-Rad chemidoc XRS). Westernblot images were quantified using the Quantity One analysis software from Bio-Rad.

**RNA isolation and assessment of mRNA abundance by RT-qPCR**

C2C12 cells were washed twice with ice-cold 1xPBS after which RLT solution containing 1% β-mercaptoethanol was added. Cells were scraped and DNA shearing was achieved by 8-10x passing the lysate through a 20G needle after which samples were stored at −80°C. Samples were further processed according to manufacturer's instructions of the RNeasy Mini Kit of Qiagen including the on-column DNase treatment. RNA was reconstituted in 30-50µl RNase free water and stored at −80°C. The RNA concentrations were measured using a Nanodrop® ND-1000 UV-Vis spectrophotometer. RNA was diluted >5x in $ddH_2O$ and 400ng of RNA was reverse transcribed to cDNA using the Transcriptor first strand cDNA synthesis kit (Roche Diagnostics GmbH, Mannheim, Germany) with anchored oligo-dT primers according to manufacturers' for generating cDNA fragment of 4kb with a final reaction volume of 20µl. RNA of genes of interest (Table 1) were determined by reverse transcription quantitative PCR (qPCR). qPCR primers were designed using Primer Express 2.0 software (Applied Biosystems, Foster City, CA), checked for both primer and amplicon secondary structures, and then obtained from Sigma Genosys (Haverhill, UK). qPCR reactions (20 µl final volume) contained 10µl Sensimix SYBR Fluorescein (Bioline, Alphen a.d. Rijn, Netherlands) 0.6µl of forward and reverse primer, and 3.8µl $H_2O$. Relative cDNA starting quantities for the samples were derived by the standard curve method. Standards curve samples were generated by serial dilution of pooled cDNA samples and had at least a $R^2$ >0.98 and an efficiency between 90-110%. The expression of the genes of interest (Table 1) were normalized with a correction factor derived by GeNorm, which is based on a combination of the expression levels of GAPDH and RPL13A, ARBP, Calnexin, β2M. RT-qPCR reactions were performed on a MyiQ single-color Real-Time thermal cycler (Bio-Rad Herculus, CA).

**Proof of concept randomized clinical trial**

63 muscle wasted male COPD patients (mean age, 66±8 yrs) with severe COPD (FEV1, 36±14% predicted) were randomized to receive either ND or placebo treatment as adjunct to a pulmonary rehabilitation program [26]. Patients were randomly allocated to receive deep intra muscular injection in the gluteus maximus muscle of 50 mg ND in 1 mL arachis oil in a double blinded setup on day 1, 15, 29, and 43. Measurements were taken at baseline and after eight weeks intervention. Fat free mass was determined by deuterium-bromide dilution [26]. As a functional read-out of muscle mass, respiratory muscle strength was assessed by maximal inspiratory mouth pressure. *Post-hoc* stratification by oral glucocorticoid use resulted in the following 4 groups: 1) no oral glucocorticoids plus placebo (Ctrl/No-GC; n=18), 2) no oral glucocorticoids plus ND (ND/No-GC; n=14), 3) oral glucocorticoids plus placebo (Ctrl/GC; n=12), and 4) oral glucocorticoids plus ND (ND/GC; n=19). Baseline blood testosterone, body composition, respiratory muscle strength, exercise capacity and reported recent weight loss were not

different between the groups. As expected, only the number of hospitalizations during the last year was significantly higher for the GC treated patients (1.63±1.75 vs. 0.66±0.70, p<0.01). For a full description of the trial we refer to the original report [26]. Baseline characteristics for the four groups after stratification are shown in Table 2. All subjects gave their informed consent in writing.

## Statistical analysis

Statistical analysis of muscle cell culture experiments was performed by t-test for independent samples with unequal variance. Significance was determined at the level of p<0.05 and data expressed as mean ± SD. Statistical analysis of the clinical trial was performed in the per-protocol group (n=29-32). Differences between the groups at baseline were analyzed by t-test for independent samples. Changes within the groups between baseline and week 8 were tested by paired t-test. Differences in the treatment response after 8 weeks of ND or placebo were tested using one-way ANOVA with *post-hoc* LSD. Significance was determined at the level of p<0.05. All data were analyzed using SPSS/PC+ (Statistical Package for the Social Sciences, Version 17.0 for Windows; SPSS; Chicago, IL).

**Table 1. Baseline patient characterization after *post-hoc* stratification according to glucocorticoid usage**

| Parameter | no glucocorticoid | | | glucocorticoid | | | |
|---|---|---|---|---|---|---|---|
| | mean ± SD | n | | mean ± SD | n | p-value | |
| Age (yr) | 65.31 ± 7.41 | 32 | | 67.55 ± 8.39 | 31 | 0.266 | |
| $FEV_1$ (% predicted) | 31.93 ± 9.20 | 32 | | 40.05 ± 16.68 | 31 | 0.019 | |
| IVC (% predicted) | 84.92 ± 15.38 | 32 | | 83.98 ± 16.61 | 31 | 0.817 | |
| Body mass index (BMI) ($kg/m^2$) | 21.65 ± 3.48 | 32 | | 21.41 ± 3.55 | 31 | 0.789 | |
| FFM index by $D_2O$ ($kg/m^2$) | 15.87 ± 1.71 | 31 | | 15.60 ± 1.36 | 30 | 0.500 | |
| Inspiratory muscle strength (cm $H_2O$) | 79.81 ± 20.19 | 32 | | 77.81 ± 21.51 | 31 | 0.704 | |
| Peak cycling workload (% predicted) | 38.43 ± 15.69 | 32 | | 38.36 ± 16.41 | 31 | 0.986 | |
| C-reactive protein (CRP) (µg/ml) | 20.74 ± 20.67 | 31 | | 22.21 ± 25.25 | 29 | 0.804 | |
| Total testosterone (nmol/l) | 13.89 ± 4.18 | 31 | | 14.38 ± 5.34 | 29 | 0.693 | |
| Reported recent weight loss | 56% | 32 | | 55% | 31 | 0.912 | |
| Number of hospitalizations during last year | 0.66 ± 0.70 | 32 | | 1.63 ± 1.75 | 30 | 0.005 | |
| Oral glucocorticosteroid dose (mg/24h) | 0 ± 0 | 32 | | 7.46 ± 2.42 | 31 | <0.001 | |

Values are means ± SD; n, no. of subjects. $FEV_1$, forced expiratory volume in 1 s; IVC, inspiratory vital capacity; BMI, body mass index; FFM, fat free mass; CRP, C-reactive protein.

**Table 2. qPCR primers sequences of genes of interest (GOI) and reference genes (RG)**

| Target | Full target name | Target Type | Genbank identifier | Sense primer | Antisense primer |
|---|---|---|---|---|---|
| REDD1 | Ddit4 | GOI | | CGGGCCGGAGGAAGACT | CTGCATCAGGTTGGCACACA |
| Glul | Glumate ammonia ligase, glutamine synthetase | GOI | | GGCCATGCGGGAGGAGA | GGTGCCTCTTGCTCAGTTTGTCA |
| KLF15 | Kruppel-like factor 15 | GOI | | TGCAGCAAGATGTACACCAAGAG | ATCGCCGGTGCCTTGAC |
| MCK | Muscle Creatine Kinase | GOI | NM_007710 | AGGTTTTCCGCCGCTTCT | CGGTGCCCAGGTTGGA |
| MyoD1 | Myoblast determination protein 1 | GOI | | GGCCGTGGCAGCGAG | CGCTGTAATCCATCATGCCAT |
| Myogenin | Myogenin | GOI | | CCCATGGTGCCCAGTGAA | GCAGATTGTGGGCGTCTGTA |
| MyHC IIB | Myosin heavy chain 2B | GOI | | ACAAGCTGCGGGTGAAGAGC | CAGGACAGTGACAAAGAACG |
| MyHC peri | Myosin heavy chain perinatal | GOI | | ACACATCTTGCAGAGGAAGG | TAAACCCAGAGAGGCAAGTG |
| ARBP | 60S acidic ribosomal protein P0 (L10E)/ Rplp0 | RG | | GGACCCGAGAAGACCTCCTT | GCACATCACTCAGAATTTCAATGG |
| RPLA13A | 60S ribosomal protein L13a (Transplantation antigen P198) (Tum-P198 antigen) | RG | NM_009438.4 | CACTCTGGAGGAGAAACGGAAGG | GCAGGCATGAGGCAAACAGTC |
| β2M | Beta-2-Microglobuline | RG | | CTTTCTGGTGCTTGTCTCACTGA | GTATGTTCGGCTTCCCATTCTC |
| Calnexin | Calnexin Precursor | RG | | GCAGCGACCTATGATTGACAACC | GCTCCAAACCAATAGCACTGAAAGG |
| GAPDH | Glyceraldehyde-3-phosphate dehydrogenase | RG | BC098095 | CAACTCACTCAAGATTGTCAGCAA | TGGCAGTGATGGCATGGA |
| Cyclophillin | Peptidylprolyl isomerase A | RG | X52803 | TTCCTCCTTTCACAGAATTATTCCA | CCGCCAGTGCCATTATGG |

## RESULTS

### Synergistic stimulation of myotube formation by glucocorticoids and IGF-I.

The synthetic GC dexamethasone (DEX) induces muscle atrophy and was used to induce GC signaling. DEX resulted in diminished morphological differentiation of C2C12 myocytes, whereas differentiation was enhanced by IGF-I compared to control (Figure 1A). Combined, DEX and IGF-I enhanced morphological differentiation compared to IGF-I alone. Quantification of morphological differentiation using the myogenic index (Figure 1B) clearly showed DEX reduced myoblast fusion. In contrast, IGF-I increased fusion, which was further exceeded by the combination of IGF-I and DEX. This not only resulted in more, but also larger myotubes (Figure 1C). Similar effects on myotube morphology were observed with equimolar as well as equipotent doses of prednisolone (PRED) (Figure 2).

### GR signaling is not impaired in presence of IGF-I.

To evaluate the effects of IGF-I on GC signaling, glucocorticoid receptor (GR) transcriptional activity was determined. A >8-fold increase was observed by DEX alone, whilst combined with IGF-I an additional (45%) increase was detected (Figure 3A). Endogenous GC-sensitive GR target genes Glul, KLF-15 and REDD1 (Figure 3B-D) were all significantly induced by DEX, ranging from 2.5-55-fold increases; regardless of absence or presence of IGF-I, confirming that active GC-induced GR-signaling is slightly attenuated, but not abrogated by IGF-I.

### Synergism between glucocorticoids and IGF-I on muscle protein abundance.

MCK is increasingly expressed during differentiation [31], which was suppressed by DEX (−50%) and increased with IGF-I (3.8-fold) or IGF-I/DEX (4.2-fold) treatment (Figure 4A). Total protein after 72h (data not shown) was lower after DEX (40%), whilst it was increased with IGF-I (2.4-fold) and IGF-I/DEX (2.0-fold). Sarcomeric protein content (Figure 4B) revealed a decrease (−50%) in response to DEX compared to control (Figure 4C-E). Conversely, IGF-I increased sarcomeric protein content (>2-fold) according to expectation. DEX and IGF-I combined increased myosin light chain (MLC) 1 and 3 abundance by 15 and 20%, respectively. MCK, MLC-1 and -3 tended to be increased for IGF-I/DEX compared to IGF-I. Similar results were also observed on muscle and in particular myofibrillar proteins when IGF-I was combined with PRED at equimolar as well as equipotent doses compared to DEX (Figure 5). In addition, in differentiating myoblasts Myosin heavy chain (MyHC) fast isoform abundance increased significantly, >2-fold when comparing IGF-I/DEX to IGF-I alone. Importantly, this synergistic effect on myotube size (not shown) and in particular myofibrillar proteins was not observed when identical concentrations of IGF-I/DEX were applied to fully differentiated myotubes (Figure 4F). Altogether, these data reveal a potentiating effect of combined DEX and IGF-I on myotube size and myofibrillar protein expression selectively during myogenic differentiation.

**DEX does not affect increased protein synthesis signaling by IGF-I.**

Considering that GC decreases protein synthesis [11, 14], it was surprising to find that increased MyHC-fast accretion was observed during differentiation by the combination of IGF-I/DEX compared to IGF-I alone. Therefore regulators of protein synthesis signaling during differentiation in the presence of IGF-I and/ or DEX were investigated next (Figure 6A). Phosphorylation of 4E-BP1 was decreased by DEX (Figure 6C), whereas it was increased by IGF-I with or without DEX. p70-S6K1 phosphorylation was strongly (70%) decreased by DEX (Figure 6B). IGF-I, even in presence of DEX, was able to increase S6K1 phosphorylation >2-fold.

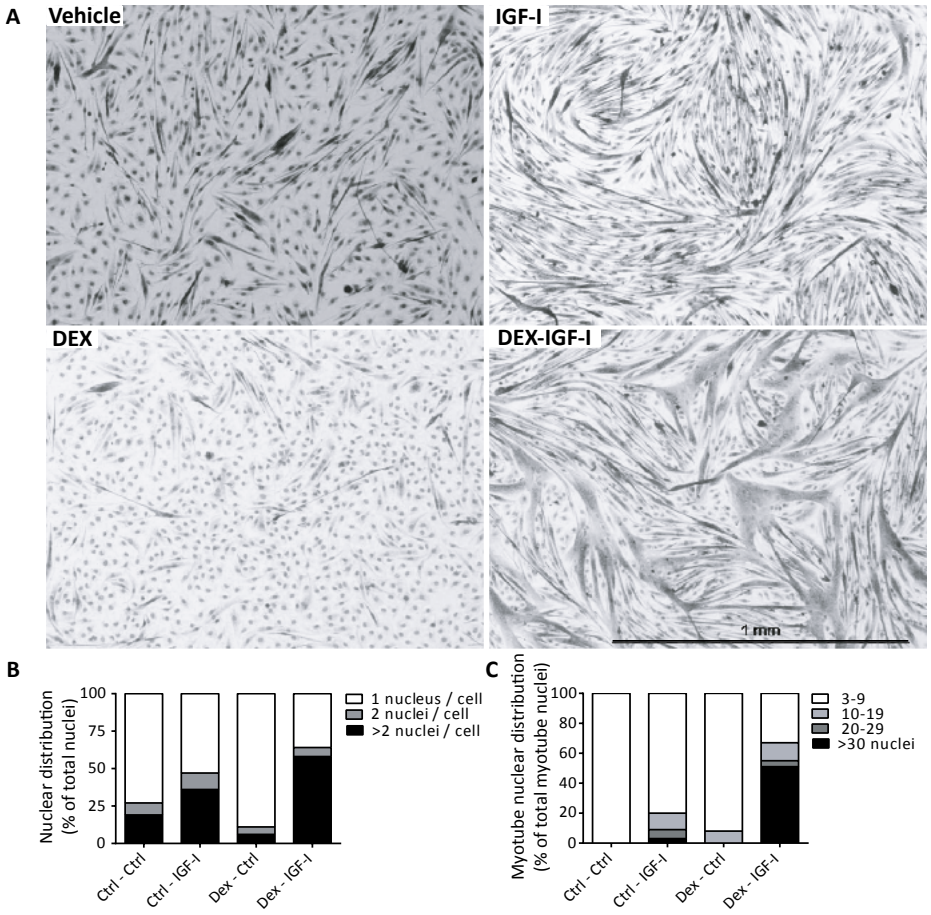


**Figure 1. Synergistic stimulation of myotube formation by DEX and IGF-I.** C2C12 cells differentiated for 72h and treated with (A) control, IGF-I (5nM), DEX (10μM) and IGF-I/DEX fixed and stained with May-Grunwald Giemsa. From these images (B) myoblast fusion was quantified by determining nuclear distribution of 1900-3800 nuclei for each separate condition, which is expressed as the % of nuclei residing in cells containing 1, 2, or >2 nuclei, reflecting mononucleated myoblasts (1 nucleus), dividing or fusing myoblasts (2 nuclei) or myotubes (>2 nuclei), respectively. Myotube nuclear distribution (C) is divided into four sizes containing 3-9, 10-19, 20-29 and 30 or more nuclei. Shown are representative data of three independent experiments.

**Glucocorticoids upregulate muscle-specific gene expression.**

As a mere restoration of protein synthesis signaling by IGF-I did not explain the synergistic effects of IGF-I and GC on myotube formation and myosin expression, muscle-specific mRNA abundance during differentiation was assessed. After 48h of differentiation muscle-specific mRNA was quantified (Figure 7A-C). MCK expression was increased by IGF-I (90%), but surprisingly also by DEX alone (70%). Combined IGF-I/DEX increased MCK expression >3-fold compared to either IGF-I or DEX alone (Figure 7A), indicating a synergistic interaction. This synergism between DEX and IGF-I during differentiation was consistently shown for MYH-4 and MYH-8 mRNA transcripts (Figure 7B, C). Since the differentiation status appeared to determine the sensitivity to this synergistic interaction, we next addressed the involvement of the muscle regulatory

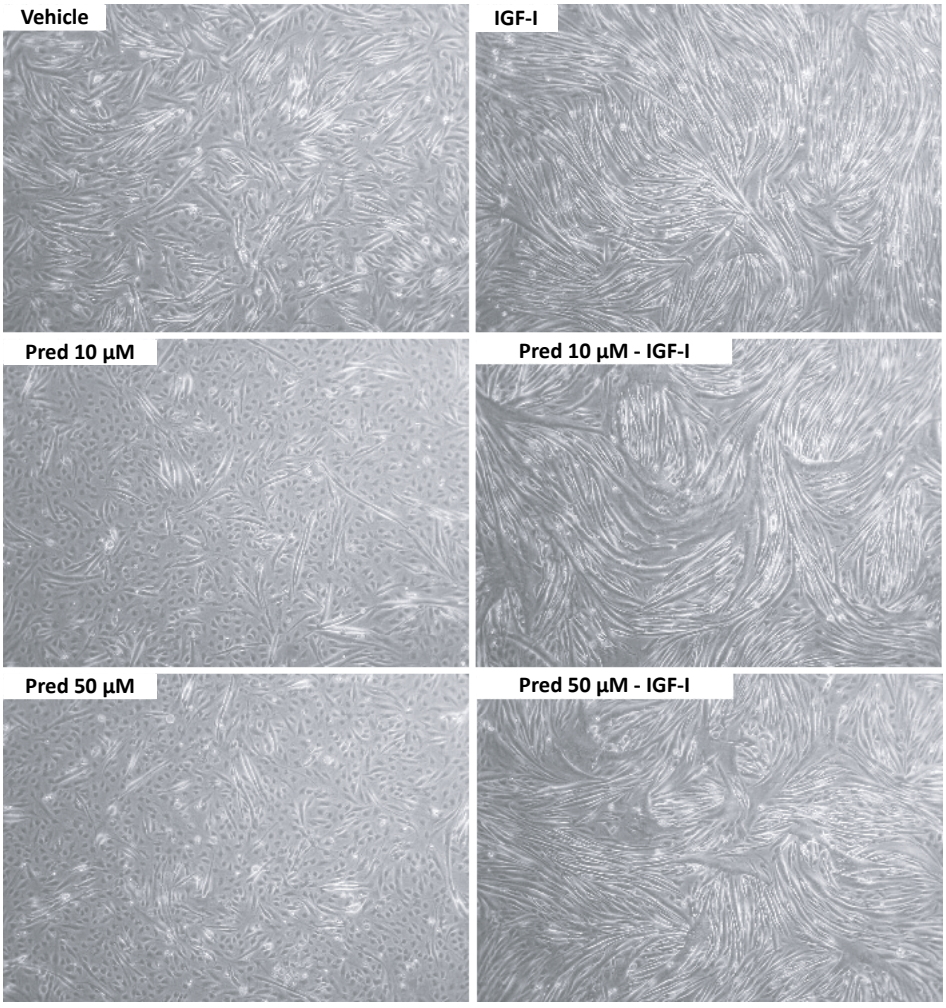


**Figure 2. Synergistic stimulation of myotube formation by prednisolone and IGF-I.** C2C12 cells differentiated for 72h and treated with vehicle, IGF-I (5nM), prednisolone (PRED) at equimolar (10µM) or equipotent, (50µM) concentrations, alone or in combination with IGF-I. Shown are representative data of three independent experiments.

factors (MRFs), which are primarily active during myogenic differentiation. mRNA expression of Myf-5 was increased by DEX and Myogenin with DEX and IGF-I combined (Figure 7D-F). MRF transcriptional activity was increased by IGF-I (2.4-fold) as expected, but even more pronounced increases were observed in response to DEX alone (3.4-fold) or DEX + IGF-I (7-fold) (Figure 7G). Altogether these data suggests that increased MRF activity in response to DEX and IGF-I, and subsequent restoration of mRNA translation by IGF-I, may lie at the basis of the potentiating interaction between IGF-I and GC on myogenic differentiation.

## Synergy between nandrolone and glucocorticoids on muscle mass recovery in COPD patients with muscle wasting.

Based on the observed results in myocytes with combined IGF and GC induced signaling during myogenesis, a *post-hoc* analysis stratifying for GC-use of our nandrolone (ND) RCT [26] was performed to address the influence of low-dose GC on ND efficacy. The ND treatment response was confirmed by reduced endogenous circulating total testosterone levels to 38±21% of baseline levels which was similar for GC and no-GC groups, whereas testosterone levels remained unchanged in the placebo (Ctrl) group. The gain in FFM (Figure 8A) was greatest and only reached statistical significance in the ND/GC (2.27±1.61kg, $p<0.05$), compared to the other groups e.g. ND/No-GC (1.10±3.03kg, p=n.s.), Ctrl/GC (0.39±2.03kg, p=n.s.), Ctrl/No-GC (0.27±1.90kg, p=n.s.). A similar trend was observed for inspiratory muscle strength (Figure 6B). Irrespective of GC medication, the placebo treated patients did not lose FFM, indicating that the rehabilitation program, which was devoid of resistance training to study the effects of anabolic steroids per se, may have prevented further muscle wasting, but did not induce muscle gain.

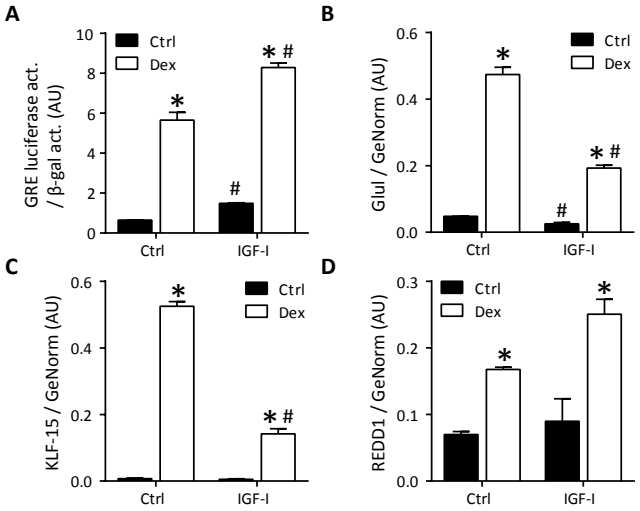


**Figure 3. GR signaling is not impaired in presence of IGF-I.** (A) C2C12 myoblasts were transfected with a GRE luciferase-reporter construct (0.9µg) and plasmid encoding β-gal (0.1µg) and cultured for 48h and treated with IGF-I (5nM) DEX 10µM or vc as indicated and harvested for glucocorticoid induced GRE-luciferase activity. (B-D) C2C12 cells differentiated for 48h and treated with IGF-I (5ng/ml) DEX 10µM or vc as indicated were harvested for mRNA quantification. Two-way t test with unequal variance * ctrl *vs.* DEX, # ctrl *vs.* IGF-I; $p<0.05$. Shown is representative data of three independent experiments.

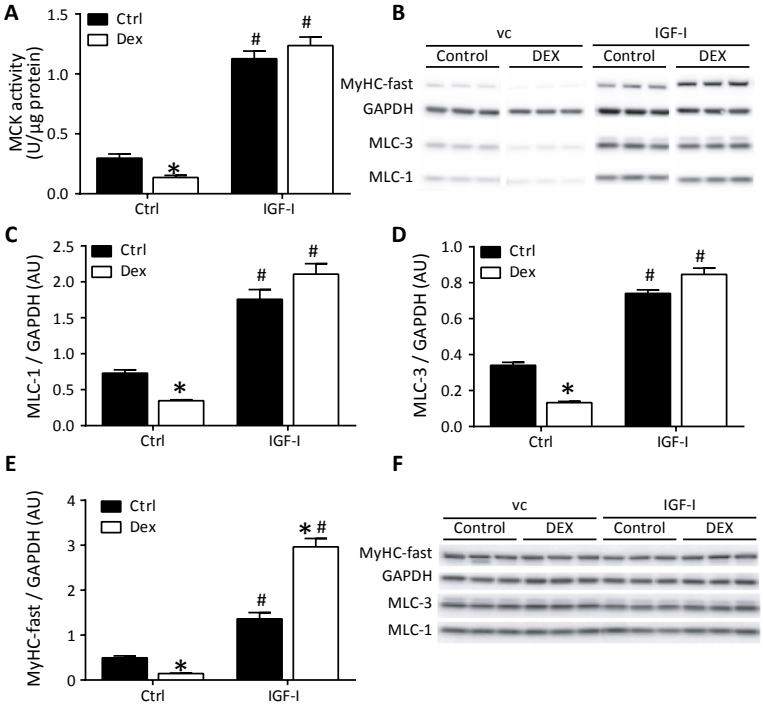


**Figure 4. Synergism between DEX and IGF-I on muscle protein abundance.** C2C12 cells differentiated for 72h and treated with IGF-I (5nM), DEX 10µM or vc as indicated. Cells were harvested to determine (A) MCK activity, or perform (B) westernblot analysis and (C-E) quantification of myofibrillar proteins. C2C12 cells were differentiated for 120h and treated with IGF-I (5ng/ml) DEX 10µM or vc as indicated for another 48h. Cells were harvested and preformed (F) westernblot. Two-way t test with unequal variance * ctrl *vs.* DEX, # ctrl *vs.* IGF-I; $p<0.05$. Shown are representative data of three independent experiments.

## DISCUSSION

GC are frequently administered to COPD patients and are known to induce muscle atrophy [9, 12, 41], but little is known regarding their influence on muscle re-growth. Yet, recovery of muscle mass is an important and main goal of pulmonary rehabilitation programs for muscle wasted COPD patients. These pulmonary rehabilitation programs are often multimodal and typically include a personalized exercise program combined with nutritional support upon indication. The last two decades several studies addressed complementation of this approach with muscle growth stimulating pharmaceuticals, including anabolic steroids [41, 42]. Many of the effects of anabolic steroids are a consequence of increased androgen receptor-mediated expression of IGF-I in skeletal muscle, which in an auto-/paracrine fashion induces local IGF-signaling and subsequent muscle growth [16-20]. Therefore, we used IGF-I to model these steroid effects on myogenesis, which is an important component of muscle re-growth. In line with previous reports, IGF-I enhanced myotube formation and muscle-specific protein expression, including sarcomeric proteins [24, 31, 43]. In contrast, the synthetic GC DEX potently inhibited all these aspects of myogenesis. Although a few studies have reported stimulatory effects of GC on myogenic differentiation [44, 45], our findings are

in agreement with most reports showing inhibitory effects of GC on myogenesis [11, 12, 14]. These contradictory effects of GC on myogenesis may reflect a concentration-dependent biphasic response. Belanto for example observed increased myoblast fusion in response to low GC levels, and we postulate that those results could be derived from an interaction between GC and anabolic factors, like IGF-I, present in the cell culturing serum [46]. This notion is in line with the synergistic stimulation of myotube formation

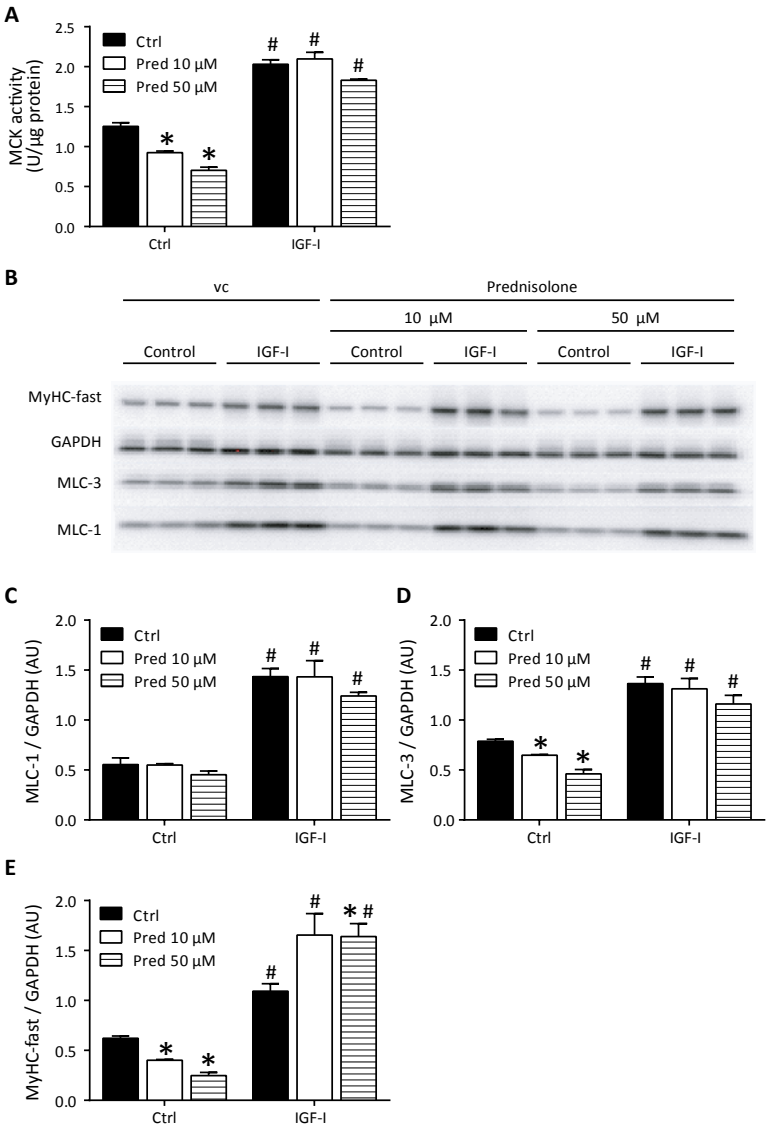


**Figure 5. Prednisolone and IGF-I synergistically increase myosin heavy chain abundance.** C2C12 cells differentiated for 72h and treated as indicated with IGF-I (5nM) Prednisolone (10 or 50µM) or vehicle (vc). Cells were harvested to determine (A) MCK activity, or perform (B) westernblot analysis and (C-E) quantification of myofibrillar proteins. Two-way t test with unequal variance * ctrl *vs.* DEX, # ctrl *vs.* IGF-I; p<0.05. Shown are representative data of three independent experiments.

in response to simultaneous addition of GC and IGF-I during myoblast differentiation reported here, and suggests that independent mechanisms lie at the basis of inhibition of myoblast fusion by GC and its synergistic stimulation when combined with IGF-I.

We and others previously demonstrated segregation between morphological and biochemical parameters of myogenic differentiation [46-48]. However, the abundance of muscle-specific, and particularly MyHC proteins was consistently increased in presence of IGF-I/GC compared to IGF-I only, indicating that the regulatory cues in control of both muscle-specific gene expression and myoblast fusion corresponded. The effects of simultaneous GC- and IGF-I signaling on myogenesis were not previously described, but

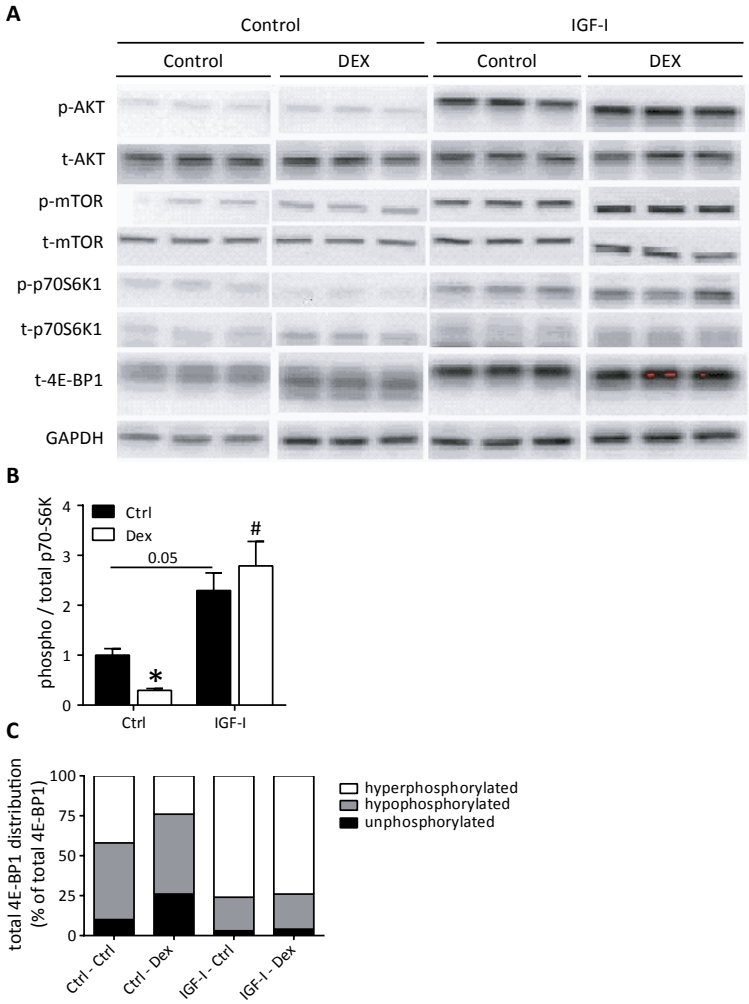


**Figure 6. DEX does not affect increased protein synthesis signaling by IGF-I.** C2C12 cells were serum starved for 18h and than treated with IGF-I (5nM) DEX 10μM or vc as indicated after 6h. (A) Cells were harvested for westernblot analysis and indicated phospho and total proteins were detected to determine protein synthesis signaling status with induction of myogenic differentiation. (B) p70-S6K1 phosphorylation and (C) determination of 4E-BP1 phospho isoform distribution were quantitatively assessed. Two-way t test with unequal variance * ctrl *vs.* DEX, # ctrl *vs.* IGF-I; p<0.05.

in mature myotubes IGF-I prevents GC-induced muscle atrophy [10, 49]. Although IGF-I and DEX at the concentrations applied in our study did not affect myofibrillar protein content in myotubes, importantly, no synergistic effect on myotube size or myofibrillar protein accretion in myotubes was observed, in contrast to differentiating myoblasts. In myotubes, prevention of GC-induced atrophy partly results from restoration of protein synthesis [10, 11, 14], as DEX-induced decreases in phosphorylation of 4E-BP1 and p70-S6K1, two key regulatory proteins of mRNA translation, is prevented in presence

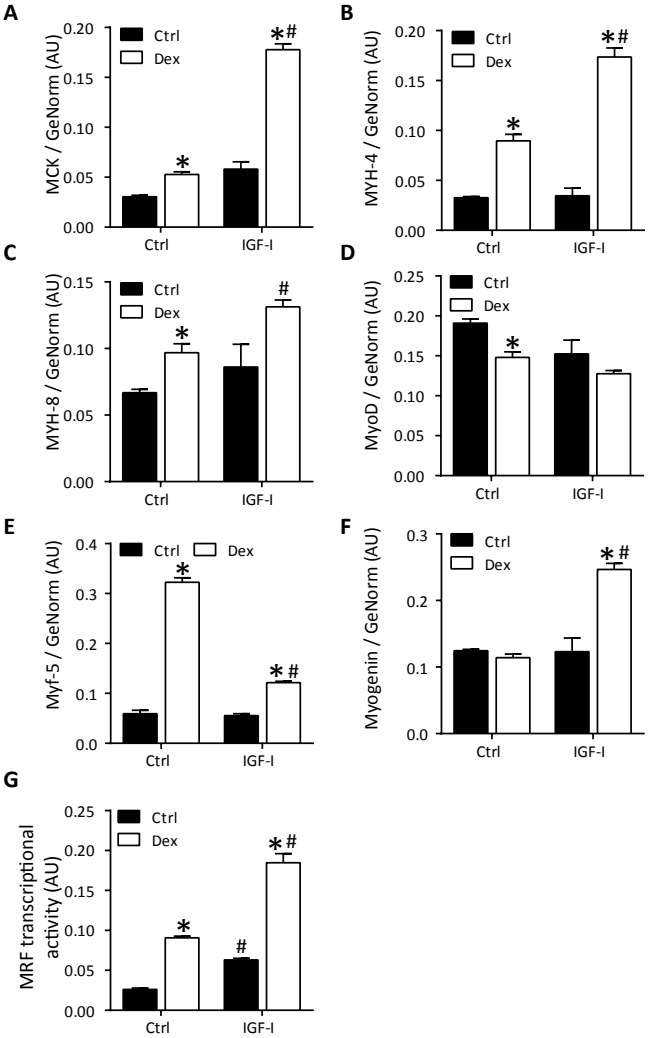


**Figure 7. Glucocorticoids upregulate muscle-specific gene expression.** (A-F) C2C12 cells differentiated for 48h and treated with IGF-I (5nM) DEX 10μM or vc as indicated were harvested for mRNA quantification. (G) C2C12 myoblasts were transfected with a 4RTK luciferase-reporter construct (0.9μg) and plasmid encoding β-gal (0.1μg) and cultured for 48h and treated with IGF-I (5nM) DEX 10μM or vc as indicated and harvested for MRF induced 4RTK-luciferase activity. Two-way t test with unequal variance * ctrl *vs.* DEX, # ctrl *vs.* IGF-I; $p<0.05$. Shown is representative data of three independent experiments.

of IGF-I. In differentiating myoblasts DEX similarly decreased 4E-BP1 and p70-S6K1 phosphorylation [11, 14], and IGF-I restored 4E-BP1 and p70-S6K1 phosphorylation levels [10]. Based on these studies and our results, regulation of protein synthesis signaling in response to GC and IGF-I appears to be conserved in myoblasts and myotubes.

As the synergism between GC and IGF-I signaling on muscle differentiation was not explained by a further stimulation of mRNA translation initiation, the stimulatory effect of simultaneous GC and IGF-I addition must stem from another regulatory level of myogenesis. Apart from inhibiting protein synthesis (signaling), GR activation also initiates the transcription of GC/GR-sensitive genes, including Glul, KLF-15 and REDD1 [14, 50]. Although IGF-I decreased both Glul and KLF-15 induction by DEX, a strong up-regulation compared to control was still detected. Furthermore GC-induced REDD1 expression [14], as well as GR-dependent reporter gene activity further increased with IGF-I addition. These differential responses of promoter transactivation and known mRNA targets of GR mediated gene transcription, could reflect a change in specific GR regulated genes in presence of IGF-I unrelated to effects on overall promoter transactivation. Nevertheless, these data reveal intact GC-induced GR-mediated signaling despite the presence of IGF-I.

IGF-I increases muscle-specific gene transcription during myogenesis [24, 31, 43], which was confirmed by a moderate increase in MCK and MYH mRNA levels in response to IGF-I. Considering the inhibitory effects of DEX on myogenesis, the elevated MYH-4, MYH-8 and MCK mRNA levels observed in response to DEX were rather surprising, although DEX-induced increases in MYH-4 and MYH-8 mRNA levels have been reported previously [33]. These results further confirm that inhibition of myogenesis by GC likely results from impaired mRNA translation, as muscle-specific mRNA transcripts were higher rather than lower in response to GC. In fact, this effect may lie at the basis for the synergism observed between DEX and IGF-I on muscle-specific protein content. As Increased accumulation of muscle-specific mRNA transcripts induced by DEX, can be effectively translated to protein in presence of IGF-I.

Still, in mature myotubes, IGF-I restores GC mRNA translation inhibition, but no synergic increases of myofibrillar protein content or myotube size have been reported [11,

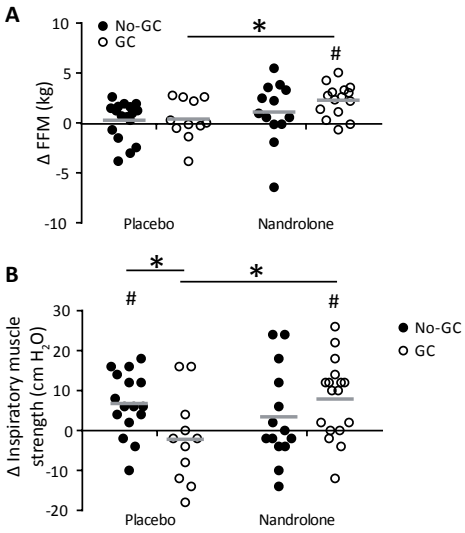


**Figure 8. Synergy between nandrolone and glucocorticoids on muscle mass recovery in COPD patients with muscle wasting.** Muscle wasted COPD patients stratified according to prednisolone use completed 8-weeks of pulmonary rehabilitation while receiving placebo or Nandrolone intramuscular-injections. The response of change in (A) FFM and (B) inspiratory muscle strength is shown as absolute change in delta of week 8 - week 0 FFM. Paired t-test week 0 *vs.* 8 #; p<0.001; one-way ANOVA with *post-hoc* LSD *; p<0.05.

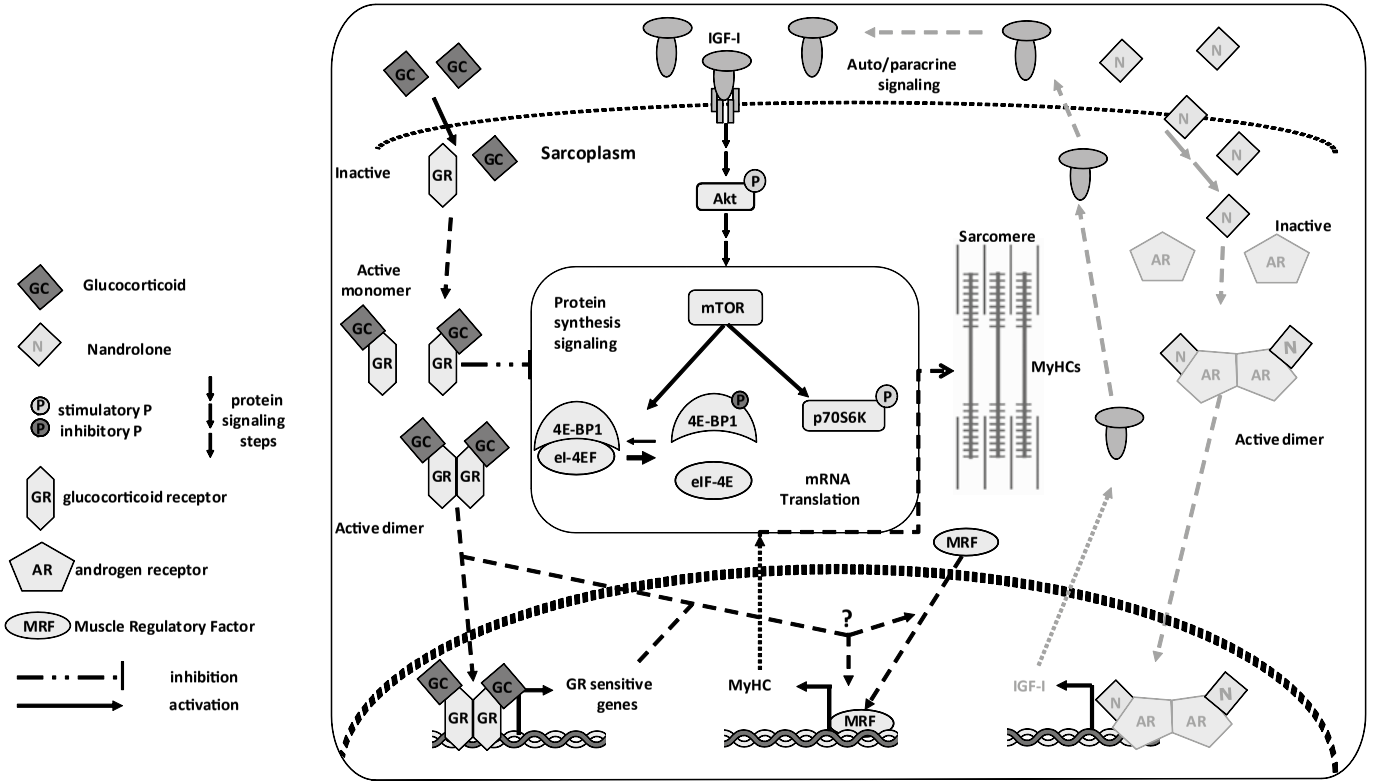


**Figure 9. Schematic representation of the proposed mechanism of glucocorticoid and IGF-I signaling synergism during myogenesis.** Glucocorticoids (GC) induce glucocorticoid receptor (GR) signaling and GR-mediated gene transcription, while reducing protein synthesis signaling. Note the unexpected stimulation of muscle specific gene transcription by GCs, which may involve increased expression or activity of the muscle regulatory factors (MRFs). Induction of androgen receptor (AR) signaling (here depicted by nandrolone (N) binding), leads to increased IGF-I expression, which in an auto-paracrine manner, stimulates mRNA translation/protein synthesis signaling. The presence of IGF-I is permissive for effective translation myogenic transcripts, which even increase in response to GC-signaling, and hence may explain the synergism between GC- and IGF-I action on myogenic differentiation.

12, 21, 51], nor observed in our own study. A striking difference between differentiating myoblasts and mature myotubes are the expression levels and activity of muscle regulatory factors (MRFs), which are essential to muscle-specific gene expression [25]. In line with previous reports, DEX potently induced Myf-5 expression in differentiating myoblasts [52, 53]. Combined DEX and IGF-I treatment synergistically increased Myogenin expression, suggesting GC alone or combined with IGF-I may affect muscle-specific transcription by increasing the expression of specific MRFs. This coincided with increased MRF transcriptional activity, as we noted elevated transactivation of a MRF-responsive promoter containing E-box sequences by GC alone, or combined with IGF-I. Whether this is the consequence of increased MRF expression or activity resulting of post-translational modification remains to be established, but these data imply positive regulation of MRFs by GC, which has not been reported previously. This increased MRF expression and activity when IGF-I and glucocorticoids are combined may also lie at the basis of their synergistic effect on myoblast fusion, considering the role of MRFs like MyoD, Myf-5 and Myogenin on this process [54]. Importantly, as DEX is not frequently applied in the treatment of COPD, key findings were reproduced using the particularly relevant GC prednisolone (PRED) at equimolar and equipotent concentrations.

Evaluating the potential clinical relevance of these findings, a *post-hoc* analysis of a RCT [26] aimed at reversal of muscle wasting in male COPD patients with Nandrolone was performed following additional stratification for GC-use (mainly prednisolone). In line with its effects on myogenesis described above, and in agreement with a previous study [8], GC negatively affected pulmonary rehabilitation outcome as no gain in FFM (indicative of impaired muscle re-growth) was detectable in approximately 55% of patients of the placebo/GC compared to only 29% of patients in the placebo/No-GC group.

ND administration in absence of GC did not result in significant gain of FFM or inspiratory muscle strength. Absence of detectable favorable androgenic steroid effects has been previously reported and may have resulted from the relatively low dose of ND and the type of pulmonary rehabilitation protocol that was used [3, 55-57]. Alternatively, these findings may reflect a relative insensitivity of the measurements to detect changes in muscle mass, which may have been subtle based on the modest effects of IGF-I alone on myogenesis detected in the muscle cell culture experiments. Conversely, ND administration in presence of GC did significantly improve gain in FFM as well as inspiratory muscle strength. This is in line with the synergistic effect of GC and IGF-I observed in cultured myocytes, which stimulated rather than inhibited myogenesis compared to GC-only, and was more potent than IGF-I alone. Furthermore, in only 13% of these patients FFM was not increased upon completion of the protocol for ND/GC group, compared to a lack of response in 55% of the Ctrl/GC group.

Limitations of the current study include the use of a *post-hoc* analysis of a previously conducted and reported RCT [26]. Despite matched numbers for GC use, there was a slightly skewed distribution of the subjects over the GC-only and ND-only groups. In addition, the original design did not include the collection of muscle biopsies which could have substantiated the accumulation of muscle-specific mRNA transcripts and inhibition of protein synthesis signaling in presence of GC and restoration by ND. These considerations would certainly be included in the design of a prospective study to verify the synergistic effects of GC and increased IGF-I signaling on muscle re-growth.

Moreover, although requirement for myonuclear addition in compensatory hypertrophy has been demonstrated [58], the relative contribution of myogenesis to muscle recovery from atrophy remains to be resolved. Nevertheless the current results indicate that it might be worthwhile to further investigate the synergy of anabolic steroids and glucocorticoids on muscle re-growth in wasted COPD patients during recovery from a severe acute exacerbation.

In conclusion, and as schematically illustrated in Figure 9, GC in presence of increased IGF-I signaling may synergistically stimulate muscle re-growth as a consequence of increased muscle-specific mRNA transcription in presence of restored protein synthesis signaling during myogenesis.

## ACKNOWLEDGEMENTS

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# Chapter 3

## Regulation of skeletal muscle plasticity by glycogen synthase kinase-3β: a potential target for the treatment of muscle wasting

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## ABSTRACT

Muscle wasting is a prevalent and disabling condition in chronic disease and cancer and has been associated with increased mortality and impaired efficacy of surgical and medical interventions. Pharmacological therapies to combat muscle wasting are currently limited but considered as an important unmet medical need. Muscle wasting has been attributed to increased muscle proteolysis, and in particular ubiquitin-proteasome system (UPS)-dependent protein breakdown. However, rates of muscle protein synthesis are also subject to extensive (patho) physiological regulation, and the balance between synthesis and degradation ultimately determines net muscle protein turnover. As multinucleated muscle fibers accommodate threshold changes in muscle protein content by the accretion and loss of muscle nuclei, myonuclear turnover may additionally determine muscle mass. Current insights in the mechanisms dictating muscle mass plasticity not only reveal intricate interactions and crosstalk between these processes, but imply the existence of signaling molecules that act as molecular switchboards, which coordinate and integrate cellular responses upon conditions that evoke changes in muscle mass. These “master regulators” of skeletal muscle mass plasticity are preferred targets for pharmacological modulation of skeletal muscle wasting. In this review Glycogen synthase kinase-3β (GSK-3β) is highlighted as a master regulator of muscle mass plasticity since, in addition to its role in UPS-mediated muscle protein degradation, it also controls protein synthesis, and influences myonuclear accretion and cell death. Moreover, the regulation of GSK-3β activity as well as currently available pharmacological inhibitors are described and discussed in the context of multimodal treatment strategies aimed at the inhibition of GSK-3β, and optimal exploitation of its potential role as a central regulator of skeletal muscle mass plasticity for the treatment of skeletal muscle wasting.

# 1. GENERAL INTRODUCTION

A gradual decline in muscle mass is common with aging, and results in decreased physical functioning [1]. Muscle wasting is also a prevalent and disabling condition in chronic diseases such as chronic heart failure (CHF) [2], chronic obstructive pulmonary disease (COPD) [3, 4] chronic renal failure (CRF), AIDS [5], rheumatoid arthritis (RA) [6, 7] and cancer [8, 9]. In these diseases, muscle wasting has been associated with increased mortality and impaired efficacy of surgical and medical intervention [10]. Maintenance of muscle mass is therefore currently considered as an important unmet medical need.

Targeted medical intervention, however, requires a better understanding of the molecular mechanisms underlying the processes of muscle atrophy, muscle hypertrophy and muscle recovery. These processes are determined by the balance between muscle protein synthesis and degradation (muscle protein turnover) and by the balance between myonuclear loss and myonuclear accretion (myonuclear turnover) [11]. This review focuses on the role and therapeutic potential of GSK-3β in the cellular and molecular regulation of skeletal muscle mass plasticity. GSK-3β, although originally isolated from muscle [12, 13], is ubiquitously expressed [14] and involved in different cellular signaling pathways [15-17], which determine tissue growth and metabolism, such as IGF-I/Akt, Wnt/β-catenin, calcineurin/NFAT and apoptosis signaling. GSK-3β is a constitutively active kinase [18-22] and its function in various cellular signaling pathways is determined by regulatory inactivation [23], which makes modulation of GSK-3β activity by inhibitors an interesting pharmacological target [24-26]. For nearly three decades, a myriad of studies have described GSK-3β in muscle protein and myonuclear turnover, and in this review we postulate that GSK-3β may be considered as a central regulator of muscle mass plasticity.

# 2. GSK-3β

## 2.1 GSK-3 isoforms

The serine/threonine kinase GSK-3 was initially described as one of the kinases capable of phosphorylating and inhibiting its namesake substrate glycogen synthase (GS), a rate-limiting enzyme involved in glycogen synthesis [12, 27]. In vertebrates GSK-3 exists as two highly homologous isoforms encoded by distinct genes known as GSK-3α and GSK-3β [28]. GSK-3α is the larger of the two isoforms with a molecular weight of 51 kDa, whereas GSK-3β is a protein of 47 kDa. This size difference is attributable to the presence of a glycine rich extension at the N-terminus of GSK-3α [28, 29]. GSK-3 has been highly conserved during evolution and orthologs exist in virtually every eukaryotic species investigated to date [30]. Intriguingly, in lower eukaryotes (e.g. nematodes, sea squirts) GSK-3 is encoded by a single gene [31]. It is believed that in higher eukaryotes the two paralogous GSK-3 isozymes split from a common precursor at the appearance of vertebrates, implying that during evolution one of the isoforms took on unique functions, possibly to compensate for a higher degree of system complexity [31]. Avian species, however, have only one copy of the GSK-3 gene and appear to have selectively lost GSK-3α [31]. GSK-3 is officially known as Shaggy (Sgg) (Gene ID 13248) in *Drosophila melanogaster* although it is also referred to a zeste-white 3 (zw3) and as human tau protein kinase (tPK1) in *Homo sapiens*. However, for the remainder of this review article we will use the term GSK-3. The overall structure of GSK-3 is shared by many kinases

and constitutes a typical two-domain kinase fold with a small N-terminal lobe, consisting mostly of β-sheets, and a large C-terminal lobe, primarily composed of α-helices. Both isoforms share a high degree of sequence similarity within their kinase domains (98% identity), but differ substantially at their N- and C-terminal domains (84% overall identity) [28]. The catalytic domain is located at the interface between the α-helical and β-strand domains [32-34] and is folded into a bi-lobal architecture containing the positively charged ATP-binding pocket and the activation loop (T-loop), which is important for kinase activity [35, 36].



## 2.2 Redundancy of GSK-3α and β

Although GSK-3 was originally isolated from muscle [12, 13], this kinase is ubiquitously expressed in all tissues, with particularly high expression levels in the brain [14]. Considering the high degree of sequence similarity, it is not surprising that both GSK-3 isoforms share similar functions. Despite this fact, GSK-3α and GSK-3β are not always functionally redundant. In 2000, Hoeflich and colleagues disrupted the GSK-3β isoform in mice, which gave rise to an embryonically lethal phenotype. Analysis of the GSK-3$\beta^{-/-}$ embryos revealed massive liver degeneration due to hepatocyte apoptosis, thought to be a consequence of a defect in nuclear factor κB (NFκB) activation [37]. The inability of GSK-3α to compensate for the loss of GSK-3β implied a specific requirement of GSK-3β in hepatocyte survival. Conversely, it was demonstrated that GSK-3α and GSK-3β appear to be entirely redundant in regulating Wnt/β-catenin signaling [37, 38]. This redundancy in function has only recently been appreciated because the role of the β-isoform has been historically overemphasized. This bias probably originated from reports suggesting that mammalian GSK-3β was more effective than GSK-3α in rescuing the zeste-white 3 mutation in *Drosophila melanogaster* [39, 40]. However, it should be noted that in these studies the expression levels of both homologs were not equalized. Recently, experiments done in mouse embryonic stem cells (ESC) clearly established that the single loss of either GSK-3α or GSK-3β did not negatively alter Wnt/β-catenin signaling, whereas GSK-3α/β double knockout ESCs displayed hyperactivated Wnt/β-catenin signaling, resulting in dramatically skewed cell differentiation [38]. Similarly, in chondrocytes the function of GSK-3α and GSK-3β appeared to be redundant or compensatory in the early stage of differentiation, as compound knockout (GSK-3$\alpha^{-/-}$; GSK-3β+/-) mice exhibited dwarfism and impaired chondrocyte differentiation, while single GSK-3$\alpha^{-/-}$, GSK-3β+/-, or cartilage-specific GSK-3β knockout mice appeared phenotypically normal [41]. Nevertheless, the two isoforms were shown to have opposite effects on the transcriptional activation of certain transcription factors [42], and GSK-3β is more potent than GSK-3α in phosphorylating phosphatase inhibitor 2 [43]. Furthermore, GSK-3α and GSK-3β play distinct roles in cardiomyocyte differentiation and cardiovascular development in mice [44-46]. Interestingly, both GSK-3 isoforms serve distinct tissue-specific roles in glycogen metabolism. MacAulay *et al.* found that global loss of GSK-3α improved glucose tolerance and insulin sensitivity in knockout mice, mainly by elevating hepatic glycogen synthesis with no observable difference in glycogen deposition in muscle [47]. Previously, it was demonstrated that muscle-specific overexpression of GSK-3β resulted in reduced muscle glycogen deposition [48], and that insulin failed to increase muscle GS activity in constitutively active GSK-3β mice [49], indicating that GSK-3α and GSK-3β exhibit divergent physiological roles in muscle

and liver tissue. Additional evidence identifying GSK-3β as a major regulator of glucose homeostasis in skeletal muscle came from the group of Woodgett, demonstrating that tissue-specific GSK-3β knockout mice displayed improved glucose tolerance and enhanced insulin sensitivity in muscle (but not liver) [50]. In a study from McManus *et al.* GSK-3β protein expression in human and mouse muscle was found to be three to four times higher than compared with GSK-3α, prompting the authors to conclude that GSK-3β, rather than GSK-3α was the predominant GSK-3 isoform in muscle [49]. In conclusion, it appears that GSK-3α and GSK-3β have both common and non-overlapping cellular functions, largely depending on the physiological context and the cell type studied. The focus of this review will be specifically on the role of GSK-3β in the regulation of skeletal muscle plasticity.

## 2.3 Substrate regulation by GSK-3β

GSK-3β is a unique multi-tasking enzyme involved in a myriad of cellular processes which is subject to multiple complex regulatory mechanisms. Unlike other kinases GSK-3β is constitutively active and may be rapidly and reversibly inactivated in response to various cellular signals, such as growth factors. Another feature that sets it apart from other kinases is that phosphorylation of its substrates generally leads to their inactivation [51, 52]. As is common with many kinases, GSK-3β catalyzes the phosphorylation of its substrates in the presence of ATP and the cofactor magnesium ($Mg^{2+}$) [53]. An intriguing feature of GSK-3β involves its requirements for substrate recognition. In general, the specificity of most kinases is largely dependent on a particular consensus sequence of amino acid residues of their substrates. GSK-3β, on the other hand, does not require a strict consensus motif, but instead has the unusual preference for target proteins that are pre-phosphorylated at a “priming” residue located four amino acids C-terminal to the site to be modified by GSK-3β [54]. Thus, the consensus sequence for GSK-3β substrates is Ser/Thr-X-X-X-X-Ser/Thr-P, where the N-terminal Ser/Thr represents the target residue and the C-terminal Ser/Thr constitutes the priming phosphorylation site. Although “X” denotes any amino acid, in the case of GSK-3β, this often concerns a proline. The phosphorylated priming site of the substrate binds to a positively charged binding pocket in GSK-3β, formed by the residues $Arg^{96}$, $Arg^{180}$ and $Lys^{205}$. This mechanism allows for proper orientation of the kinase domains, and places the substrate at the correct position within the catalytic groove for optimal kinase activity [20, 55, 56]. Mutation of $Arg^{96}$ to an uncharged alanine disrupts the positively charged pocket which prevents any interaction with the negatively charged phosphate and sulfate ions, precluding primed substrate binding [56]. However, in spite of this mutation the enzyme retains its activity and can still phosphorylate unprimed substrates. Though not strictly required, priming phosphorylation significantly augments the efficiency of GSK-3β substrate phosphorylation by 100-1000 fold [57]. Nevertheless, several substrates such as β-catenin, tau and axin do not require a priming step prior to phosphorylation by GSK-3β [52].

## 2.4 Regulation of GSK-3β enzymatic activity

The discovery of the crystal structure of GSK-3β in 2001 has had a profound impact on our understanding of the regulatory mechanisms of this extraordinary molecule [20, 55, 58]. Few enzymes exert more influence over cellular function than GSK-3. In fact,

GSK-3 is one of the kinases with the most identified substrates in the cell [59], including a wide variety of metabolic signaling molecules, structural proteins and over a dozen transcription factors [60, 61]. The ability of GSK-3β to accurately execute this astoundingly diverse set of functions is due to the unique and intricate regulatory mechanisms this kinase is subject to. Tight regulation of GSK-3β's activity can be achieved by a variety of mechanisms that are each dependent on specific signaling pathways. These include regulation mediated by a) phosphorylation b) cellular localization c) protein-protein interactions and d) proteolytic cleavage.



### *2.4.1 Regulation by phosphorylation*

The best-defined mode of GSK-3β regulation is inhibition of its activity by phosphorylation of the regulatory $Ser^{9}$ residue in the N-terminus ($Ser^{21}$ in GSK-3α) [23]. Insulin/IGF-I-mediated activation of the phosphatidylinositol 3-kinase (PI-K)/Akt signaling pathway is a major regulator of GSK-3β activity in which activated Akt/protein kinase B (PKB) (hereinafter termed Akt) directly phosphorylates GSK-3β on this inhibitory residue [23, 62, 63]. Upon phosphorylation, the N-terminus becomes a primed pseudo-substrate that binds intra-molecularly to the positively charged binding pocket and the active site, occupying the same binding site as the priming phosphate of a substrate thereby hindering substrate phosphorylation [51, 64]. Important to note is that this inhibition mechanism is competitive, implying that elevated concentrations of primed substrates will outcompete pseudo-substrate inhibition [56]. Bioinformatics analyses of pseudo-substrate sequences in GSK-3β have pinpointed two highly conserved amino acid residues, namely $Arg^{4}$ and $Arg^{6}$. Mutation of any of these residues to alanine enhanced basal GSK-3β activity and prevented autoinhibition by the pseudo-substrate, even upon phosphorylation at $Ser^{9}$ [65]. More specifically, $Arg^{4}$ and $Arg^{6}$ were found to facilitate the interaction of the pseudo-substrate with the catalytic core [65]. In addition to insulin and IGF-I, numerous other stimuli lead to GSK-3β inactivation through $Ser^{9}$ phosphorylation, including growth factors Platelet-derived growth factor (PDGF) and Epidermal growth factor (EGF) that stimulate the GSK-3β inactivating kinase $p90^{RSK}$ via Mitogen-activated protein kinases (MAPK) [61]. Moreover, amino acids can activate p70S6K which in turn reduces GSK-3β activity [66, 67] via $Ser^{9}$ phosphorylation [67]. Other kinases capable of phosphorylating GSK-3β at $Ser^{9}$ include Protein kinase A (PKA) and some isoforms of Protein kinase C (PKC) [68]. GSK-3β activity can also be regulated by phosphatases which activate the enzyme by dephosphorylation. The serine/threonine Protein phosphatase 1 (PP1) dephosphorylates the N-terminal serine residues of GSK-3β [69]. In addition, Protein phosphatase 2A (PP2A) may activate GSK-3β directly by dephosphorylation, or indirectly by dephosphorylation and subsequent inhibition of Akt [70].

In contrast to the inhibitory effect obtained after GSK-3β phosphorylation at $Ser^{9}$, the enzymatic activity of GSK-3β can be enhanced by a stimulatory phosphorylation on $Tyr^{216}$ [21]. Recent crystallographic studies and molecular dynamics simulation techniques have clearly demonstrated that $Tyr^{216}$ phosphorylation renders the kinase active by stabilization of the activation loop (T-loop), allowing full substrate accessibility [18, 71]. Hughes *et al.* reported that this phosphorylation is constitutive in resting cells [21], and it was suggested that in mammalian species the phosphorylation of $Tyr^{216}$ was a chaperone-dependent autocatalytic event regulated by Hsp90 [19]. Additional support for this intramolecular autophosphorylation model, at least in mammals, was provided

by Lochhead *et al.*, who showed that newly synthesized GSK-3β becomes phosphorylated on $Tyr^{216}$ [22]. Overall, it appears that T-loop phosphorylation at $Tyr^{216}$ may facilitate substrate phosphorylation but it is not strictly mandatory for kinase activity [20]. Murai and co-workers reported that prolonged growth factor stimulation decreased both the activity and $Tyr^{216}$ phosphorylation of GSK-3β [72]. However, these findings were contradicted by another report claiming that the insulin-induced decrease in GSK-3β activity was strictly due to increased phosphorylation at $Ser^{9}$, rather than the involvement of a phosphotyrosine phosphatase acting on GSK-3β [73]. In line with the latter study, Simon *et al.* revealed that pharmacological GSK-3β inhibition using lithium did not alter phosphotyrosine levels, despite effective kinase inhibition [74]. Moreover, Bhat *et al.* reported that in neurons proapoptotic stimuli increased nuclear activity of GSK-3β by increasing phosphorylation at $Tyr^{216}$, arguing against the autophosphorylation model. In concordance with the study by Simon *et al.* pharmacological GSK-3β inhibition induced $Ser^{9}$ phosphorylation, resulting in diminished GSK-3β activity without reducing $Tyr^{216}$ phosphorylation [75]. It is evident that due to the conflicting nature of these reported findings more research is needed to unravel the exact role of $Tyr^{216}$ phosphorylation. In muscle, too, the physiological relevance and the mechanisms regulating $Tyr^{216}$ phosphorylation remain poorly defined and there is a paucity of data pertaining to the identification of the GSK-3β tyrosine kinases and phosphotyrosine phosphatases involved. Recently, two other regulatory phosphorylation sites have been described in GSK-3β. First, a phosphorylation event at $Thr^{43}$ was shown to be catalyzed by Erk and resulted in GSK-3β inhibition [76]. Likewise, p38 MAPK-mediated phosphorylation of GSK-3β on $Ser^{389}$ and $Thr^{390}$ was associated with reduced kinase activity [77]. In both instances, phosphorylation of these residues increased the propensity of $Ser^{9}$ to be phosphorylated, as opposed to promoting direct kinase inhibition.

### *2.4.2 Regulation by protein-protein interactions*

GSK-3β activity can also be determined by its presence in protein complexes. The best characterized example is the Wnt/β-catenin signaling pathway, also known as the canonical Wnt-signaling pathway (for review see, i.e. [78]). Wnt/β-catenin signaling requires, in part, GSK-3 inactivation [38]. Under basal conditions, β-catenin levels are regulated by a protein complex, termed the degradation complex containing Axin (the scaffolding protein), adenomatous polyposis coli (APC, also a target of GSK-3 [79]), GSK-3β and casein kinase 1α (CK1α). In this complex CK1α primes β-catenin by phosphorylation which elicits further N-terminal phosphorylation by GSK-3 [80], thereby marking it for degradation by the ubiquitin-proteasome system (UPS) [81]. Upon binding of canonical Wnts the formation of a complex with membrane receptors Frizzled (Fz), a seven-transmembrane protein, and the low-density lipoprotein receptor-related protein 5/6 (LRP5/6) leads to activation of Wnt/β-catenin signaling [82]. Dishevelled (Dvl) becomes activated and recruits the degradation complex to the membrane [83] where GSK-3β phosphorylates the receptor LRP5/6 [84, 85], which prevents subsequent GSK-3β-mediated β-catenin phosphorylation, resulting in β-catenin stabilization [86-88]. Accumulated cytoplasmic β-catenin is involved in cell-cell interactions in a complex with cadherins [89, 90], while increased nuclear β-catenin levels induce the expression of growth-associated genes such as c-Myc and cyclin D1 (see Figure 3) by interacting with transcriptional co-activators such as members of the T-cell factor/Lymphocyte-

enhancement factor-1 (Tcf/Lef-1) family [91]. Thus, GSK-3 appears to play a dual role, being involved in both the activation of Wnt/β-catenin signaling, through activating phosphorylation of LRP5/6, as well as in its inhibition via phosphorylation of β-catenin, illustrating the intricate regulation of this signaling cascade. GSK-3-binding protein (GBP), also known as FRAT, is another complex that has been associated with GSK-3 [83, 92]. The formation of such protein complexes enables the cell to control the actions of GSK-3β within subcellular compartments. For instance, the protein-kinase interaction of GSK-3-binding protein GBP with GSK-3 appears to facilitate nuclear export of GSK-3 as a mutant form of GSK-3 that cannot bind FRAT accumulates in the nucleus [93].

***2.4.3 Regulation by subcellular localization***

Even though GSK-3β is traditionally viewed as a cytosolic protein, it has also been found in nuclei and mitochondria [94]. In contrast, under basal conditions GSK-3α does not shuttle between the cytosol and the nucleus. Yet, upon activation of the calpain pathway in response to increased calcium levels or in absence of growth factors, GSK-3α has been shown to accumulate in the nucleus [95]. This process was not mediated by N-terminal cleavage of GSK-3α (*vide infra*). Instead, nuclear accumulation was governed by binding of the N-terminus of GSK-3α to a calpain-sensitive protein [95]. As mentioned earlier, GSK-3β regulates a host of substrates, many of which are transcription factors. For instance, nuclear GSK-3β is capable of phosphorylating NFATc, thereby negatively affecting its DNA binding activity [96]. However, surprisingly little is known about the regulation of the intracellular compartmentalization of GSK-3β. As early as 1998 Ragano-Caracciolo *et al.* reported that GSK-3β was enriched in a glycogen fraction associated with the nuclear envelope [97]. Not soon thereafter, studies by the group of Diehl demonstrated that there were dynamic cell cycle-dependent changes in the intracellular localization of GSK-3β. Specifically, they found increased levels of nuclear GSK-3β during the S phase, related to proteasomal degradation of cyclin D1 [98, 99]. Furthermore, heat shock was shown to evoke nuclear GSK-3β accretion [100]. It was also reported that activated Akt decreased nuclear GSK-3β levels [101], while in mitochondria GSK-3β was inactivated in an Akt-dependent fashion without affecting the mitochondrial level of GSK-3β [68]. In cardiomyocytes, GSK-3β is present in both the nucleus and the cytosol, and endothelin was reported to enhance nuclear localization of GSK-3β [102]. Consistent with the previously established role of GSK-3β in NFκB activation [37], highly activated GSK-3β was found to accumulate in the nuclei of pancreatic cancer cell lines, positively affecting NFκB-mediated proliferation of cancer cells [103]. Although the exact mechanisms regulating the subcellular localization of GSK-3β remain elusive, recent advances (*vide supra*) in this field have ascribed an important function to GSK-3β binding proteins in the regulation of subcellular trafficking.

***2.4.4 Regulation by proteolytic cleavage***

Recently, a novel mechanism of GSK-3 regulation was proposed. It involves calpain-1-mediated truncation of both GSK-3 isoforms. This type of cleavage removes a considerable portion of the N-terminal region including the regulatory $Ser^{9}$ and $Ser^{21}$ residues, which results in a non-reversible activation of the enzyme [104]. This proteolytic cleavage appeared to be mediated by extracellular calcium levels and could be inhibited by memantine [105], a N-methyl-D-aspartate (NMDA) receptor antagonist

which had previously been demonstrated to increase the serine phosphorylation of both GSK-3 isoforms [106]. In the former study, GSK-3 truncation was observed in mouse and human post mortem brain tissue [105]. Matrix metalloproteinases (MMPs) are traditionally known for their role in extracellular matrix remodeling. However, several lines of evidence have recently revealed additional substrates and novel biological roles for these proteases [107, 108]. Interestingly, in cardiomyoblasts matrix metalloproteinase-2 (MMP-2) is capable of cleaving the N-terminal portion of GSK-3β, including the regulatory $Ser^9$, resulting in increased GSK-3β activity [109]. In this study, hydrogen peroxide-induced MMP-2 activity led to increased GSK-3β activity, which in turn contributed to cardiac dysfunction as a result of oxidative stress injury.

***2.4.5 Pharmacological modulation of GSK-3***

A large body of evidence has confirmed the practicability and therapeutic benefit of pharmaceutical GSK-3 inhibitors, making GSK-3 a prime target in drug discovery. A detailed analysis of all these inhibitors is beyond the scope of this manuscript. Instead, we will focus on the most important types of GSK-3 inhibitors and their *modus operandi*.

Most GSK-3 inhibitors function either as ATP-competitive, non-ATP-competitive or substrate-competitive inhibitors. GSK-3 inhibitors come in a diversity of chemotypes, including cations, synthetic small molecules and inhibitors isolated from natural sources. The alkali metal lithium ($Li^+$) was the first GSK-3 inhibitor to be discovered [110, 111]. Lithium possesses the intriguing capability of inhibiting GSK-3 in both a direct and indirect fashion. As mentioned earlier, enzymatic GSK-3 activity is ATP-dependent and magnesium is required as a cofactor [53]. First, lithium functions as a direct reversible inhibitor of GSK-3 by competing for magnesium ions [112]. Second, GSK-3 inhibition is achieved indirectly via enhanced serine phosphorylation and autoregulation [69, 113-115]. Increased phosphorylation of GSK-3 in response to lithium is the result of decreased phosphatase activity, which normally reactivates GSK-3 by removing the inhibitory phosphate from the regulatory serine residue ($Ser^9$ in GSK-3β) [114, 116]. When used in cultured cells or *in vivo*, this dual mode of action of lithium probably accounts for a cumulative suppression of GSK-3 activity.

Of note, regulation of GSK-3β activity by lithium does not always occur through increased GSK-3β phosphorylation [117]. This divergence between GSK-3β phosphorylation at $Ser^9$ and GSK-3β kinase activity has also been reported for chemically unrelated GSK-3β inhibitors [115, 118, 119] and is consistent with other described modes of GSK-3β inactivation [55]. Although lithium has been widely used as a GSK-3 inhibitor for decades, it is not completely specific in its actions and high therapeutic concentrations are required [120]. The therapeutic range of lithium is 0.5 - 1.5 mM and its $IC_{50}$ towards GSK-3 is 1 – 2 mM [110]. Other direct molecular targets of lithium include, amongst others, inositol monophosphatases (IMPAs), cyclooxygenase (COX) and β-arrestin 2 (βArr2) [121]. The fact that magnesium and lithium share similar periodic properties, may explain the wide array of biochemical effects exerted by lithium, as magnesium is a cofactor for multiple enzymes [122]. In addition to lithium, several other metal ions such as beryllium, zinc, mercury and copper are considered *bona fide* GSK-3 inhibitors [123, 124].

To date, most of the protein kinase inhibitors are ATP-competitors. However, the clinical use of these compounds is limited because of serious limitations in their

specificity. This is due to the fact that the human kinome constitutes more than 500 kinases that all share a significant degree of sequence homology in the catalytic site, especially within the ATP-binding pocket [125]. The quest for optimization of selectivity is therefore one of the most important challenges in the discovery and development of kinase inhibitors [125, 126]. Some of the first synthetic small molecule GSK-3 inhibitors included the purine analog aminopyrimidines CHIR98014, CHIR98023 and CHIR99021 [127], which together with the maleimides derivatives SB-216763 and SB-41528 displayed selective inhibition against both GSK-3 isoforms [128]. Despite the fact that these are all "less specific" ATP-competitive GSK-3 inhibitors, systematic analysis of profiled kinase inhibitors revealed high selectivity of these compounds toward GSK-3 in the nanomolar concentration range [129].

Generally, physiological inhibition of GSK-3 in response to insulin [130] or IGF-I [131] reduces GSK-3 activity by 40% and 20%, respectively, indicating that moderate inhibition using pharmacological approaches may be sufficient to correct or influence GSK-3β-dependent signaling processes. For example, pharmacological GSK-3 inhibition using lithium was shown to reduce GSK-3β activity by 30 and 35% in cultured muscle cells and muscle tissue, respectively [117, 131]. In other words, restoration of physiological GSK-3 activity levels could be sufficient to yield important therapeutic effects in pathologies characterized by aberrant GSK-3 regulation [18, 132]. Moreover, over-inhibition of GSK-3 under normal circumstances might have detrimental effects, such as tumorigenesis [133], cardiac hypertrophy [102, 134] or the induction of neurodegenerative markers [135].

Non-ATP-competitive GSK-3 inhibitors bind to unique regions within the kinase, offering a more selective and subtle mode of enzymatic inhibition, as opposed to simply blocking ATP-binding. Several organic (synthetic) compounds have been reported that do not rely on ATP competition in their action against GSK-3. Some examples include the small heterocyclic thiadiazolidinones (TDZD) inhibitors such as TDZD-8 and NP00111, which were both shown to be potent GSK-3 inhibitors [136, 137]. Little is known about their exact mechanism of action but it was suggested that these compounds interact with $Cys^{199}$, a key residue located at the entrance to the ATP binding site of GSK-3 [53]. The halomethylketone (HMK) derivatives have recently been described as the first irreversible non-ATP-competitive GSK-3 inhibitors [138, 139]. The irreversible nature of this inhibition is due to the formation of a covalent sulfur-carbon bond between $Cys^{199}$ of GSK-3 and the HMK moiety [140].

It has proven difficult to identify substrate-competitive inhibitors by means of high-throughput screening, mainly due to the weak binding interaction with the enzyme (similar to the binding properties of the substrate). Nevertheless, substrate-competitive inhibitors are more selective than ATP-competitive molecules and may therefore be a more suitable choice for clinical use. In addition, chemical modification of substrate-competitive inhibitors have the potential of improving their specific binding affinity and pharmaceutical applicability [141]. Current research efforts focus on the use of peptides as potential protein kinase inhibitors [142]. As GSK-3 predominantly binds primed substrates, a set of phosphorylated peptides patterned after known GSK-3 substrates was generated and found to inhibit the enzyme in the micromolar range. The synthesized peptide L803 is derived from heat shock factor-1 (HSF-1) and displayed the best inhibitory properties [143]. Other examples of specific GSK-3 peptide inhibitors

include FRATide and Axin GID, which were synthesized based on the GSK-3β substrates FRAT and Axin, respectively [132].

In conclusion, the use of non-ATP-competitive GSK-3 inhibitors could be relevant for therapeutic use because of better kinase selectivity and lower $IC_{50}$ values which is paramount to avoid toxicity. The future challenge will be to design selective inhibitors toward GSK-3α and GSK-3β. This effort could prove challenging as both GSK-3 isoforms are highly conserved within their kinase domains [15]. An alternative strategic approach could involve exploiting the unique properties of both isozymes, such as cellular localization or specific protein-protein interactions.



## 3. GSK-3β IN SKELETAL MUSCLE PLASTICITY

As mentioned previously, GSK-3 was originally isolated from muscle as a kinase capable of phosphorylating and inactivating GS, a key enzyme that promotes glycogen assimilation [12, 144]. Apart from a role in muscle metabolism, GSK-3β is also involved in the maintenance and plasticity of skeletal muscle mass. A study by Schakman and co-workers implicated an important role of GSK-3β in skeletal muscle atrophy *in vivo* [145]. Muscle atrophy brought about by the synthetic glucocorticoid dexamethasone was associated with alterations in the Akt/GSK-3β signal transduction pathway. Previous data from the same group established that glucocorticoid-induced muscle atrophy and the associated reduction in muscle IGF-I-signaling could be restored by local IGF-I overexpression into muscle [146]. In the former study these findings were extended by local overexpression of caAkt which completely blocked dexamethasone-induced atrophy and resulted in marked muscle fiber hypertrophy. This phenotype was associated with increases in the phosphorylation status of p70S6K and GSK-3β, indicative of reduced GSK-3β activity. Similarly, overexpression of a dnGSK-3β induced a modest muscle fiber hypertrophy and completely prevented muscle atrophy induced by glucocorticoids. A study by the group of Booth investigated the effect of pharmacological GSK-3β inhibition on myotube size [147]. Remarkably, lithium supplementation increased the C2C12 myotube surface area by 85%, while inclusion of IGF-I in the culture medium reduced levels of phosphorylated GSK-3β and caused a similar increase in myotube area (80%). In order to understand the involvement of GSK-3β in muscle atrophy, hypertrophy and regeneration, the determinants of these processes, i.e. changes in muscle protein- and myonuclear turnover [11] will be briefly discussed below, and subsequently the involvement of GSK-3β in protein synthesis and degradation, myonuclear accretion and apoptosis during muscle mass plasticity will be addressed individually.

### 3.1 Processes controlling skeletal muscle mass plasticity

Muscle protein turnover is determined by the balance between protein synthesis (anabolism) and degradation (catabolism). A shift in favor of synthesis over degradation will result in muscle hypertrophy. Conversely, when muscle protein degradation exceeds depressed or even increased protein synthesis, muscle atrophy may ensue. Initiation of mRNA translation is one of the rate limiting steps of protein synthesis, and many regulatory pathways of protein synthesis converge at this level, including the IGF-I/Akt/mTOR and IGF-I/Akt/GSK-3β signaling pathways [148, 149]. Increased proteolysis during atrophy relies on the orderly activation of three main cellular proteolytic systems

including the ubiquitin 26S-proteasome system (UPS), the autophagy-lysosomal pathway (ALP) and the calcium-dependent calpains [11, 150-153]. The ALP is involved in clearance of long-lived proteins and dysfunctional organelles [154, 155], while the UPS mainly targets myofibrillar and short-lived proteins [156-159]. Interestingly, crosstalk exists at multiple levels in the signaling pathways that determine muscle protein synthesis and degradation, and this reciprocal interaction is essential in the control of protein turnover (see Figure 1).

The balance between myonuclear accretion and apoptosis determines myonuclear turnover, which is involved in the control of adult muscle fiber hypertrophy, atrophy and regeneration. Multinucleated muscle fibers are formed by the fusion of mono-nucleated muscle cells and these cells stem from the embryonic myotome [160], which will subsequently form mature muscle fibers [161]. The remaining non-fusing single nucleated cells form a specialized cellular niche and are termed “satellite cells” [162]. This name is derived from their location in between the epimysium, the membrane surrounding the muscle fibers, and the adult muscle fibers [163]. Satellite cells form a reservoir of new myonuclei, which are recruited during muscle plasticity responses [164] and upon activation, these cells proliferate, differentiate and fuse with or into (existing) muscle fibers (see Figure 1).

Muscle fiber nuclei (myonuclei) regulate gene expression within a certain cytoplasmic range referred to as the myonuclear domain. The current paradigm implies that when reaching the lower limit, myonuclei disappear from the muscle fiber, while when reaching the upper limit of the domain, nuclei provided by the satellite stem cell pool are incorporated into the myofiber [164, 165], although the latter was recently disputed [166-168]. The loss of myonuclei appears to involve apoptosis, and increased apoptotic signaling in atrophying skeletal muscle has been reported in aging [169-171], but has also been linked to conditions such as muscle disuse [172], burn injury [173], muscle denervation [174], unweighting [175] and cancer cachexia [176]. Nonetheless, nuclear apoptosis in muscle does not inevitably lead to myofiber cell death [170], and it is unknown how many nuclei may be lost before an entire muscle fiber undergoes apoptosis [177]. Further, accretion of new myonuclei may occur during muscle fiber hypertrophy when the upper limit of myonuclear domain of the present myonuclei in a muscle fiber is exceeded. Alternatively, myonuclear accretion also occurs in regenerating muscle fibers following damage or during recovery from atrophy [178, 179]. These new myonuclei are provided by the satellite-cell population [180, 181] (see Figure 1). In order to appreciate the role of GSK-3β in the regulation of muscle mass maintenance and plasticity, the current insights of the involvement of GSK-3β in the control of muscle protein synthesis and degradation, as well as myonuclear accretion and apoptosis will be individually discussed below.

## 3.2 Muscle protein turnover

### *3.2.1 Muscle protein synthesis and GSK-3β*

Protein synthesis is a tightly controlled process that requires orderly recruitment and functioning of a plethora of signaling molecules. Protein translation initiation appears to be the rate-limiting step in muscle protein synthesis, and is essentially controlled by eukaryotic initiation factors (eIFs). The activity of a number of these eIFs is

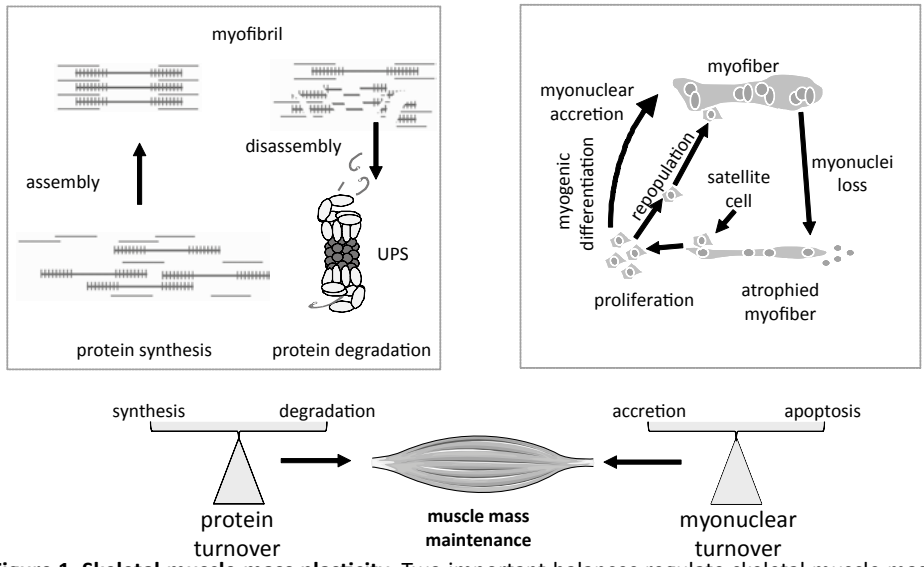


**Figure 1. Skeletal muscle mass plasticity.** Two important balances regulate skeletal muscle mass plasticity 1) Protein turnover, which comprises protein synthesis and degradation. Disassembly of contractile proteins from the myofibrillar apparatus may involve other proteolytic systems in addition to the UPS prior to their degradation. 2) Myonuclear turnover, which consists of myonuclear accretion and myonuclear loss. Myonuclear accretion occurs upon satellite cell activation and proliferation, which is followed by myogenic differentiation and myonuclear fusion. Loss of myonuclei mediated by apoptosis may occur during myofiber atrophy.

controlled by growth factor (e.g. IGF-I) and hormonal (e.g. insulin) signaling pathways. Protein translation is initiated by the binding of eIF2 to the activated initiator tRNA ($tRNA_i^{Met}$), which leads to the formation of a ternary complex that binds to the 40S ribosomal subunit to ultimately form the 43S preinitiation complex [182]. The ability of eIF2 to engage in the formation of this ternary complex depends on the activity of another initiator factor, namely eIF2B. eIF2Bε is the largest of the five eIF2B subunits and functions as a catalyst responsible for the GDP/GTP exchange reaction of eIF2 [134, 182]. Activated GSK-3β is responsible for an inhibitory phosphorylation on eIF2Bε at $Ser^{540}$, which results in the loss of guanine nucleotide exchange activity, and is associated with a decrease in the initiation of protein synthesis [119, 183, 184]. Conversely, inactivation of GSK-3β by its upstream kinase Akt leads to decreases in the phosphorylation of eIF2Bε, which facilitates mRNA translation initiation (see Figure 2).

Akt has been identified as a nodal point which transduces extracellular cues (e.g. growth factors, nutrients) and mechanical stimuli to its downstream signaling cascades through the phosphorylation of a myriad of substrates, resulting in the integration of anabolic, catabolic and mechanical responses [185, 186]. Constitutive Akt1 activation results in pronounced muscle hypertrophy in transgenic mice [166, 187], while Akt1/Akt2 double-knockout mice display severe skeletal muscle atrophy [188]. Anabolic factors such as insulin or IGF-I stimulate PI-3K signaling resulting in an activating phosphorylation of two Akt residues [189, 190]. Phosphoinositide-dependent kinase 1 (PDK1) activates Akt by phospholipid binding and activation loop phosphorylation at $Thr^{308}$, whereas PDK2/rictor-mTOR (mTORC2) phosphorylates Akt at $Ser^{473}$ [191-193]. Activated Akt, in turn,

stimulates the mammalian target of rapamycin complex 1 (mTORC1) and in parallel inactivates GSK-3β through phosphorylation of $Ser^{9}$ [52]. Of note, phosphorylation of Akt on $Thr^{308}$ was proven to be essential and sufficient for the regulation of GSK-3β by Akt [193]. Further, the IGF-I/insulin-induced activation of mTORC1 through Akt leads to phosphorylation of its downstream targets p70S6K and 4E-BP1, which contribute to stimulation of protein synthesis by enhancing mRNA translation capacity and mRNA translation initiation via eIF4, respectively (mTOR; reviewed in detail by Proud [194] and Glass [195]). Activated p70S6K can in turn inactivate GSK-3β [67]. Conversely, in the absence of growth factor signaling activated GSK-3β can cooperate with adenosine monophosphate-activated protein kinase (AMPK) to negatively regulate mTORC1 by activating the mTORC1 inhibitor Tuberous sclerosis protein 2 (TSC2) [196]. Similarly, activated GSK-3β can hinder Akt signaling by destabilizing the insulin receptor substrate 1 (IRS-1), an essential adaptor molecule for PI-3K, which is required for the relay of Akt activating signals downstream of the IGF-I/insulin receptor (IGF-IR) (IR) [197-199]. Furthermore, a recent study by Kelly *et al.* described a novel interaction between GSK-3β and the IGF-IR [200]. It was postulated that IGF-IR phosphorylation at $Ser^{1248}$ was mediated by GSK-3β in the absence of growth factors, resulting in restrained IGF-IR kinase activity. In addition, a kinome wide siRNA screen gave evidence of novel bi-directionality in the Akt/GSK-3β interaction, whereby genetic ablation of GSK-3β significantly reduced Akt phosphorylation [201] (see Figure 2).

Much of the early evidence pertaining to a role for GSK-3β in muscle protein synthesis was derived from studies in cardiac muscle cells. In 2000 Haq *et al.* demonstrated that overexpression of a constitutively active GSK-3β (caGSK-3β) (S9A mutant) in neonatal rat cardiomyocytes was sufficient to suppress protein synthesis and to block key features of cardiac hypertrophy [102]. By contrast, inhibition of GSK-3β with lithium increased protein synthesis, measured by radioactively labeled leucine incorporation. In addition, increased GSK-3β activity appeared to be associated with impaired cardiomyocyte growth, as transgenic mice overexpressing wild-type (WT) GSK-3β developed hypotrophic hearts [202].

In 2001 Bodine *et al.* demonstrated that both Akt and GSK-3β phosphorylation were increased in a rat model of compensatory skeletal muscle hypertrophy [149]. In addition to the decreased phosphorylation status of GSK-3β, its specific activity was also reduced upon functional overload of the muscle. Concomitantly, mTORC1 signaling was stimulated, as illustrated by increased phosphorylation of p70S6K and 4E-BP1. Furthermore, overexpression of a constitutively active form of Akt (caAkt) in mice resulted in hypertrophy of normal muscle fibers and prevented muscle fiber atrophy upon denervation [149]. The authors contributed the hypertrophy phenotype to increased protein synthesis signaling, based on phosphorylation and subsequent inhibition of GSK-3β and through activation of mTORC1, respectively. In the same year, a report by Rommel *et al.* extended these findings by showing that expression of a dominant-negative form of GSK-3β (dnGSK-3β) caused profound myotube hypertrophy [203]. The dnGSK-3β (K85R mutant) is a catalytically inactive (kinase dead) form of GSK-3β where lysine at position 85 of the ATP-binding site is mutated to arginine [203, 204]. Treatment of C2C12 myotubes with IGF-I resulted in phosphorylation and inactivation of GSK-3β, via a mechanism that could be blocked by the PI-3K inhibitor LY294002, but not by rapamycin; a selective mTORC1 inhibitor. It was concluded that

GSK-3β constituted a pivotal downstream target of the PI-3K/Akt pathway, and its inhibition contributes to hypertrophy in an mTORC1-independent manner via relief of GSK-3β-mediated suppression of the translation initiator factor eIF2B, resulting in increased protein synthesis. This was the first study to assess the contribution of the individual Akt/mTORC1 and Akt/GSK-3β pathways to muscle protein synthesis during muscle hypertrophy.

As described below, previous studies investigating the role of lithium and GSK-3β in the regulation of protein turnover in atrophic conditions primarily assessed the role of this kinase in governing muscle protein degradation [115, 205, 206]. However, there is a paucity of data pertaining to the potential contribution of GSK-3β to muscle atrophy via suppression of the muscle protein synthesis rate. Recent work by Bertsch *et al.* addressed this gap in the literature [117]. In line with previous studies investigating the role of GSK-3β in muscle protein synthesis during hypertrophy [102, 147], the incubation of control muscle with lithium significantly increased protein synthesis, measured by incorporation of radioactively labeled phenylalanine in excised muscle tissue, whereas this anabolic effect was absent in muscle tissue from septic rats [117]. Further, protein synthesis was decreased in septic muscle, due to diminished phosphorylation of 4E-BP1 and S6. Sepsis increased phosphorylation and inhibition of eIF2Bε, consequent to markedly increased GSK-3β activity, thereby decreasing protein translation initiation [183, 207]. Moreover, lithium was capable of reversing the sepsis-induced increase in eIF2Bε phosphorylation, whereas the phosphorylation of S6 or 4E-BP1 remained unaltered in either control or septic muscles incubated with lithium. Interestingly, lithium decreased GSK-3β activity by 35% in control muscle, while the sepsis-induced increase in GSK-3β kinase activity was absent in the presence of lithium.

### *3.2.2 Muscle proteolysis signaling and GSK-3β*

Much of the early evidence linking GSK-3β to the proteolysis of skeletal muscle proteins was collected in models of burn-induced muscle proteolysis. Considering its previously established role as a negative regulator of skeletal muscle hypertrophy [147] Sugita *et al.* hypothesized that uninhibited increased GSK-3β activity, associated with perturbed Akt signaling, could play a role in muscle wasting in burns [208]. Not soon after, a study by the group of Hasselgren clearly established a role for the PI-3K/Akt/GSK-3β signaling pathway in the anticatabolic effects of IGF-I in a rat model of burn injury, as IGF-I-treatment resulted in pronounced phosphorylation (inactivation) of GSK-3β and inhibition of protein degradation. Likewise, pharmacological GSK-3β inhibition, using lithium or TDZD-8, blocked burn-induced muscle protein breakdown, which was quantified by the release of trichloroacetic acid-soluble radioactivity from proteins prelabeled with $^{3}$H-tyrosine. Concretely, lithium and TDZD-8 treatment reduced the tyrosine release rates, measured *ex vivo* in *Extensor digitorum longus* (EDL) muscles [205].

In a subsequent study, the potential mechanisms involved in the IGF-I-induced inhibition of protein degradation were examined *in vitro*. Since increased levels of glucocorticoids had been associated with a number of pathological catabolic states [209, 210], Li *et al.* employed the synthetic glucocorticoid dexamethasone to mimic the catabolic response observed in muscle after burn injury or during sepsis [211, 212]. Treatment of rat L6 myotubes with dexamethasone resulted in an approximately 20%

increase in protein degradation, determined by reduced radioactively labeled tyrosine release rates. Moreover, supplementation of IGF-I to myotubes significantly reduced the basal protein degradation rate and completely abolished the dexamethasone-induced increase in protein breakdown [211, 212]. Exposure of dexamethasone-treated myotubes to the GSK-3β inhibitors lithium or TDZD-8 resulted in a dose-dependent reduction of protein degradation comparably to IGF-I, suggesting that IGF-I-induced inhibition of protein degradation, at least in part, reflected inhibition of GSK-3β [212].



Fang *et al.* were the first to report that burn injury increased GSK-3β kinase activity in atrophying muscle [206]. Formerly, GSK-3β activity was determined indirectly by measuring tissue levels of GSK-3β phosphorylated at $Ser^9$, equating reduced GSK-3β phosphorylation in muscle from burned and septic rats with an increase in its activity [115, 205, 207]. Burn injury significantly lowered Akt kinase activity, which was confirmed by reduced levels of phosphorylated Akt. The authors concluded that GSK-3β activation in catabolic conditions reflected reduced phosphorylation of GSK-3β secondary to reduced Akt activity. To support the claim that increased GSK-3β activity could be involved in the regulation of protein degradation in catabolic muscle, siRNA targeted against GSK-3β was used to further investigate its role in dexamethasone-induced proteolysis. Despite a relatively modest reduction of GSK-3β protein (25%), transfected L6 myoblasts were more refractory to glucocorticoid-induced protein degradation, providing additional evidence that GSK-3β contributed to muscle proteolysis during skeletal muscle wasting [206]. These results were in support of their previous work in which GSK-3β inhibitors blocked burn-induced muscle protein degradation [205]. Similarly, in a recent report by Bertsch *et al.* [117] increased rates of protein breakdown in septic muscle were completely antagonized by lithium, confirming previous findings by the group of Hasselgren [115, 205, 206].

*3.2.2.1 UPS-mediated muscle proteolysis*

The stimulation of proteolysis observed during skeletal muscle atrophy appears to rely on increased protein degradation via the ubiquitin 26S-proteasome system (UPS) [213, 214]. UPS-mediated protein degradation is a selective process and involves two distinct and successive series of steps, which include the covalent attachment of multiple ubiquitin molecules to the target protein and the subsequent degradation of the tagged substrate by the 26S-proteasome [215]. Conjugation of ubiquitin to the substrate proceeds via a three-step enzymatic mechanism and the E3 ubiquitin ligases catalyze the rate-limiting step in this process [216]. Substrate specificity is achieved by several hundred E3s which are expressed in a tissue-specific manner. The expression of the muscle-specific E3s, atrogin-1 (MAFbx) and muscle RING finger 1 (MuRF1) is upregulated under a variety of atrophic conditions [217, 218] and genetic knockout studies have shown that the null deletion of MuRF1 and atrogin-1 attenuates muscle atrophy following denervation [219, 220]. Importantly, MuRF1 and atrogin-1 are essential for UPS-mediated proteolysis of myofibrillar proteins [156, 157], muscle-specific enzymes [221] and muscle-specific transcription factors [222] (see Figure 2).

The first report that implicated GSK-3β in the regulation of UPS-mediated protein degradation came from the group of Hasselgren [115]. In this study the contribution of GSK-3β activity to increased proteolysis was assessed *in vivo* in a model of sepsis-induced muscle wasting. Rats were made septic by cecal ligation and puncture (CLP).

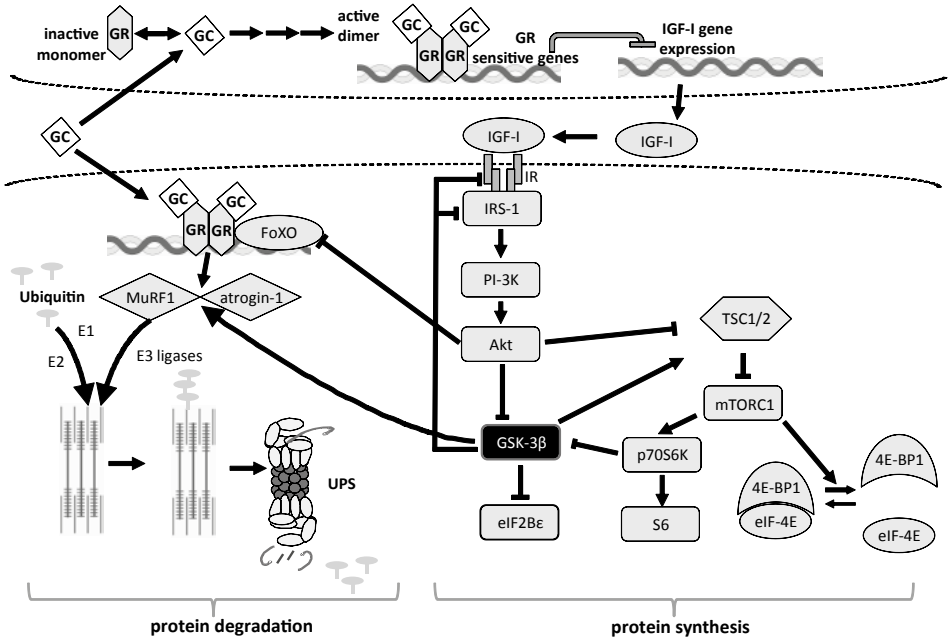


**Figure 2. Schematic representation of the signaling pathways involved in muscle protein turnover and central role of GSK-3β.** Anabolic factors such as insulin or IGF-I stimulate PI-3K signaling which results in activation of Akt. Phosphorylated Akt, in turn, inactivates GSK-3β and in parallel stimulates mTORC1 signaling, leading to enhanced protein synthesis. Conversely, under catabolic conditions (which may include glucocorticoid (GC) signaling) Akt activity is reduced, thereby stimulating the expression of the E3 ligases MuRF1 and atrogin-1 through FoXO and GR, leading to enhanced UPS-mediated proteolysis of muscle-specific proteins. Activation of GSK-3β, consequent to reduced Akt activity, is required for increases in E3 ubiquitin ligase expression, and may further decrease PI-3K/Akt signaling via a negative feedback loop.

Remarkably, incubation of EDL muscles of septic rats with either lithium or TDZD-8 significantly reduced basal and sepsis-induced protein breakdown rates [115]. To model sepsis-induced skeletal muscle atrophy *in vitro*, L6 myotubes were exposed to dexamethasone either in the presence or absence of lithium or one of the GSK-3β inhibitors SB-216763 or SB-415286. In analogy with previous results GSK-3β inhibition markedly decreased myotube proteolysis in response to dexamethasone. Furthermore, lithium-treatment increased GSK-3β phosphorylation at $Ser^{9}$ in myotubes as well as in incubated muscles. Given the fact that dexamethasone had previously been shown to increase the activity of the UPS [223], the expression of the atrophy markers atrogin-1 and MuRF1 was also assessed. The induction of these E3 ubiquitin ligases in response to dexamethasone was completely prevented by insulin and chemical GSK-3β inhibition at concentrations that inhibited protein degradation [115]. Previously, Sandri *et al.* demonstrated that overexpression of caAkt in cultured myotubes inhibited expression of atrogin-1 [224], while overexpression of caGSK-3β in cultured muscle cells resulted in increased atrogin-1 expression and promotor activation [224], further illustrating the pivotal role of GSK-3β's kinase activity in the regulation of skeletal muscle proteolysis.

Interestingly, Bertsch *et al.* reported that incubation of septic muscle with lithium tended to reverse the elevated 26S-proteasome activity, but this reduction failed to reach statistical significance and was not associated with a concomitant decrease in atrogin-1 and MuRF1 mRNA content. Besides, lithium had previously been shown to directly reduce 26S-proteasome activity in a GSK-3β-independent fashion [225]. Interestingly, recent data from our group suggests that UPS-dependent degradation of myofibrillar proteins requires GSK-3β. Genetic ablation of GSK-3β in C2C12 myotubes using siRNA revealed that loss of endogenous GSK-3β protein suppressed both basal and atrophy stimulus-induced atrogin-1 and MuRF1 transcript levels. Moreover, GSK-3β ablation resulted in specific sparing of myosin heavy chain fast (MyHC-f), myosin light chains 1 (MyLC-1) and -3 (MyLC-3) protein abundance and myotube size in response to dexamethasone treatment or impaired IGF-I/Akt signaling, demonstrating that muscle atrophy resulting from increased proteolysis signaling requires GSK-3β [226]. Furthermore, gene expression analysis revealed that enzymatic GSK-3β inhibition, using either lithium or CHIR99021, only attenuated atrophy stimulus-induced atrogin-1 expression, while MuRF1 expression levels remained unaffected. The notion that atrogin-1 and MuRF1 expression was differentially regulated in response to GSK-3β inactivation was not in agreement with findings by Evenson *et al.* [115], who found that in response to dexamethasone both atrogin-1 and MuRF1 mRNA levels were suppressed following GSK-3β inhibition. Furthermore, pharmacological suppression of only atrogin-1 expression was not sufficient to prevent or attenuate loss of myofibrillar protein abundance, suggesting that MuRF1 was the predominant E3 ubiquitin ligase responsible for the loss of contractile proteins in both atrophy models. In the light of these findings, it was concluded that the preferential depletion of contractile proteins in response to dexamethasone or LY294002 likely occurred in a UPS-dependent manner, as previous reports by Clarke *et al.* [156] and Cohen *et al.* [157] clearly implicated MuRF1 in the targeted destruction of several key myofibrillar proteins during atrophy. Moreover, MuRF1 knockdown studies (sh-MuRF1) confirmed the results in the above-mentioned studies as genetic ablation of MuRF1 completely abolished LY294002 -or dexamethasone-induced loss of myofibrillar proteins, similar to the observations made in GSK-3β-deficient (sh-GSK-3β) myotubes in response to these atrophy stimuli [226]. These data provided causal evidence to support the hypothesis that in a catabolic state, suppression of GSK-3β exerts a protective effect on myofibrillar protein content by downregulating MuRF1-driven UPS-mediated muscle proteolysis.

*3.2.2.2 ALP-mediated muscle proteolysis*

Autophagy is a highly conserved bulk degradation process implicated in the clearance of long-lived proteins and dysfunctional organelles [154, 155]. This proteolytic system is distinguished by the formation of phagophores, which elongate and fuse while engulfing a portion of the cytoplasm, to ultimately form double-membraned vesicles, called autophagosomes. These structures, in turn, merge with endosomes that later fuse with acidic lysosomes, which degrade the cytosolic contents [227]. Autophagosome formation requires the orderly recruitment and assembly of various autophagy-related proteins onto membrane phospholipids, but only the small ubiquitin-like molecules, including LC3 and Gabarapl1, bind covalently to phosphatidylethanolamine [228]. To date, the contribution of the ALP to skeletal muscle wasting is unclear, despite ample

evidence suggesting a clear role for lysosomal degradation in atrophying muscles [228-230]. Interestingly, basal autophagy is constitutively active in skeletal muscle and appears to be necessary to maintain myofibers in a healthy state under normal physiological conditions. Suppression of autophagy by muscle-specific deletion of the essential autophagy gene Atg7 amounts to severe muscle atrophy, and Atg7 null muscles display accumulation of abnormal mitochondria and protein aggregates [231]. However, several recent studies have reported that autophagy is strongly induced in skeletal muscle upon starvation [228, 232], denervation [229, 230] and oxidative stress [233], substantially aggravating muscle loss during these catabolic circumstances. The autophagic flux appears to be a double-edged sword, and tight control of ALP-related gene expression is therefore indispensable to prevent unbalanced autophagy activation and subsequent muscle loss.

In serum-deprived human prostate cancer cells both genetic as well as chemical modulation of GSK-3β activity stimulated an extensive autophagic response, which eventually led to necrotic cell death [234]. It was postulated that under serum-free conditions GSK-3β acted as a survival factor with the ability to fine-tune the autophagy process to avoid excessive autodigestion and subsequent cell death [234]. Conversely, in mesangial cells it was shown that pharmacological inhibition or genetic ablation of GSK-3β was sufficient to decrease cadmium-induced autophagy [235]. Overexpression of GSK-3β potentiated autophagy in the presence of cadmium and, in addition, cadmium-induced increases in reactive oxygen species (ROS) decreased GSK-3β phosphorylation at $Ser^{9}$, indicative of increased GSK-3β activity [235]. Sarkar and coworkers demonstrated that pharmacological GSK-3β inhibition attenuated autophagy and mutant huntingtin clearance by activating mTORC1 [236]. Recently, an elegant study by Lin *et al.* unraveled a novel autophagy signaling cascade involving GSK-3β [237]. It was reported that GSK-3β was activated upon growth factor withdrawal and that activated GSK-3β catalyzed the phosphorylation of TIP60, which in turn increased acetylation and kinase activity of ULK1, an important autophagy gene (Atg). Similarly, co-expression of GSK-3 increased phosphorylation of TIP60 on $Ser^{86}$, while treatment with the GSK-3 inhibitors SB-216763, SB-415286 or lithium impeded TIP60 phosphorylation, blocking serum deprivation-induced autophagy [237]. Although these recent reports imply regulation of autophagy by GSK-3β, currently, there is no convincing evidence to implicate GSK-3β in the regulation of autophagy during muscle proteolysis.

Although discussed separately above, both proteolytic systems, i.e. the ALP and the UPS, may interact to coordinately facilitate muscle protein degradation. For decades both these pathways were viewed as independent of each other. However, it is becoming increasingly more clear that crosstalk between the UPS and the ALP occurs at several levels [238]. It was proposed that impairment of the UPS leads to increased autophagic function [239, 240]. Furthermore, during selective autophagy certain substrates may be specifically targeted for destruction and specificity is achieved by substrate ubiquitylation [241]. Moreover, the seminal work by the group of Sandri demonstrated that the ALP and the UPS are coordinately regulated during muscle wasting, and that FoXO3a functions as a critical liaison molecule governing transcription of autophagy-related genes (e.g. LC3, Gabarapl1, Bnip3), as well as the UPS-related E3 ligases atrogin-1 and MuRF1 [224, 242, 243]. As GSK-3β appears to regulate UPS-mediated contractile protein breakdown [226], and considering the extensive crosstalk between the UPS and the ALP, it is not

improbable that GSK-3β might also converge on the ALP, possibly through a direct or indirect interaction with FoXO3a.

*3.2.2.3 Calpain and caspase-mediated muscle proteolysis*

The proteasome cannot degrade intact myofibrils, so the first step in the degradation of myofibrillar proteins, such as actin and myosin, during atrophy requires the release of myofilaments from the sarcomere [159, 244]. These initial steps in myofibrillar proteolysis appear to rely on the calpain and caspase systems. Several lines of evidence have indicated that the activation of the calpain system constitutes an additional mechanism contributing to skeletal muscle proteolysis during atrophy [245-247]. Although, actin and myosin are poor calpain substrates, several proteins important for the structural integrity of the sarcomere, such as nebulin, titin and filamin are readily cleaved by calpain [153, 248].

Calpains are non-lysosomal, calcium-dependent cysteine proteases, which cover a broad range of physiological functions, including proteolysis of proteins involved in the cell cycle, apoptosis, signal transduction and cytoskeletal organization [249]. Under basal conditions calpains are typically in an inactive state, and binding of calcium results in conformational changes which activates the protease [153]. Calpain activity can also be regulated by the endogenous inhibitor calpastatin [247]. Calpain activity was found to be increased during atrophic conditions like disuse [250], denervation [251], sepsis [245, 252] and glucocorticoid treatment [246]. However, these reports only provided associative support for a role of calpains in muscle wasting. Other data came from studies that employed calpain inhibitors to study the contribution of calpain activity to muscle proteolysis [253, 254]. However, due to their lack of specificity it was not possible to pronounce upon the direct role of calpains during muscle wasting [255]. In contrast, Tidball and Spencer established a causal role for calpains in muscle atrophy by overexpressing calpastatin in mice [256]. These transgenic mice exhibited a lower susceptibility to muscle atrophy induced by unloading [256]. Although the former study did not verify the contribution of proteolysis to muscle atrophy, transient overexpression of calpastatin in cultured muscle cells reduced protein degradation following dexamethasone treatment [257].

Caspases are calcium-independent cysteine-aspartic proteases that are well-known for their essential role as “executioner” proteins in apoptosis [258]. Intriguingly, besides its role in apoptosis, caspase-3 has been demonstrated to function during the initial phase of myofibrillar protein degradation [259, 260], comparably to calpains. Du *et al.* proposed that increased caspase-3 activity, following a catabolic insult (e.g. reduced PI-3K signaling during acute diabetes, sepsis or starvation), plays a critical role in the dissociation of actomyosin complexes, prior to UPS-mediated clearance of myofibrillar proteins [259]. Similarly, caspase-3 activity was required during the first two weeks of denervation-induced skeletal muscle atrophy [260]. In summary, the current paradigm of skeletal muscle proteolysis encompasses an early “upstream” dissociation of the myofibrils by calpains (possibly acting in concert with caspase-3), resulting in the release myofibrillar proteins which are subsequently ubiquitinated and targeted for destruction by the 26S-proteasome [159, 247].

A study by Smith *et al.* found that increased calpain activity and calpain-dependent protein degradation in calcium-treated muscles was associated with

reduced Akt activity [261]. Moreover, this reduction in Akt activity was accompanied by reduced phosphorylation of several downstream signaling molecules, including GSK-3β. Considering previous evidence demonstrating the essential role of the inhibition of Akt activity during skeletal muscle atrophy [195, 262], these findings suggest that calpain-mediated proteolysis not only contributes to muscle atrophy by facilitating myofibrillar protein degradation, but may also affect the control of muscle protein turnover, as reduced Akt activity will allow nuclear translocation and transcriptional activation of FoXO transcription factors, which are vital in regulating the expression and activity of atrogin-1 and MuRF1, and thus, initiation of UPS-mediated muscle proteolysis [195, 224, 263]. In addition, attenuated Akt signaling, as observed following calpain activation [261], will result in increased GSK-3β activity, which in turn contributes to enhanced protein degradation [115, 206, 226], and, parallel to decreased mTORC1 activity, will suppress protein synthesis [264]. Future studies should address the role of calpain activity in the activation of FoXO and GSK-3β, and the inhibition of mTORC1 in the context of skeletal muscle loss during catabolic conditions. Moreover, calpains are renowned for their ability to cleave signaling molecules and transcription factors [247], and several of these substrates such as NFκB [265], C/EBP-β [266] and STAT [267] have been linked to the control of muscle mass. Thus, in addition to the disruption of the sarcomere, increased calpain activity may relay or interrupt cellular signaling cues involved in the regulation of skeletal muscle plasticity.

A recent study by Ebert *et al.* focused on the role of growth arrest and DNA damage-inducible 45a (Gadd45a) in reprogramming skeletal muscle gene expression during atrophy [268]. Gadd45a expression was shown to be under the control of ATF-4, a transcription factor whose upregulation had previously been associated with muscle fiber atrophy [269]. The authors unraveled a novel stress-induced pathway in which Gadd45a stimulated multiple interconnected atrophy mechanisms [268]. Specifically, overexpression of Gadd45a resulted in an increase in GSK-3β activity and caspase-3-mediated proteolysis [268]. The concurrent activation of GSK-3β and caspase-3 in this study may concern more than an association, given the previous reports demonstrating a causal role for GSK-3β in caspase-3 activation [270-272]. Moreover, Hildesheim *et al.* described an interaction between Gadd45a and GSK-3β, i.e. Gadd45a associated with GSK-3β and promoted its dephosphorylation at $Ser^{9}$, essential for GSK-3β activation [273]. Although there is no causal evidence to support a direct link between GSK-3β and caspase-3 dependent protein degradation during muscle wasting, it is conceivable that Gadd45a-mediated increases in GSK-3β activity in response to cellular stress, may enhance caspase-3 activity in muscle, which in turn could cause disruption of the sarcomere.

Of note, several lines of evidence have postulated that there is a crosstalk between the cysteine proteases and UPS-mediated proteolysis. As indicated, calpain cleavage products become substrates for UPS-mediated protein breakdown, and the availability of these substrates may enhance UPS-activity [274]. Similarly, caspase-3 activation stimulated proteasome activity in differentiated muscle cells, and increased caspase-3 activity exerted a feed-forward amplification of protein degradation in muscle in concert with the UPS [275]. It is tempting to suggest that GSK-3β governs an additional level of crosstalk between the UPS and the cysteine proteases, as increased GSK-3β activity could either directly or indirectly relay signaling cues to stimulate caspase-3 and calpain-

mediated myofibril dissociation, while simultaneously regulating atrogin-1 and MuRF1 expression [226], resulting in enhanced UPS-mediated degradation of myofibrillar proteins.

### *3.2.3 Integration of muscle protein synthesis and degradation regulation by GSK-3β*



As highlighted above, activation of the Akt pathway, and subsequent signaling through its downstream targets GSK-3β and mTORC1, results in increased protein synthesis [148, 149]. Although, this pathway is suppressed in various models of skeletal muscle wasting, this does not simply imply that muscle atrophy is solely due to decreased protein synthesis. Activation of the UPS-mediated protein degradation during muscle atrophy is associated with the induction of a distinct set of genes, including the E3 ubiquitin ligases MuRF1 and atrogin-1, which are inversely regulated by IGF-I/Akt signaling [276]. Thus, crosstalk between muscle protein synthesis and the UPS occurs at multiple levels. Based on these and other studies, Akt has been proposed as a central regulator of skeletal muscle plasticity [148, 149, 263, 276].

Coordination between muscle protein synthesis and degradation not only occurs during physiological circumstances, but also appears to be maintained under pathological conditions, and several of these are associated with increased levels of circulating glucocorticoids [277, 278]. The direct effects of glucocorticoids on skeletal muscle depend to a large extent on activation of the glucocorticoid receptor (GR) [279], and the administration of synthetic glucocorticoids induces muscle atrophy [145, 226]. Conversely, endogenous glucocorticoids appear indispensable for the induction of protein degradation in several atrophy models, as adrenalectomy or treatment with the GR antagonist RU-486 attenuates muscle proteolysis and atrophy [224, 280-282]. In addition to its role in proteolysis, GR activation by glucocorticoids reduces protein synthesis at multiple levels. A non-genomic mechanism by which GR impairs protein synthesis involves competition of GR with PI-3K for association with IRS-1, resulting in suppression of Akt/mTORC1 and Akt/GSK-3β signaling [280, 283]. In addition, GR activation results in the transcriptional repression of muscle IGF-I expression [284], whereas transcriptional activation of REDD1 [285] concerns another genomic action of GR activation responsible for suppression of protein synthesis via the inhibition of mTORC1. Furthermore, glucocorticoids decrease translation initiation through increased availability of dephosphorylated eIF4E binding protein 1 (4E-BP1) [286] and reduced activity of p70S6K [287]. Recently, a mutually exclusive signaling crosstalk between GR and mTORC1 was revealed [288], providing further evidence of coordination between the protein synthetic and degradation machinery in response to (pathological) stimuli that affect muscle mass.

Based on its positioning as a direct downstream target of Akt, it is tempting to speculate that, GSK-3β may constitute an additional reciprocal link between the processes that govern muscle protein degradation and synthesis. Interestingly, GSK-3β was shown to directly phosphorylate the human GR, which significantly altered the repertoire of GR-regulated gene expression [289]. Since transcriptional regulation of FoXO1 [288] , MuRF1 [279] as well as of REDD1 [285], and suppression of IGF-I expression are dependent on genomic actions of GR, its regulation by GSK-3β will coordinately affect UPS-mediated proteolysis and protein synthesis. This warrants further investigation of a potential interaction between GSK-3β and the GR in the control of muscle protein synthesis and degradation during muscle wasting.

## 3.3 Myonuclear turnover

### *3.3.1 GSK-3β and myonuclear loss*

Myonuclear apoptosis may contribute to myofiber plasticity. The apoptosis machinery is classically divided into the "mitochondrial, intrinsic apoptotic pathway" and the "death-receptor-mediated, extrinsic apoptotic signaling pathway" [17, 290], and either internal or external signals can induce caspase activity resulting in cleavage of specific proteins required for cell viability [258, 291]. The intrinsic pathway may be induced by DNA damage [292, 293], ER stress [294], and hypoxia [295] and GSK-3β increases its activity by enhancing signals for mitochondrial disruption like Bax [296], Bim [297], MLC-1 [298] or modulation of transcription factors like β-catenin [299], p53 [293], and NFκB [300].

Conversely, GSK-3β suppresses the extrinsic pathway in response to TNF, Fas ligand, or TRAIL by upstream blockage of caspase-8 [301, 302] or caspase-3 [301, 303] activation. The molecular bases for these inhibitory actions of GSK-3 on apoptosis are currently unclear. This implies that inhibition of GSK-3β activity may attenuate or prevent apoptosis via the intrinsic pathway, while increasing the susceptibility to apoptosis mediated by the extrinsic pathway. Despite this emerging role for GSK-3β in the regulation of apoptosis, currently no literature exists addressing the involvement of GSK-3β in the regulation of skeletal muscle cell apoptosis. Myonuclear apoptosis has been predominantly described during muscle atrophy in both cachectic patients and models of muscle wasting [304-306]. Plant *et al* demonstrated that denervation-induced muscle atrophy is caspase-3 dependent [260]. However, the involvement of caspase-3 in prevention of myofiber atrophy was attributed to caspase-3-dependent degradation of myofibrillar proteins rather than myonuclear apoptosis. Nevertheless, caspase-3 activity in skeletal muscle is PI-3K dependent [307] involving GSK-3β [17, 308], i.e. phosphorylation of GSK-3β abrogates capsase-3 activation. Moreover, IGF-I/Akt signaling inhibits apoptosis of cardiomyocytes and skeletal muscle cells/myofiber nuclei [263, 305, 309], implying the potential involvement of GSK-3β (see Figure 3).

### *3.3.2 GSK-3β and myonuclear accretion*

GSK-3β plays an integrative role in several signaling pathways that are involved in the cellular processes that direct proliferation and myogenic differentiation following satellite cell activation of and their subsequent incorporation into mature muscle fibers. Notch signaling is active in early satellite cell proliferation [310] and during cell type commitment toward the myoblast lineage, Notch signaling decreases and activation of Wnt/β-catenin signaling occurs, which is an essential step in the formation of myocytes capable of fusion [311, 312]. GSK-3β appears to be involved in both pathways as inhibition of Notch signaling leads to increased GSK-3β activity, while activation of Wnt/β-catenin signaling results in GSK-3β inactivation. GSK-3β can associate with Notch thereby decreasing Notch-dependent gene expression [313]. However active GSK-3β has also been positively linked with increased Notch-dependent gene expression [314]. This dualistic role of GSK-3β in Notch signaling may rely on the cellular context. Wnt/β-catenin signaling is essential during embryogenesis [315, 316] and appears to be important for postnatal muscle growth and regeneration [317-319]. In addition to promoting satellite cell proliferation [320], Wnt signaling induces location-specific expression of muscle regulatory factors (MRFs) [321]. These MRFs, such as MyoD and myogenin, in

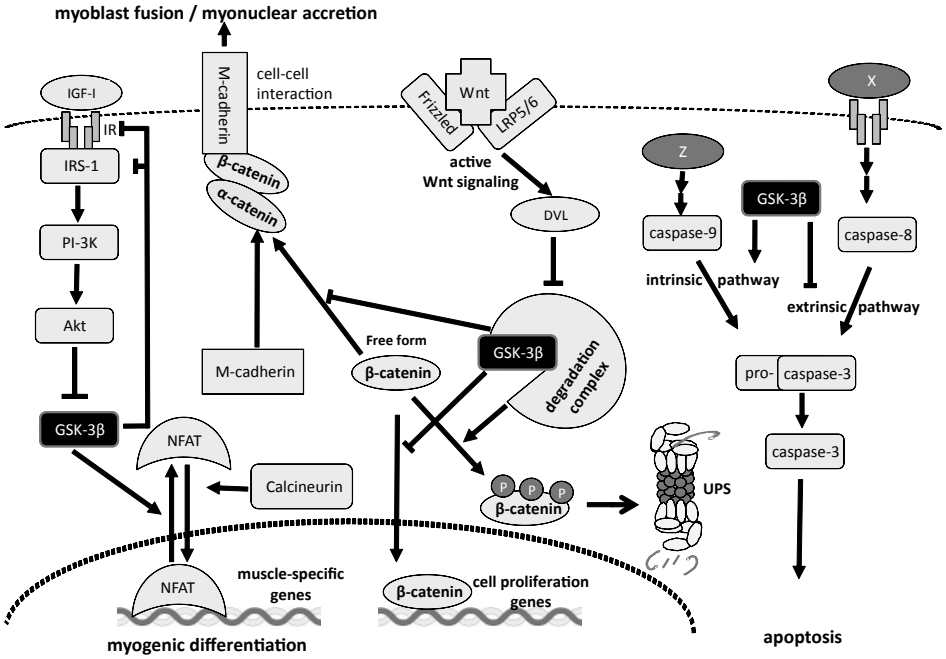


**Figure 3. Schematic representation of the cellular signaling pathways involved in myonuclear turnover.** IGF-I signaling leads to inactivation of GSK-3β resulting in increased NFAT-dependent gene transcription and myogenic differentiation. Wnt signaling leads to inactivation/relocation of the degradation complex resulting in redistribution of cellular β-catenin content. This increases β-catenin-dependent (mitotic) gene transcription as well as its association with (M-) cadherin, thereby improving cell-cell interactions and subsequent myoblast fusion and myonuclear accretion. Apoptosis may contribute to myonuclear loss and GSK-3β differentially affects intrinsic ("Z", e.g. DNA-damage oxidative or ER stress) and extrinsic ("X", e.g. FasL, TRAIL, DR4/5, TNF) apoptosis signaling.

turn, induce further muscle-specific gene expression [322-324], which results in muscle protein accretion [325]. Inhibition of Wnt/β-catenin signaling can abrogate myogenic differentiation [326], while increasing Wnt signaling with canonical Wnt-3a leads to enhanced myogenic differentiation [327] (see Figure 3).

A recent report from our group demonstrated that Wnt-3a supplementation markedly improved myoblast fusion and myotube formation, and incubation of myoblasts with lithium yielded similar results, suggesting that GSK-3β inhibition accounted for these stimulatory effects on morphological differentiation [327]. Although inhibition of GSK-3 activity was accompanied by increased β-catenin protein content and β-catenin dependent gene expression, increased β-catenin dependent transcriptional activity did not appear to account for improved myoblast fusion upon Wnt-3a or lithium-treatment [327]. Increased myotube formation may rather be a consequence of enhanced myoblast fusion due to increased cell-cell contact sites upon β-catenin accumulation. β-catenin is an essential binding partner for cadherin cytoplasmic tail, such as Muscle (M)-cadherin [328-330]. β-catenin co-localizes with M-cadherin at the cell–cell contact sites in membranes which are important during myoblast fusion [330-332]. Interestingly, although pharmacological inhibition of GSK-3β (lithium) increases muscle-specific gene

expression in addition to myoblast fusion, the former was not observed in response canonical Wnt mediated GSK-3β inhibition. Canonical Wnts suppress GSK-3β-mediated β-catenin phosphorylation and degradation through sequestration by Axin and Dishevelled (DVL) [333-335], whereas lithium does not only inhibit GSK-3β associated with the β-catenin degradation complex, but also the "free" GSK-3β involved in other cellular signaling pathways like the IGF-I/Akt signaling cascade.

Muscle regeneration following injury or during recovery from muscle atrophy involves satellite cell activation, increased autocrine/paracrine IGF-I/Akt signaling [336] and inhibition of GSK-3β enzymatic activity through $Ser^{9}$ phosphorylation [337]. In addition, stimulation with IGF-I has been repeatedly shown to improve myogenic differentiation including enhanced muscle-specific gene expression [131, 338, 339], and either pharmacological inhibition or genetic ablation of GSK-3β is sufficient to recapitulate this stimulatory effect on myogenic gene expression during differentiation [131, 340]. Suppression of muscle-specific gene expression by GSK-3β likely occurs through phosphorylation and nuclear exclusion of the transcriptional regulator nuclear factor of activated T cells (NFAT) [340-342] as NFAT transcriptional activity is regulated through dephosphorylation by calcineurin allowing nuclear import [343-345], while GSK-3β phosphorylation leads to nuclear export [96, 346], thereby preventing NFAT dependent gene transcription.

Consequently, pharmacological inactivation of GSK-3β will affect both the gene regulatory as well as the cellular remodeling processes involved in myogenic differentiation, by activating/stabilizing two GSK-3β substrates, namely NFAT and β-catenin, in distinct signaling pathways. However, the crosstalk between these pathways appears to be minimal [347] or absent [327] in skeletal muscle based on studies using either IGF-I or Wnt to accomplish ligand-specific inactivation of GSK-3β [83, 327, 334, 348, 349]. A possible explanation for these ligand-specific effects of GSK-3 lies in the modus of GSK-3 inactivation by Wnt, i.e. through sequestration by Axin and DVL [333-335], which, in contrast to IGF-I signaling, is not dependent on inhibitory $Ser^{9}$ phosphorylation of GSK-3β [49, 348]. Thus, two distinct signaling routes involved in control of specific aspects of myogenic differentiation, rely on different and independent modes of modulating GSK-3 activity [131, 327] reiterating the potential effect of pharmacological inhibition of GSK-3. However, it also indicates that knowledge on the precise role of GSK-3β in the various signaling pathways is of great importance when considering this kinase as a pharmacological target. Therefore, further *in vivo* dissection of the involvement of GSK-3β in myonuclear turnover and its contribution to skeletal muscle mass plasticity is imperative.

## 4. THERAPEUTIC POTENTIAL OF GSK-3β MODULATION

Therapeutic interventions aimed at the treatment of muscle wasting will either target the impaired signaling that lies at the basis of disturbed muscle mass homeostasis, or may aim at modulation of master regulators of the processes that govern muscle mass plasticity. GSK-3 activity levels or its downstream substrates have not systematically been included in the biochemical analyses of muscle tissue obtained in clinical studies that investigate the signaling pathways controlling skeletal muscle mass plasticity. Nevertheless, muscle biopsy analyses have provided some degree evidence for altered GSK-3β activity

in patients with muscle atrophy. Doucet *et al.* reported similar GSK-3β phosphorylation levels in skeletal muscle of COPD patients with muscle atrophy compared to healthy controls, despite increased Akt phosphorylation, which may suggest aberrant GSK-3β regulation in wasted muscle [350]. In chronic complete spinal cord injury, a condition characterized by profound muscle atrophy, muscle biopsy analyses revealed strongly reduced GSK-3β phosphorylation levels, indicative of reduced endogenous inactivation [351]. Interestingly, similar observations were made in pre-cachectic cancer patients [352]. Altogether, there is currently insufficient evidence to conclusively imply or rule out aberrant GSK-3β regulation and signaling during pathological conditions associated with muscle wasting. Apart from the limited number of studies measuring GSK-3 activity and signaling in patient muscle biopsies, this is in part due to methodological limitations including heterogeneity of studied patient populations, timing of muscle biopsies and the variability of biochemical analyses in human muscle tissue subjects. Cross-sectional study designs could be optimized by careful phenotyping and matching of study groups, and by trying to capture the acute wasting process [353].

Alternatively, rather than comparing GSK-3β signaling under basal conditions, studying it in the context of physiological relevant anabolic responses may be more appropriate and sensitive in order to expose aberrant GSK-3 regulation. One such example is the response to resistance exercise, as several studies have clearly indicated physiological alterations of GSK-3 activity levels post-exercise [354-356]. Nevertheless, even in absence of conclusive evidence obtained from human muscle biopsies implying impaired GSK-3β regulation and/or signaling as a primary cause of muscle wasting, the pre-clinical research described above may provide sufficient ground to progress into proof of concept clinical trials with GSK-3β inhibitors to reverse muscle atrophy.

Interestingly, pharmacological modulation of GSK-3β has been clinical practice for several decades already, as lithium is an FDA-approved drug for the treatment of psychiatric conditions including bipolar disorder and manic depression [357]. Unfortunately, the studies addressing efficacy of lithium salts in these types of disorders do not typically include any parameters related to muscle mass or function. As currently a number of registered clinical trials (www.clinicaltrials.gov) apply GSK-3β inhibitors in psychiatric disorders, inclusion of muscle mass or muscle strength using simple non-invasive techniques may provide useful information with respect to muscle mass plasticity following systemic pharmacological GSK-3β inhibition.

Thus far, there have been no reports in literature that have associated long term lithium usage to increased incidence of cancer or cancer-related deaths [357]. Nevertheless, the treatment time and the dose of pharmacological GSK-3β inhibitors are best kept to a minimum. An important feature of GSK-3β to consider for the purpose of pharmacological inhibition, is that physiological stimuli including insulin, IGF-I or exercise typically reduce GSK-3 activity only to 20 - 50% of basal levels in skeletal muscle [130, 131, 358], indicating that complete inhibition of this molecule may not be needed and desirable. Secondly, the pleiotropic actions of GSK-3β, apart from its involvement in muscle plasticity, warrant care with the applied dose of GSK-3β inhibitors considering potential side effects. An important field for further exploration concerns the non-ATP-competitive GSK-3β inhibitors because of their superior kinase selectivity and lower $IC_{50}$ values. Indeed, some preclinical results suggest that prolonged GSK-3 inhibition using TDZDs was not associated with adverse effects [359, 360]. Substrate-competitive

inhibitors also deserve further development and characterization, as these will allow superior specificity towards downstream signaling of GSK-3β related to processes involved in muscle plasticity. Finally, systemically administered inhibitor doses may be further reduced by sensitizing skeletal muscle by means of physical activity or tailored exercise, as the latter enhances local drug delivery due to increased blood flow, and more importantly, in itself already reduces muscle GSK-3β activity levels [355]. Therefore, the integration of pharmacological and physical training modalities into a multimodal treatment strategy aimed at inhibition of GSK-3β will allow optimal exploitation of its potential role as a central regulator of skeletal muscle mass plasticity for the treatment of skeletal muscle wasting.



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# Chapter 4

## Segregation of myoblast fusion and muscle specific gene expression by distinct ligand-dependent inactivation of GSK-3β

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## ABSTRACT

Myogenic differentiation involves myoblast fusion and induction of muscle-specific gene expression, which are both stimulated by pharmacological (LiCl), genetic or IGF-I-mediated GSK-3β inactivation. To assess whether stimulation of myogenic differentiation is common to ligand-mediated GSK-3β inactivation, myoblast fusion and muscle-specific gene expression were investigated in response to Wnt-3a. Moreover, crosstalk between IGF-I/GSK-3β/NFATc3 and Wnt/GSK-3β/β-catenin signalling was assessed. While both Wnt-3a and LiCl promoted myoblast fusion, muscle-specific gene expression was increased by LiCl, but not by Wnt-3a or β-catenin over-expression. Furthermore, LiCl and IGF-I but not Wnt-3a increased NFATc3 transcriptional activity. In contrast, β-catenin-dependent transcriptional activity was increased by Wnt-3a and LiCl but not IGF-I. These results for the first time reveal a segregated regulation of myoblast fusion and muscle-specific gene expression following stimulation of myogenic differentiation in response to distinct ligand-specific signalling routes of GSK-3β inactivation.

## INTRODUCTION

Satellite cells are muscle precursor cells located between the basement and sarcolemmal membrane in skeletal muscles [1]. These mononuclear cells are activated in response to injury or recovery from atrophy, which is required for efficient regeneration and restoration of muscle mass [2, 3]. Proliferating satellite cells are referred to as myoblasts, which subsequently differentiate to fuse with existing myofibers or form new myofibers [4]. In addition to myoblast fusion, myogenic differentiation is characterized by increased transcriptional activity of the Muscle Regulatory Factors (MRFs), which promote expression of muscle specific genes. Examples of muscle specific genes are structural proteins of the contractile apparatus such as Troponin-I (TnI) and myosin heavy chain (MyHC), and enzymes involved in muscle energy metabolism such as muscle creatine kinase (MCK) [5].

Post-natal muscle growth is stimulated by Insulin-like growth factor-I (IGF-I), and IGF-I is known to promote myogenic differentiation [6, 7]. IGF-I induces an increase in muscle specific gene expression during myogenic differentiation, which involves activation of Akt/PKB, hereafter referred to as Akt, and inactivation of glycogen synthase kinase-3β (GSK-3β) [8]. Genetic or pharmacological inactivation of GSK-3β also promote muscle specific gene expression and myotube fusion in differentiating myoblasts, suggesting a central role for GSK-3β in myogenic differentiation [9]. This notion is further supported by *in vivo* evidence revealing that markers of muscle differentiation and regeneration are inversely related to GSK-3β activity during skeletal muscle regrowth [10]. Of note is that pharmacological inhibition of GSK-3β activity by lithium (LiCl) has a more striking effect on myoblast fusion and myotube formation than IGF-I [8]. This could be related to the ability of LiCl to mimic Wnt/β-catenin signaling [11, 12].

Wnt/β-catenin signaling in part depends on the inactivation of GSK-3, which leads to an accumulation of the transcriptional co-activator β-catenin [13, 14]. Under basal conditions, β-catenin levels are regulated by a protein complex containing Axin, adenomatous polyposis coli (APC), GSK-3β and Casein kinase 1α (CK1α), hereafter termed the 'degradation complex'. This degradation complex N-terminally phosphorylates β-catenin [15] and thereby labeling it for degradation by the ubiquitin–proteasome pathway [16]. When Wnt ligands form a ternary complex with membrane receptors Frizzled (Fz), a seven-transmembrane protein, and the low-density lipoprotein receptor-related protein 5/6 (LRP5/6) [17], Dishevelled (Dvl) is activated. Activated Dvl recruits Axin from the degradation complex to the membrane [18] preventing GSK-3β from phosphorylating β-catenin, resulting in its stabilization [19]. Cytoplasmic accumulated β-catenin is involved in cell-cell interactions in complex with cadherins [20, 21]. Increased nuclear concentrations of β-catenin result in induction of growth-associated genes such as c-Myc and cyclin D1 by interacting with transcriptional co-activators such as members of the T-cell factor / Lymphocyte-enhancement factor-1 (Tcf/Lef-1) family [22].

Wnt/β-catenin signaling is essential in embryogenesis [23, 24] including muscle development, as Wnt ligands regulate the specification of skeletal myoblasts in the paraxial mesoderm [25-27], and induce location-specific expression of MRFs [28]. However, Wnt/β-catenin signaling not only contributes to embryonic but also to post-natal muscle formation, as it has been implicated in muscle regeneration [29] and hypertrophy [30]. Recently, it has been shown that Wnt's 1, 3A and 5A are expressed in activated satellite cells. Moreover, ectopic exposure of satellite cells to these Wnts

enhanced proliferation [31], while Wnt's 4 and 6 reduced this process [31]. Conversely, inhibition of Wnt signaling abrogated differentiation [32]. In this study, we evaluated the effects of Wnt/β-catenin signaling induced by Wnt-3a on differentiating myoblasts. We hypothesized that Wnt-3a would induce β-catenin signaling, and promote myogenic differentiation in a similar manner as observed following pharmacological inhibition of GSK-3β. Improved understanding of the processes regulating myogenic differentiation can be applied to therapeutic approaches to stimulate effective skeletal muscle regeneration following muscle trauma or atrophy.



## MATERIALS AND METHODS

### Cell culture

The murine skeletal muscle cell line C2C12 (ATCC # CRL1772) and the murine fibroblast control L-cells (ATCC # CRL2648) and Wnt-3a secreting L-cells (ATCC # CRL2647) (LGC Promochem, Teddington, UK) were cultured in growth medium (GM). This was composed of low (for C2C12 cells) or high (for L-cells) glucose Dulbecco's Modified Eagle Medium (DMEM) containing antibiotics (50U/ml Penicillin and 50µg/ml Streptomycin) and 9% (v/v) Fetal Bovine Serum (FBS) (all from Gibco, Rockville, MD). Conditioned medium (CM) containing Wnt-3a CM was obtained from Wnt-3a secreting L-cells (or control-CM from control L-cells) by culturing them on high glucose DMEM containing 5% FBS without supplementation of antibiotics for 7 days until approximately reaching confluency. Medium was collected after 4 and 7 days, pooled, spun down at 1500g for 3 minutes, filter sterilized (20µm) and frozen at −20 °C in aliquotes. C2C12 Cells were plated at 104/cm2 and cultured in GM for 24h. To induce spontaneous differentiation by growth factor withdrawal [33], GM was replaced with differentiation medium (DM), which contained low glucose DMEM with 1.0% heat-inactivated FBS and antibiotics. Alternatively, differentiation was induced with conditioned medium (CM) of control (control-CM) or Wnt-3a secreting L-cells, diluted in DM. C2C12 cells were grown on Matrigel (BD Biosciences, Bedford, MA) coated (1:50 in DMEM low glucose) dishes as described previously [34]. Murine IGF-I (Calbiochem, La Jolla, CA) or LiCl (Sigma, St. Louis, MO) was added directly after induction of differentiation and again 24h later when the cells were provided with fresh DM and every 48hrs thereafter. The anti-mouse Wnt-3a antibody (MAB1324, R&D systems, Abingdon, UK) was used to neutralize Wnt-3a conditioned medium effects by incubated Wnt-3a conditioned medium with anti-Wnt-3a at 37°C for 1h prior to dilution with DM and addition to the C2C12 cells.

### Stable cell lines

For the assessment of β-catenin-assisted T-Cell Factor / Lymphocyte Enhancer Factor (TCF/LEF)-dependent transcriptional activation, or the Troponin I (TnI) promoter activity during differentiation, stable C2C12 cell lines were created carrying a genomic TCF/LEF promoter-luciferase reporter gene [35] or a TnI promoter-luciferase reporter gene [36]. To determine luciferase activity, cells were lysed in luciferase lysis buffer (Promega, Madison, WI) and stored at −80°C. Prior to analysis lysates were spun at 16,000 × g and 4°C, and the soluble fraction was used. Luciferase activity was measured according to the manufacturers' instructions and expressed after correcting for total protein in the soluble fraction. Total protein concentration was assessed by Bio-Rad

protein assay kit (Bio-Rad, Herculus CA) according to manufacturer's instructions, $R^2$ values were >0.98 for the standard curve.

### Transfections and plasmids

Transient transfections were performed using Nanofectin (PAA, Pasching, Austria) and in all cases included co-transfection with pSV-β-gal to correct for differences in transfection efficiency (Promega, Madison, WI). According to manufacturers' instructions 1.0μg plasmid per 3.2μl nanofectin was used. Per transfection 1.0-2.5μg DNA of expression plasmids or empty vector controls was used per 35mm-dish. The transfection mix was added 6h prior to differentiation induction. TCF/LEF luciferase reporter plasmid was used to measure β-catenin dependent transcriptional activity, the Troponin I (TnI)-luciferase plasmid was used as a reporter for the activity of muscle specific transcription factors and the 4RTK [37, 38] MRF-sensitive luciferase plasmid was used to determine MRF transcriptional activity. Additionally an NFAT-sensitive luciferase plasmid [39] was co-transfected with a plasmid encoding NFATc3 [40] to evaluate effects on NFATc3 transcriptional activity. Plasmids encoding WT or K85R (kinase dead) GSK-3β [41], β-catenin [35], and MyoD [9] were transfected in combination with the TCF/LEF, TnI or 4RTK- reporter as indicated. To determine luciferase and β-galactosidase activity, cells were lysed in luciferase lysis buffer and stored at –80°C. Luciferase (Promega, Madison, WI) and β-galactosidase (Tropix, Bedford, MA) were measured according to the manufacturers' instructions.

### Muscle Creatine Kinase activity

Myogenic differentiation was assessed biochemically via determination of muscle creatine kinase (MCK) activity. Cells were grown on Matrigel coated dishes. After induction of differentiation with DM alone or in combination with LiCl, control-CM, or Wnt-3a for 72h, cells were washed twice in cold PBS, lysed in 0.5% Triton X-100, and scraped from the dish with a rubber policeman. Lysates were centrifuged for 2 min at 16,000g and 4°C, and the supernatant was stored in two aliquots at −80°C for determination of protein content or MCK activity in presence of 1.25%BSA. MCK-activity was measured by using a spectophotometric–based [42] kit from Stanbio (Stanbio, Boerne, TX). Specific MCK activity was calculated after correction for total protein concentration [43], $R^2$ values were >0.98 for the standard curve.

### May-Grunwald Giemsa Staining

C2C12 cells were grown on Matrigel coated 60mm dishes and after induction of differentiation with DM, LiCl, control-CM or Wnt-3a for 24 or 72h, cells were washed twice in PBS (RT), fixed in methanol and stained in May-Grunwald Giemsa (Sigma, Saint Louis, MO) according to the manufacturer's instructions. Pictures were taken at 40x and 100x magnifications using a microscope connected to a digital camera (DXM 1200F), both from Nikon (Nikon, Kanagawa, Japan). The 100x magnified images were taken in series of four with fixed overlap.

### Nuclei count and myogenic index

The total number of nuclei of 4 or more fields (100x magnification) were counted to determine the number of nuclei present after 24 and 72h of differentiation. The myonuclear distribution was assessed by counting all nuclei within four 100x magnified linked images. Counted nuclei were assigned to one of three classes: single nucleated myoblasts, dividing or fusing bi-nucleated myoblasts or multi-nucleated (>2) myotubes. Per condition 800-1800 nuclei were counted and assigned. Of the nuclei contained in myotubes a subdivision was made in four groups with 3-9, 10-19, 20-29 or >30 nuclei per myotube.



### Western blotting

The C2C12 cells were washed twice with ice-cold 1xPBS after which they were scraped and lysed in a whole cell lysate (WCL) buffer (20mM Tris, pH 7.4; 150mM NaCl; 1% Nonidet P-40; 1mM DTT; 1mM $Na_3VO_4$; 1mM PMSF; 10µg/ml Leupeptin and 1% aprotenin) using rubber policemen. Next, crude lysates were incubated on ice for 30 minutes, followed by a 30 minute centrifugation step at 16.000g and 4°C. A portion of the supernatant was saved for protein determination, prior to the addition with 4x Laemmli sample buffer (0.25M Tris-HCl pH 6.8; 8% (w/v) SDS; 40% (v/v) glycerol; 0.4M DTT and 0.04% (w/v) Bromophenol Blue). The samples were boiled for 5 minutes at 95°C and stored at −20°C. Total protein concentration was assessed by Bio-Rad DC protein assay kit (Bio-Rad, Herculus CA) according to manufacturer's instructions. For SDS-PAGE 0.5µg -20µg of protein was loaded per lane and separated on a Criterion™ XT Precast 4-12% Bis-Tris gel (Bio-Rad, #3450124), followed by transfer to a 0.45µm Whatman® Protran® Nitrocellulose Transfer membrane (Whatman GmbH, #7324007) by electroblotting (Bio-Rad Criterion Blotter) (Bio-Rad, Hercules, CA, USA). The membrane was blocked for 1h at room temperature in 5% (w/v) NFDM (non-fat dried milk) (ELK, Campina, the Netherlands) diluted in TBS-Tween-20 (0.05%). Nitrocellulose blots were washed in TBS-Tween-20 (0.05%) on a rotating platform, followed by overnight (o/n) incubation at 4°C with specific antibodies directed against: p-GSK-3β ($Ser^9$) (#9336), GSK-3β (#9332), p-Akt ($Ser^{473}$) (#9271), Akt (#9272), GAPDH (#2118) (all from Cell Signaling Technology, Inc., Danvers, MA,) all were diluted 1/1000 in TBS-Tween-20 (0.05%). After 3 washing steps of 10 minutes each, the blots were probed with a peroxidase conjugated secondary antibody (Vector Laboratories, #PI-1000), and visualized by chemiluminesence using Supersignal® WestPico Chemiluminescent Substrate (Pierce Biotechnology, Inc.) according to the manufacturers' instructions and exposed to film (Biomax light film, KODAK). Westernblot-films were imaged and quantified using the Quantity One analysis software from Bio-Rad

### RNA isolation and assessment of mRNA abundance by RT-qPCR

C2C12 cells were washed twice with ice-cold 1xPBS after which Total RNA was isolated using the Totally RNA™ kit (Ambion, Austin, TX) according to the manufacturer's instructions. After isolation RNA was dissolved in 1mM Na-citrate (pH 6.4) and stored at −80°C. The RNA concentrations were measured spectrophotometrically using a Nanodrop® ND-1000 UV-Vis spectrophotometer. RNA was diluted >5x in ddH2O and 400 ng of RNA was reverse transcribed to cDNA using the Transcriptor first strand cDNA synthesis kit (Roche Diagnostics GmbH, Mannheim, Germany) with anchored oligo-

dT primers according to manufacturor's for generating cDNA fragment of 4kb with a final reaction volume of 20µl. RNA of genes of interest (Table 1) were determined by reverse transcription quantitative PCR (qPCR). qPCR primers were designed using Primer Express 2.0 software (Applied Biosystems, Foster City, CA), checked for both primer and amplicon secundairy structures, and than obtained from Sigma Genosys (Haverhill, UK). qPCR reactions (20 µl final volume) contained absolute qPCR SyBr Green Fluorescein Mix (Abgene,Leusden, NL) and primers (600nM). Relative cDNA starting quantities for the samples were derived by the standard curve method. Standards curve samples were generated by serial dilution of pooled cDNA samples and had at least a $R^2$ >0.98 and an efficiency between 90-110%. The expression of the genes of interest were normalized with a correction factor derived by geNorm, which is based on a combination of the expression levels of β-actin, cyclophillin A, GAPDH and RPL13A. RT-qPCR reactions were performed on a MyiQ single-color Real-Time thermal cycler (Bio-Rad Herculus, CA).



**DNA content determination**

After treatment, cells were washed twice with cold PBS. Three hundred µl Tris-EDTA buffer was added followed by a freeze-thaw cycle after which dishes were scraped and the lysate was collected. DNA content was determined in the cell lysates using the Quant-iT™ PicoGreen® dsDNA assay kit (Molecular Probes) according to manufacturers' protocol.

**Immunohistochemical staining**

C2C12 cells were grown on glass coverslips coated with Matrigel (BD Biosciences, Bedford, MA) (1:50 in DMEM low glucose) as described previously [34]. After 72h of differentiation cells were washed twice with cold PBS fixed with 4% PFA, permeabilized with 0.1 % Triton x-100 in PBS and non-specific binding was blocked with 1% BSA in 0.1% Triton in PBS. MyHC-fast obtained from Sigma-Aldrich (Saint-Louis, MO USA) was used as a primary antibody 1:250 in 1% BSA / 0.1% Triton / PBS and Alexa fluor 488 goat anti IgG from InVitrogen was used as a secondary antibody 1:1000 in 1% BSA / 0.1% Triton / PBS washed counter stained with DAPI (20µg/ml) and mounted with Dako mounting medium. Pictures were taken at 200x magnification using a fluorescence microscope connected to a digital camera (DXM 1200F), both from Nikon (Nikon, Kanagawa, Japan).

**Statistical analysis**

All values are means ± SE. Raw data was statistically analysed for one-way t-test with unequal variance. Results are considered significantly different with $p<0.05$ or smaller.

**Table 1. Genes of interest real-time quantitative PCR primers**

| Gene name | Accession number (Ensembl) | Primers |
|---|---|---|
| β-actin | ENSMUST00000052678 | FW: 5'-CTGAATGGCCCAGGTCTGA-3' |
| | | RV: 5'-CCCTCCCAGGGAGACCAA-3' |
| cyclophilin A | ENSMUST00000090749 | FW: 5'-TTCCTCCTTTCACAGAATTATTCCA-3' |
| | | RV: 5'-CCGCCAGTGCCATTATGG-3' |
| GAPDH | ENSMUST00000118875 | FW: 5'-CAACTCACTCAAGATTGTCAGCAA-3' |
| | | RV: 5'-TGGCAGTGATGGCATGGA-3' |
| RPL13A | ENSMUST00000102669 | FW: 5'-CACTCTGGAGGAGAAACGGAAGG-3' |
| | | RV: 5'-GCAGGCATGAGGCAAACAGTC-3' |
| Cyclin D1 | ENSMUST00000093962 | FW: 5'-CATTCCCTTGACTGCCGAGAAGTT-3' |
| | | RV: 5'-TTGTTCACCAGAAGCAGTT CCATTT-3' |
| PCNA | ENSMUST00000028817 | FW: 5'-CCAAATCAAGAGAAAGTTTCAGACTATGA-3' |
| | | RV: 5'-TCACCCGACGGCATCTTTATT-3' |
| c-Myc | ENSMUST00000022971 | FW: 5'-ACCACCAGCAGCGACTCTGA -3' |
| | | RV: 5'-GCCCGACTCCGACCTCTTG-3' |
| MCK | ENSMUST00000003643 | FW: 5'-AGGTTTTCCGCCGCTTCT-3 |
| | | RV: 5'- CGGTGCCCAGGTTGGA-3 |
| MyHC 2B | ENSMUST00000018632 | FW: 5'-ACAAGCTGCGGGTGAAGAGC-3 |
| | | RV: 5'- CAGGACAGTGACAAAGAACG-3 |
| MyHC peri (-natal) | ENSMUST00000019625 | FW: 5'-ACACATCTTGCAGAGGAAGG-3 |
| | | RV:5'- TAAACCCAGAGAGGCAAGTG-3 |
| Axin 2 | ENSMUST00000052915 | FW: 5'-CTCAGCAAAAAGGGAAATTACAGGTAT-3' |
| | | RV: 5'-ACTGTCTCGTCGTCCCAGATCTC-3' |

# RESULTS

## Wnt-3a or pharmacological inactivation of GSK-3β promote myoblast fusion during differentiation

C2C12 myoblasts were differentiated in DM with or without LiCl, or in DM containing diluted medium conditioned (CM) by Wnt-3a-secreting or control L-cells (Control-CM). During the first 48h of differentiation, alignment (Supplemental Figure 1), which precedes fusion, appeared to be enhanced by the presence of Wnt-3a compared to control, whereas no effects on myoblast survival were observed (Supplemental Figure 2). Similar to differentiation with DM, in control-CM treated myoblasts, myotubes were forming at 72h. Differentiation in presence of Wnt-3a markedly promoted myoblast fusion resulting in larger myotubes (Figure 1A). Pharmacological inhibition of GSK-3β using LiCl also enhanced myotube formation as reported previously [8]. Staining for MyHC-fast confirmed that the multinucleated fused cells were genuine myotubes in all conditions (Supplemental Figure 3). Myoblast fusion and myotube formation was quantified by determining the number of nuclei in each cell. A greater percentage of nuclei residing in multinucleated cells was observed when myoblasts were differentiated in presence of LiCl or Wnt-3a compared to their respective controls (Figure 1B). In addition, LiCl and Wnt-3a promoted the formation of larger myotubes containing more nuclei compared with their respective controls (Figure 1C). The stimulatory effect of Wnt-3a conditioned medium on myoblast fusion and myotube formation was reduced by co-incubation with a Wnt-3a specific antibody (Figure 1B inset). Transcript levels of genes associated with proliferation significantly decreased compared to GM irrespective of treatment conditions (Figure 1D), indicating that the observed increase in number of nuclei per myotube following LiCl or Wnt-3a were not likely the result of prolonged myoblast proliferation, which was further supported by the absence in changes in total nuclei number or total DNA of adherent cells (Supplemental Figure 2).

## Muscle specific gene expression during differentiation is stimulated by pharmacological inactivation of GSK-3β but not by Wnt-3a

Next we investigated whether enhanced myotube formation by Wnt-3a was associated with increased muscle specific gene expression. Muscle creatine kinase (MCK) activity was increased by LiCl compared with control after 72h (Figure 2A), which is in line with previous results [8]. In contrast, MCK activity was not increased by Wnt-3a when compared to controls (Figure 2A). Similarly, mRNA expression levels of MCK, but also myosin heavy chain (MyHC)-IIB and MyHC-perinatal at 72h were only increased by LiCl, but not by Wnt-3a treatment (Figure 2B). Differentiation-induced transcriptional activation, of the Troponin I (TnI) promoter, evaluated in a stable reporter cell line, was increased by LiCl but not Wnt-3a (Figure 2C) as increasing concentration of Wnt-3a even slightly decreased TnI-promoter transactivation. In line with this, over-expression of β-catenin, did not promote TnI-promoter transactivation (Supplemental Figure 4A). Next, MRF activity was assessed with a transiently transfected, MyoD-sensitive (supplemental Figure 4B), 4RTK luciferase reporter. Only LiCl induced an increase in MRF transcriptional activity compared to control, whereas Wnt-3a did not affect MRF activity (Figure 2D), in line with the absence of a stimulatory effect on muscle-specific gene expression.

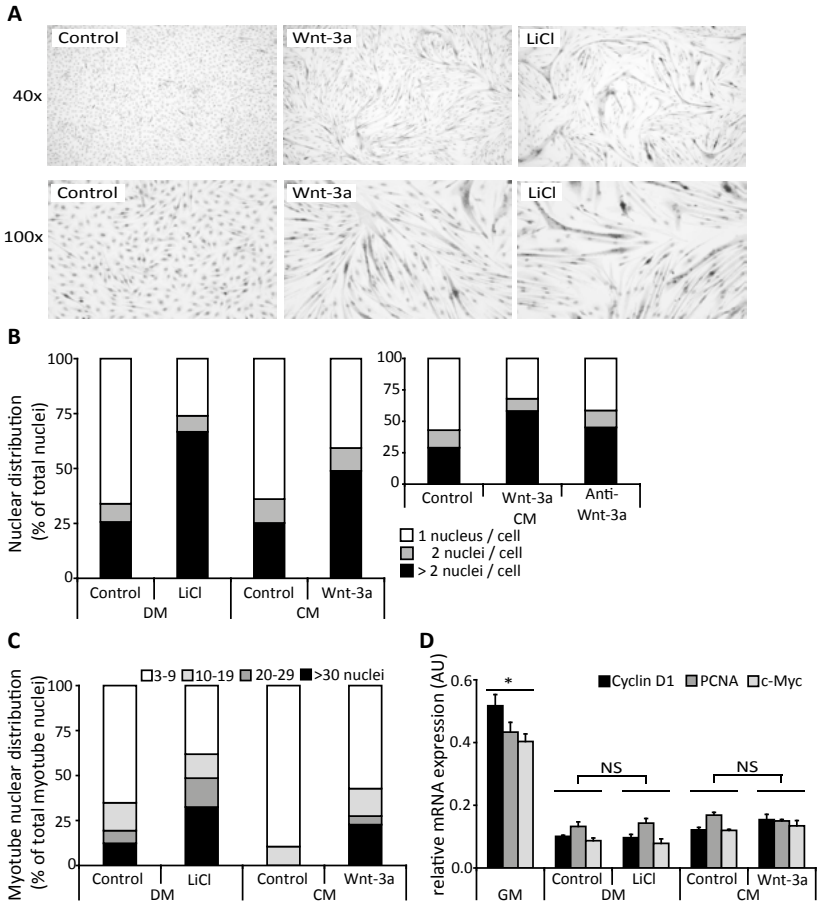


**Figure 1. Wnt-3a or LiCl promote myoblast fusion during differentiation.** C2C12 myoblasts were differentiated for (A) 72h in control-CM or Wnt-3a-CM (each diluted 1/10 in DM), or DM in presence or absence of LiCl (10mM), fixed and stained with May-Grunwald Giemsa to determine myoblast fusion and myotube formation or for 24h to determine mRNA expression. Shown are representative pictures of >10 independent experiments at 40x and 100x magnification. From these pictures (B) myoblast fusion was quantified by determining nuclear distribution of 800-1800 nuclei for each separate condition, which is expressed as the % of nuclei residing in cells containing 1, 2, or >2 nuclei, reflecting mononucleated myoblasts (1 nucleus), dividing or fusing myoblasts (2 nuclei) or myotubes (>2 nuclei), respectively shown is representative data of three independent experiments. (B inset) Wnt-3a-CM was pre-incubated with an anti-Wnt-3a antibody, and cells were cultured for 120h and myonuclear distribution was assessed. (C) Stratification of myonuclear content in myotubes of LiCl or Wnt-3a treated cultures. (D) C2C12 myoblasts were differentiated for 24h and RNA was extracted for assessment of proliferation-associated mRNA transcripts Shown are (C, D) representative data of 3 independent experiments (N=3 ±SEM), *: $p<0.01$ and NS: non-significant.

### Wnt-3a or pharmacological inhibition of GSK-3β induce β-catenin stabilization and β-catenin-dependent transcriptional activity in differentiating myoblasts

To investigate whether Wnt-3a induces Wnt/β-catenin signaling in C2C12 myoblasts, β-catenin protein abundance was assessed. A clear increase (>2.5 fold) in cellular β-catenin protein content was observed following 24h differentiation in DM containing 1/10 diluted Wnt-3a-CM compared to control-CM (Figure 3A). To assess whether β-catenin accumulation was accompanied by increases in its functional activity as a transcriptional co-activator, β-catenin-dependent TCF/LEF transcriptional activity was assessed in C2C12 cells stably transfected with a TCF/LEF-sensitive promoter reporter construct. Wnt-3a induced a concentration (Figure 3B) and time (Figure 3C) dependent increase in TCF/LEF transcriptional activity. Moreover, Wnt-3a induced TCF/LEF transcriptional activity was abrogated by co-incubation with a Wnt-3a specific, but not isotype control, antibody (Figure 3D). In addition to Wnt-3a, pharmacological inhibition of GSK-3β activity by LiCl also induced TCF/LEF transcriptional activity (Figure 3D). Finally, to assess endogenous β-catenin mediated gene expression, Axin 2 mRNA levels were assessed. Axin 2 mRNA expression was increased by LiCl (>2.5 fold) and Wnt-3a (>14 fold) compared with their respective controls (Figure 3E).

### GSK-3β activity directly inhibits β-catenin-dependent TCF/LEF transcriptional activity

To address GSK-3β regulation of β-catenin-dependent transcriptional activity in skeletal muscle cells, C2C12 myoblasts were transiently transfected with a TCF/LEF responsive promoter luciferase reporter plasmid. Simultaneous over-expression of β-catenin resulted in increased TCF/LEF transcriptional activity (Figure 4). In contrast, over-expression of wild-type (WT-)GSK-3β led to suppressed β-catenin-induced TCF/LEF transcriptional activity (Figure 4), whereas over-expression of a dominant negative (dn-) GSK-3β (K85R) mutant actually enhanced β-catenin-dependent transcriptional activity (Figure 4). Therefore inhibition of β-catenin mediated transcriptional activation requires enzymatically active GSK-3β.

### Wnt-3a and IGF-I regulate distinct GSK-3β substrates in differentiating myoblasts

In contrast to Wnt-3a, inactivation of GSK-3β by IGF-I did not result in β-catenin accumulation after 24h (Figure 5A), or affect β-catenin dependent transcriptional activity after incubation 24h (Fig. 5B), 48, 96, or 120h of incubation (data not shown). GSK-3β inactivation following IGF-I signaling results from phosphorylation GSK-3β at Ser-9 [44]. GSK-3β phosphorylation was increased by IGF-I (Figure 5C), while no effect was observed in response to Wnt-3a. Similar results were seen for Akt phosphorylation (Supplemental Figure 5). This indicates, that Wnt/β-catenin signaling in skeletal muscle cells is not dependent on GSK-3β phosphorylation at Ser-9, in line with previous reports [45, 46]. Increased muscle specific gene expression in response to genetic or pharmacological GSK-3β inhibition was previously shown to be mediated by the GSK-3β phospho-substrate and transcription factor NFATc3 [9]. Evaluation of NFATc3 transcriptional activity using a transiently transfected NFAT luciferase reporter construct, revealed increased NFAT-dependent transcription following either IGF-I or LiCl, but not in response to Wnt-3a or over-expression of β-catenin (Figure 5D).

## DISCUSSION

Recent work identified GSK-3β as a negative regulator of myogenic differentiation controlled by IGF-I signaling [8]. Wnt/β-catenin signaling also involves regulation of GSK-3β, and is reported to participate in the initiation of pre-myogenic cell differentiation [47]. Besides its well-established role in skeletal muscle formation during embryogenesis [23], recent studies propose the involvement of Wnt/β-catenin signaling in postnatal skeletal muscle hypertrophy [48, 49]. However, whether Wnt/β-catenin signaling affects myogenic differentiation during skeletal muscle growth was not assessed in these studies. Our results reveal a potential role for Wnt/β-catenin signaling in myogenic differentiation, as Wnt-3a induced a marked increase in C2C12 myoblast fusion and myotube formation during differentiation. This is in line with findings describing stimulation of myotube formation by co-culture of myoblasts on Wnt1-presenting monolayers [50]. In our study however, myoblasts were provided with cell-free conditioned medium from Wnt-3a-secreting L-cells to rule out producer-cell-myoblast interaction effects. To confirm that



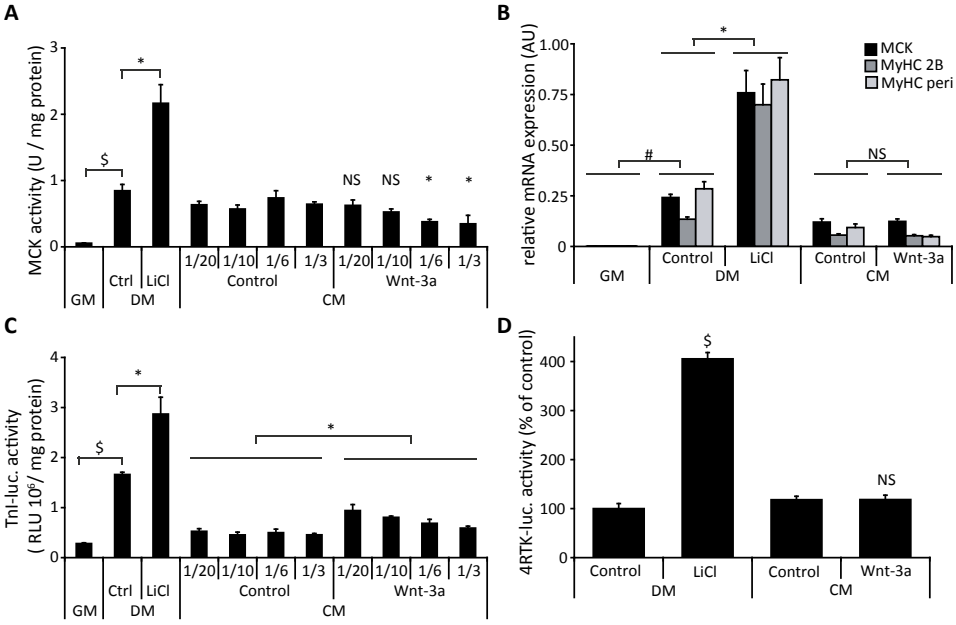


**Figure 2. Muscle-specific gene expression during differentiation is stimulated by LiCl but not by Wnt-3a.** C2C12 myoblast cells were cultured in DM with or without LiCl (10mM), Control or Wnt-3a CM (diluted 1/10, or as indicated, in DM). (A) After 72h lysates were prepared for determination of muscle creatine kinase activity and total protein. Results are expressed as specific enzyme activity (units/mg protein). (B) After 72h lysates were prepared to determine mRNA expression levels of MCK, MyHC IIB and perinatal. (C) Myoblasts containing a stable genomically integrated Troponin I (TnI) luciferase reporter construct were cultured for 48h in DM, +/- LiCl (10mM), or Wnt-3a or Control-CM (1/10 diluted in DM). Alternatively, (D) C2C12 myoblasts were transfected with a 4RTK luciferase-reporter construct and plasmid encoding b-gal (0.25mg each) and cultured in DM with or without LiCl (10mM), Control or Wnt-3a CM (diluted 1/10 in DM). Lysates were prepared for luciferase and β-galactosidase enzyme activity (RLU / mg protein) or (RLU luciferase / β-gal activity). Shown are representative graphs of 3 independent experiments (N=3 ±SEM), *: p<0.05, #: p<0.01, $: p<0.001 and NS: non-significant.

stimulation of fusion (Figure 1B inset) and TCF/LEF transcriptional activity (Figure 3E) was attributable to Wnt-3a, the conditioned medium was incubated with a Wnt-3a-specific antibody, which partly neutralized Wnt-3a-mediated effects. Finally, opposed to the study by Rochat *et al.*, we did not supplement our cells with insulin, as one of our aims was to distinguish between effects of GSK-3β inhibition by Wnt-3a and IGF-I/insulin signaling.

Improvement of myoblast fusion by Wnt-3a, reflected by an increased myogenic index, was similar to stimulation of fusion following GSK-3β inhibition by LiCl [8]. Although

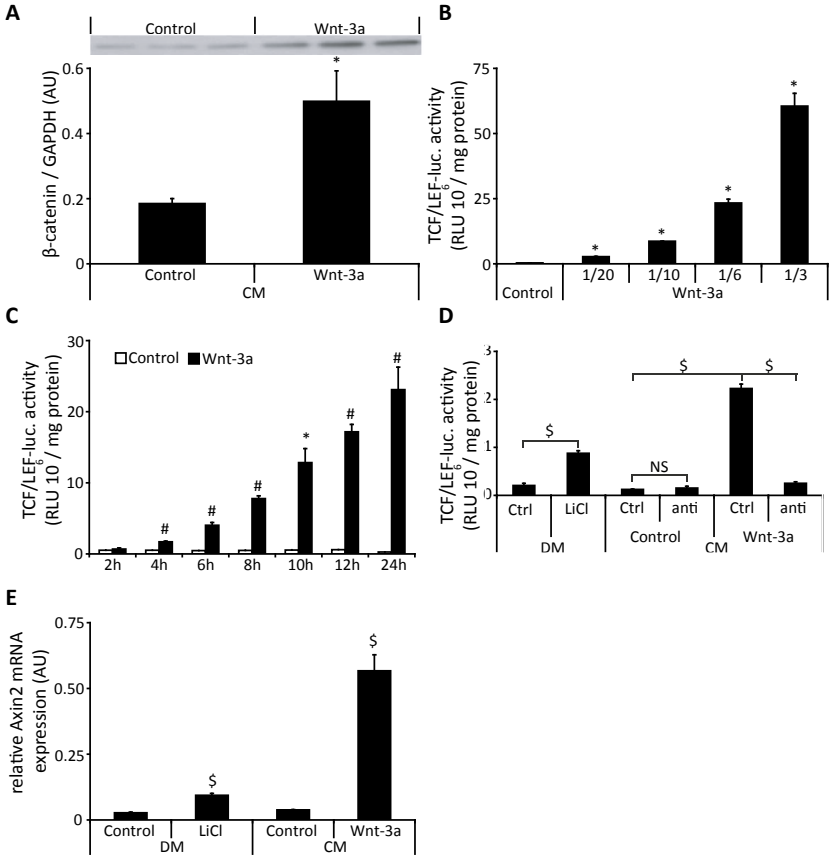


**Figure 3. Wnt-3a or LiCl induce β-catenin stabilization and β-catenin-dependent transcriptional activity in differentiating myoblasts.** C2C12 myoblasts were differentiated for (A) 24h in control or Wnt-3a CM (each diluted 1/10 in DM), lysates were prepared and cellular β-catenin and GAPDH protein content were visualized and quantified. Myoblasts containing a stable genomically integrated TCF/LEF luciferase reporter construct were cultured for (B) 24h in DM, Wnt-3a CM diluted as indicated, or (C) or for the indicated time in control or Wnt-3a CM diluted 1/10 in DM. C2C12 myoblasts were cultured for (D) 24h in DM with or without LiCl (10mM), control or Wnt-3a CM (pre-incubated with an Wnt-3a specific or isotype control antibody for 1h at 37°C) diluted 1/10 in DM. After the indicated times lysates were prepared for specific enzyme activity (RLU / mg protein). Myoblast were (E) harvested 24h after induction of differentiation for determination of endogenous Axin 2 mRNA levels. Shown are representative graphs of 3 independent experiments (N=3 ±SEM), *: p<0.05, #: p<0.01, $: p<0.001 and NS: non-significant.

Wnt-3a can induce expression of genes promoting proliferation like Cyclin-D1 and c-Myc [51, 52], the increased myogenic index did not result from sustained cell division, as the decrease in proliferation marker mRNA levels (two-fold or more) following induction of differentiation was not affected by Wnt-3a or LiCl. Alternatively, improved myoblast survival could contribute to an increased myogenic index, as inhibition of GSK-3β reduces caspase activity and cellular apoptosis [53]. Determination of DNA content and total nuclear count of the adherent cells 24-48h after induction of differentiation revealed no changes in presence of either LiCl or Wnt-3a. This leads to the observation that the increased myogenic index did not result from either increased proliferation or survival of myoblasts, but is likely attributable to improved cell-cell interactions and subsequent myoblast alignment. Following the improved alignment larger myotubes after Wnt-3a or LiCl stimulation are observed which could results from either faster or enhanced myoblast-myoblast, myoblast-myotube or myotube-myotube fusion [54, 55]. We can however, not discern at present which of these processes results in those larger myotubes. Although they are already observed 3 days after induction of differentiation.

Wnt-3a as well as LiCl-induced GSK-3 inactivation stabilized and increased cellular levels of β-catenin leading to β-catenin-dependent TCF/LEF transcriptional activity in differentiating myoblasts, which is in agreement with previous reports [11, 56, 57]. In addition, over-expression of WT-GSK-3β in myoblasts confirmed direct inhibition of β-catenin-induced TCF/LEF transcriptional activity by GSK-3β. Conversely, inhibition of endogenous GSK-3 activity or over-expression of dominant-negative (dn)-GSK-3β (K85R) increased TCF/LEF reporter activity, the latter likely resulting from competition with endogenous GSK-3 for participation in the degradation-complex to phosphorylate β-catenin [58, 59].

GSK-3β inactivation by IGF-I [8] or LiCl (shown here) resulted in increased muscle-specific gene expression during differentiation, such as increased MCK and MyHC mRNA expression, and TnI-promoter activity. Wnt-3a mediated GSK-3 inactivation stimulated myoblast fusion, which coincided with increased β-catenin-dependent transcriptional activity. Wnt/β-catenin signaling is shown to precede MRF expression, which control myogenic differentiation [5, 26, 60], and some reports suggest Wnt signaling may increase MyoD expression [61, 62]. Therefore, we expected Wnt-3a to increase muscle-specific gene expression. To our surprise however, Wnt-3a-mediated GSK-3 inactivation did not increase muscle-specific protein abundance or mRNA transcripts, muscle-specific promoter transactivation or MRF transcriptional activity. The latter results appear to be in contrast to postulated effects of Wnt signaling on MRF expression. However, very different experimental settings were used to obtain these results, including over-

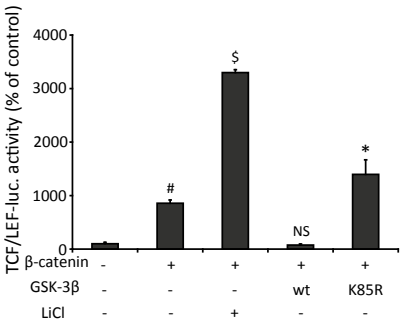


**Figure 4. GSK-3β activity directly inhibits β-catenin-dependent TCF/LEF transcriptional activity.** C2C12 myoblasts were transiently transfected with a TCF/LEF luciferase reporter plasmid and plasmids encoding β-gal (0,25μg each), β-catenin (0,5μg), WT or kinase dead (K85R)-GSK-3β (1,0μg each). After 24h incubation in DM with or without LiCl (5mM), cells were lysed to measure luciferase and β-gal activity. Shown is representative graph of 3 independent experiments (N=3 ±SEM), *: p<0.05, #: p<0.01, $: p<0.001 and NS: non-significant.

expression of Wnt ligands in combination with other stimulatory factors. Nevertheless, as any biologically significant effects on MRF expression should result in altered MRF activity, we are confident Wnt does not alter MRF functionally in differentiating myoblasts, as MRF-dependent transcriptional activity was not altered in presence of Wnt-3a. Previously, increased muscle-specific gene expression following GSK-3β inhibition was shown to involve increased NFATc3 transcriptional activity [9]. In line with the absence of a stimulatory effect on muscle-specific gene expression and in contrast to IGF-I or LiCl, Wnt-3a did not induce NFATc3 transcriptional activity. Furthermore, β-catenin over-expression did not affect NFATc3 transcriptional or TnI-promoter activity.

Overall, these data indicate that stimulation of muscle-specific gene expression corresponds with increased transcriptional activity of IGF-I but not Wnt-3a signaling associated GSK-3β phospho-substrates.

In contrast to our findings, increased MyHC-IIB mRNA expression by IGF-I was reported to in part depend on nuclear β-catenin accumulation [63]. However, the ~7-fold



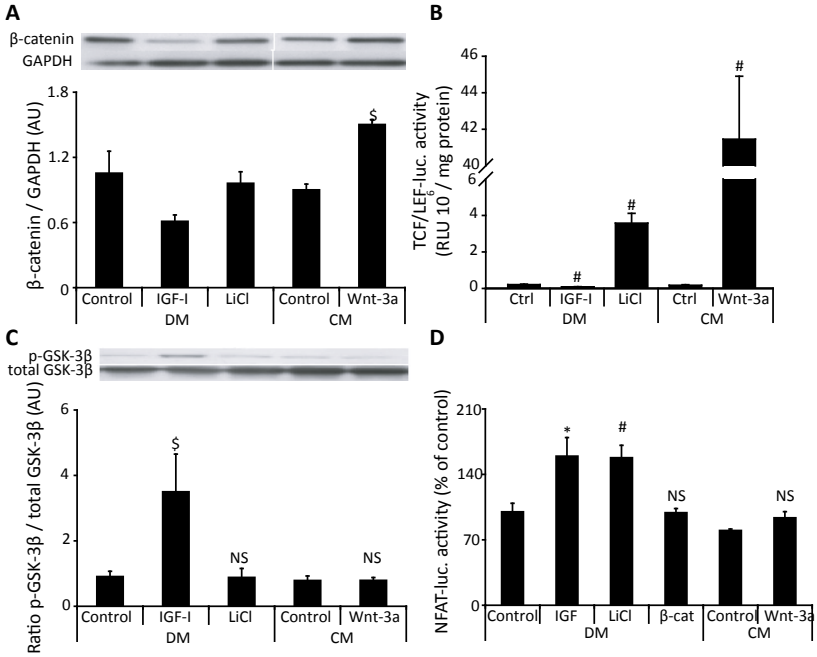


**Figure 5. Wnt-3a and IGF-I regulate distinct GSK-3β substrates in differentiating myoblasts.** C2C12 myoblasts were differentiated for (A) 24h in DM with or without IGF-I (5nM) or LiCl (10mM), control or Wnt-3a CM (each diluted 1/10 in DM), lysates were prepared and cellular β-catenin, and GAPDH protein content were visualized and quantified. Alternatively, (B) myoblasts containing a stable genomically integrated TCF/LEF luciferase reporter construct were cultured for 24h under the same conditions. (C) C2C12 myoblasts were treated for 2h in DM with IGF (5nM) or LiCl (10mM), or 2h incubated in control or Wnt-3a CM (each diluted 1/10 in DM). Lysates were prepared and cellular phospho-GSK-3β and total GSK-3β protein content were visualized and quantified. (D) C2C12 myoblasts were transfected with a NFAT-sensitive promoter-luciferase reporter plasmid and plasmids encoding β-gal (0,25μg each), NFATc3 (1,0μg each) β-catenin, or empty vector (1,0μg each) and treated as indicated. After 48h incubation cells were lysed to measure luciferase and β-galactosidase activity. Shown are representative graphs of 3 independent experiments (N=3 ±SEM), *: p<0.05, #: p<0.01, $: p<0.001 and NS: non-significant.

lower IGF-I concentrations used in our studies, did not affect β-catenin protein content or β-catenin transcriptional activity (Figure 5A, B), despite a robust increase in GSK-3β phosphorylation (Figure 5C) or muscle-specific gene expression [8] in differentiating myoblasts. Based on this we postulate that β-catenin presence may aid IGF-I stimulated MyHC-IIB promoter activity [63], but that stabilization of β-catenin is not sufficient to induce muscle-specific gene expression.

Nevertheless, most literature suggests that GSK-3β inactivation by IGF-I/Akt signalling is not sufficient to stabilize β-catenin or induce β-catenin-dependent transcription [18, 45, 46, 64]. The inability of IGF-I to affect the GSK-3β substrate β-catenin transcriptional activity, likely relates to the unique signalling route by which Wnt inactivates GSK-3, i.e. via its sequestration by Axin [46, 57, 65]. Moreover, Wnt-mediated GSK-3 inactivation is not dependent on the Akt phosphorylation sites of GSK-3 [45, 66]. In agreement with this, Wnt-3a did not induce GSK-3β phosphorylation in differentiating myoblasts, nor did it affect NFATc3 transcriptional activity, as opposed to IGF-I mediated GSK-3β inactivation. These data support the notion that the differential inactivation of GSK-3β by IGF-I and Wnt/β-catenin signaling constitutes the molecular basis to differentiate between GSK-3β substrates [46, 64].

During myogenic differentiation β-catenin and NFATc3 may represent the GSK-3 substrates subject to independent regulation by the distinct GSK-3 signaling pools, and be responsible for the separate stimulation of myoblast fusion and muscle-specific gene expression by Wnt-3a and IGF-I, respectively. In line with this, LiCl, which inhibits GSK-3 enzymatic activity towards both β-catenin and NFAT-c3, promoted both myoblast fusion and myogenic gene expression. Although the segregation of these aspects of myogenic differentiation has been reported previously [67, 68], this is the first report to imply the dissociation of myoblast fusion and gene expression may be controlled by these distinct modes of GSK-3β inactivation.

The observed effect of Wnt-3a on increased myoblast fusion may relate to the function of β-catenin as an essential binding partner for the cytoplasmic tail of various cadherins, including M-cadherin [69]. β-catenin co-localizes with M-cadherin at the cell–cell contact sites in membranes and is essential for proper fusing of myoblasts [70-72]. Therefore, increased myoblast fusion observed in response to Wnt-3a or LiCl inactivation of GSK-3 may rather relate to the function of β-catenin in cell-cell contact than its activity as a transcriptional co-regulator. This idea is supported by the observation that LiCl caused a less potent induction of TCF/LEF transcriptional activation or Axin-2 expression compared to 1/10 diluted Wnt-3a, despite similar effects on myoblast fusion, thereby demonstrating that the effects of β-catenin activity modulation do not linearly correlate with β-catenin-dependent transcriptional activity.

In conclusion, our results demonstrate that Wnt-3a induces β-catenin signaling in differentiating myoblasts, which can be mimicked by pharmacological, but not IGF-I-dependent GSK-3 inhibition. Moreover, Wnt-3a strongly promotes myoblast fusion and myotube formation without enhancing muscle-specific gene expression. These data demonstrate that two distinct signaling routes controlling GSK-3 activity independently regulate myoblast fusion and muscle-specific gene expression during myogenic differentiation.

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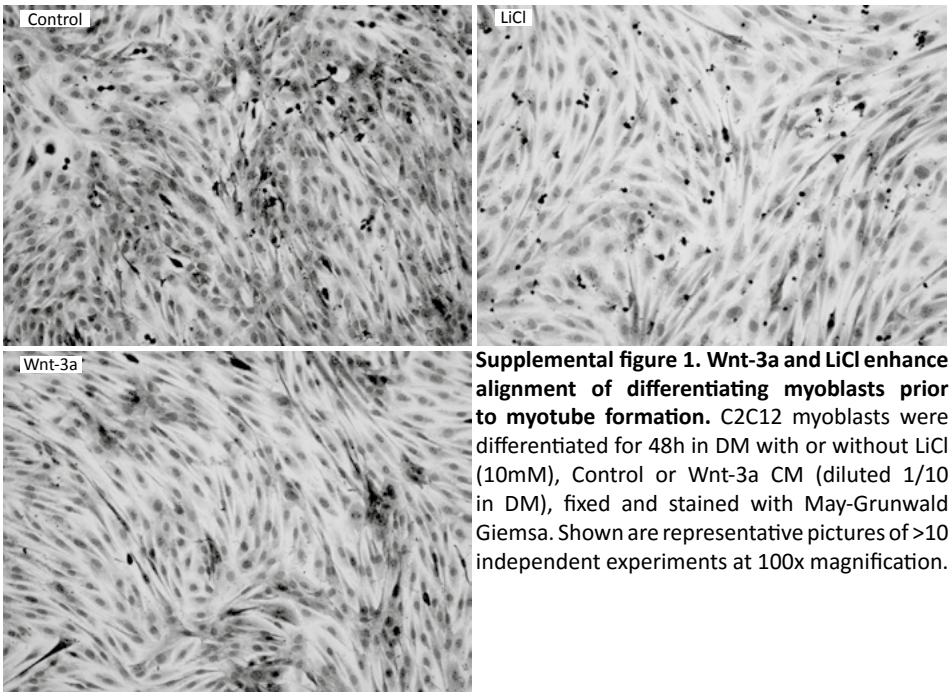
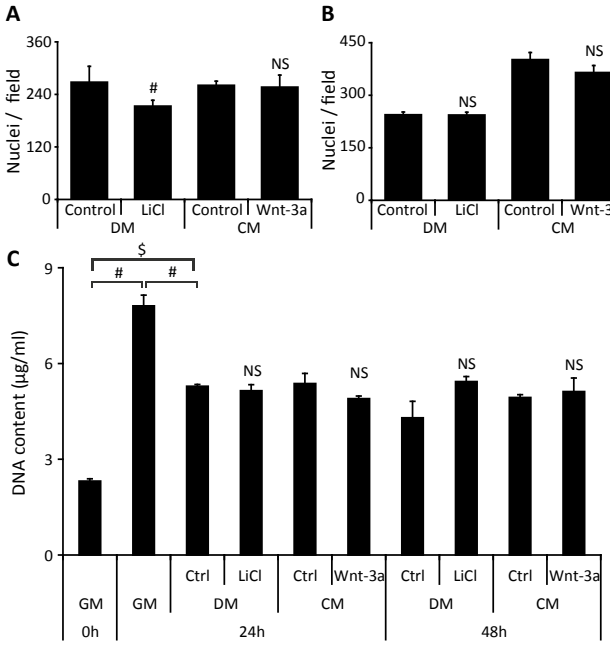
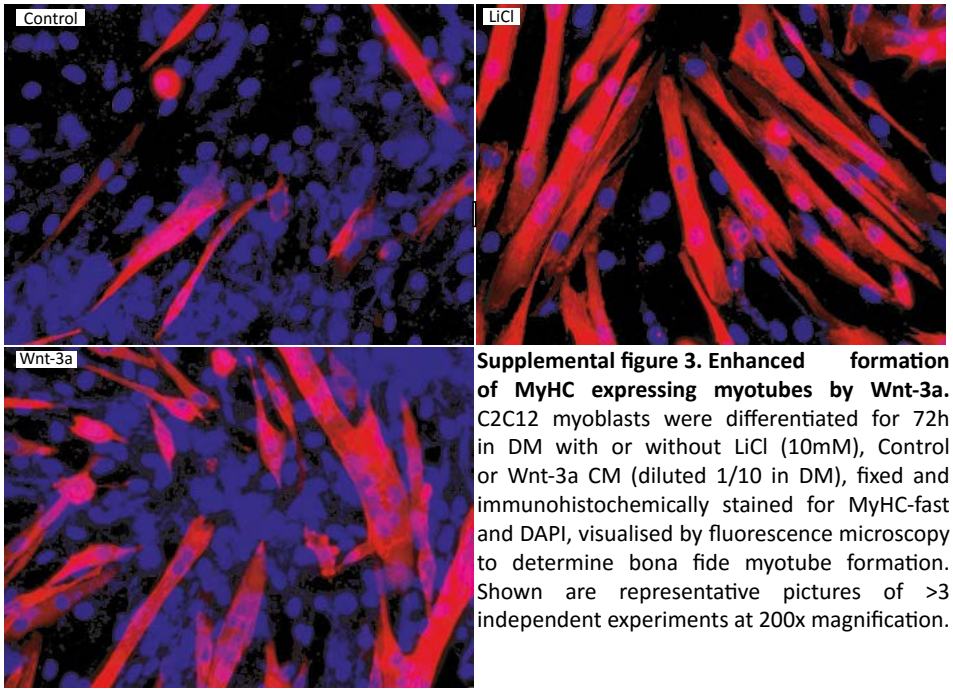
# CHAPTER 4 SUPPLEMENTAL FIGURES



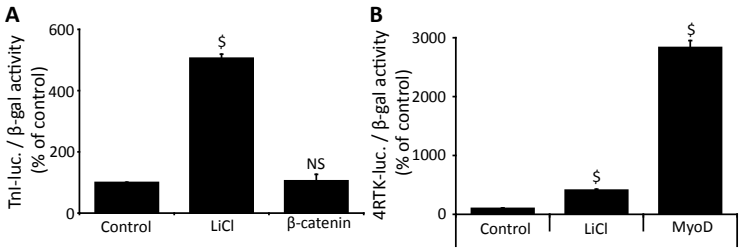

**Supplemental figure 1. Wnt-3a and LiCl enhance alignment of differentiating myoblasts prior to myotube formation.** C2C12 myoblasts were differentiated for 48h in DM with or without LiCl (10mM), Control or Wnt-3a CM (diluted 1/10 in DM), fixed and stained with May-Grunwald Giemsa. Shown are representative pictures of >10 independent experiments at 100x magnification.



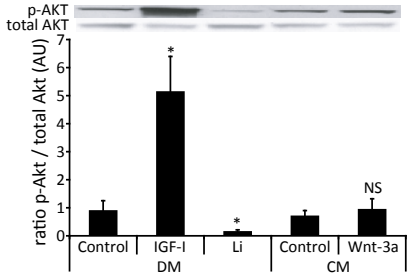

**Supplemental figure 2. L i C l or Wnt-3a does not affect cell survival in differentiating myoblasts.** C2C12 myoblasts were differentiated for (A) 24h (B) 72h in DM with or without LiCl (10mM), Control or Wnt-3a CM (diluted 1/10 in DM), fixed and stained with May-Grunwald Giemsa to determine number of nuclei per 100x magnified microscopic field and >4 microscopic field per condition were quantified. (C) C2C12 myoblasts were differentiated for 0, 24 and 48h in in DM with or without LiCl (10mM), Control or Wnt-3a CM (diluted 1/10 in DM). Adherent cells were lysed and total DNA content was determined as a measure for total cell number. Shown are representative graphs of 3 independent experiments (N=3 ±SEM), *: $p<0.05$, #: $p<0.01$, $: $p<0.001$ and NS: non-significant.




**Supplemental figure 3. Enhanced formation of MyHC expressing myotubes by Wnt-3a.** C2C12 myoblasts were differentiated for 72h in DM with or without LiCl (10mM), Control or Wnt-3a CM (diluted 1/10 in DM), fixed and immunohistochemically stained for MyHC-fast and DAPI, visualised by fluorescence microscopy to determine bona fide myotube formation. Shown are representative pictures of >3 independent experiments at 200x magnification.




**Supplemental figure 4. Muscle-specific gene expression during differentiation is stimulated by LiCl and MyoD but not by β-catenin.** (A) C2C12 myoblasts were transfected with a TnI-promoter luciferase-reporter construct and plasmids encoding b-gal (0.25mg each), b-catenin or empty vector (0.5mg each) and cultured +/- LiCl (5mM). (B) C2C12 myoblasts were transfected with a 4RTK luciferase-reporter construct and plasmids encoding b-gal (0.25mg) and pEMSV-MyoD or empty pcDNA3.1 (1.0µg) and cultured in DM with or without LiCl (10mM), Control or Wnt-3a CM (diluted 1/10 in DM). Lysates were prepared for luciferase and β-galactosidase enzyme activity. Shown are representative graphs of 3 independent experiments (N=3 ±SEM), *: p<0.05, #: p<0.01, $: p<0.001 and NS: non-significant.




**Supplemental figure 5. Wnt-3a does not affect Akt phosphorylation in differentiating myoblasts.** C2C12 myoblasts were treated for 2h in DM with IGF (5nM) or LiCl (10mM), or 2h incubated in control or Wnt-3a CM (each diluted 1/10 in DM). Lysates were prepared and cellular phospho-Akt and total Akt protein content were visualized and quantified. Shown are representative graphs of 3 independent experiments (N=3 ±SEM), *: p<0.05 and NS: non-significant.

# Chapter 5

## Muscle mass recovery following disuse-induced atrophy does not require GSK-3 inactivation

Nicholas A.M Pansters, Annemie M.W.J. Schols, Koen J.P. Verhees, Chiel C. de Theije, Rick H.P. van Gorp, Marco C.J.M. Kelders,Onne Ronda, Kei Sakamoto, Astrid Haegens and Ramon C.J. Langen
*Submitted*

## ABSTRACT

Muscle wasting impairs physical performance, increases mortality and reduces medical intervention efficacy in chronic diseases and cancer. Intervention strategies should not only focus on reducing or preventing muscle atrophy, but also encompass stimulation of muscle mass recovery. This requires improved understanding of the molecular mechanisms governing muscle mass, determined by the balance between muscle protein synthesis and degradation balance, and myonuclear accretion and loss. IGF-I is known to stimulate net protein accretion and myonuclear accretion/myogenesis. Inactivation of GSK-3, which occurs in response to IGF-I signalling, similarly affects protein and nuclear turnover in *in vitro* studies. Previous work by our group revealed that muscle mass recovery during reloading of unloaded hindlimb muscle is accompanied by IGF-I signaling and GSK-3 inactivation. We hypothesized that inactivation of GSK-3 is required for muscle mass recovery from disuse-induced muscle atrophy. This hypothesis was addressed by using KI mice in which the wild-type (WT) GSK-3α and GSK-3β alleles were replaced by inactivation-resistant 'constitutively active' (CA) GSK-3α and GSK-3β alleles. At baseline, directly after hindlimb suspension (HS)-induced muscle atrophy, and during reloading-induced (RL) muscle mass recovery, markers of muscle mass, protein synthesis and proteolysis signalling as well as post-natal myogenesis signalling were monitored. Despite subtle but consistent differences indicative of a reduced protein turnover at baseline, reloading-associated changes in muscle protein turnover were not affected by CA GSK-3. In contrast, post-natal myogenic proliferation and differentiation during RL appeared to be increased by CA GSK-3. Soleus muscle mass recovery during RL was increased in the presence of CA GSK-3, but this was not accompanied by restoration of soleus muscle fiber cross-sectional area. In conclusion, this study indicates that muscle mass recovery following disuse atrophy does not require GSK-3 inactivation.

# Chapter 6

## Muscle-specific GSK-3β ablation accelerates regeneration of disuse-atrophied skeletal muscle

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*Biochimica et Biophysica Acta. 2015. 1852(3): p. 490-506*

## ABSTRACT

Muscle wasting impairs physical performance, increases mortality and reduces medical intervention efficacy in chronic diseases and cancer. Developing proficient intervention strategies requires improved understanding of the molecular mechanisms governing muscle mass wasting and recovery. Involvement of muscle protein- and myonuclear turnover during recovery from muscle atrophy has received limited attention. The insulin-like growth factor (IGF)-I signaling pathway has been implicated in muscle mass regulation. As glycogen synthase kinase 3 (GSK-3) is inhibited by IGF-I signaling, we hypothesized that muscle-specific GSK-3β deletion facilitates recovery of disuse-atrophied skeletal muscle. Wild-type mice and mice lacking muscle GSK-3β (MGSK-3β KO) were subjected to a hindlimb suspension model of reversible disuse-induced muscle atrophy and followed during recovery. Indices of muscle mass, protein synthesis and proteolysis, and post-natal myogenesis which contributes to myonuclear accretion, were monitored during reloading of atrophied muscle. Early muscle mass recovery occurred more rapidly in MGSK-3β KO muscle. Reloading-associated changes in muscle protein turnover were not affected by GSK-3β ablation. However, coherent effects were observed in the extent and kinetics of satellite cell activation, proliferation and myogenic differentiation observed during reloading, suggestive of increased myonuclear accretion in regenerating skeletal muscle lacking GSK-3β. This study demonstrates that muscle mass recovery and post-natal myogenesis from disuse-atrophy is accelerated in absence of GSK-3β.

# 1 INTRODUCTION

Chronic diseases, like congestive heart failure [1], rheumatoid arthritis [2, 3], chronic renal failure, AIDS [4], chronic obstructive pulmonary disease (COPD) [5, 6] and cancer [7, 8] are associated with muscle wasting. Muscle wasting impairs physical performance, increases mortality and reduces efficacy of medical intervention [9]. Currently, muscle mass maintenance is an unmet-medical-need, which should be integrated in the management of chronic diseases and cancer. Developing proficient intervention strategies requires improved understanding of the molecular mechanisms governing muscle atrophy, hypertrophy, as well as muscle mass recovery-associated muscle regeneration. These mechanisms are regulated by balancing muscle protein synthesis and degradation, and potentially myonuclear loss and accretion, referred to as muscle protein-, and myonuclear turnover, respectively [10]. Involvement of these processes in muscle mass recovery following muscle atrophy has received limited attention.

Muscle protein synthesis can be initiated through insulin-like growth factor-I (IGF-I)-mediated activation of the PI-3K signaling cascade leading to phosphorylation of Akt/PKB at $Thr^{308}$/$Ser^{473}$ [11-13]. Activated Akt can subsequently phosphorylate both the mammalian target of rapamycin (mTOR)-raptor complex 1 (mTORC1) [14, 15] and the two isoforms of GSK-3 [16, 17], thereby initiating mRNA translation resulting in increased protein synthesis [18, 19]. More specifically, activated mTOR, when phosphorylated at $Ser^{2448}$, phosphorylates p70-S6K1 at $Thr^{389}$ to stimulate translation capacity and hyper-phosphorylates eIF4E binding protein 1 (4E-BP1) to inhibit suppression of translation initiation [15, 20-22]. In addition, Akt phosphorylation of GSK-3β at $Ser^{9}$ and GSK-3α at $Ser^{21}$ suppresses GSK-3 function including its inhibitory phosphorylation of eIF2Bε at $Ser^{540}$ [23, 24], which allows eIF2Bε to interact with eIF2-complex and facilitate mRNA translation initiation [25, 26]. Muscle proteolysis involves several proteolytic systems, including the ubiquitin (Ub) 26S-proteasome system (UPS) and the autophagy-lysosomal pathway (ALP) [10, 27, 28]. UPS entails selective proteolysis through multi-ubiquitination of marked proteins by rate-limiting E3 ubiquitin ligases [29], which results in degradation of the tagged proteins by the 26S-proteasome [30]. The E3 ligases muscle-specific atrogin-1/muscle atrophy F-box (MAFbx) (hereafter termed atrogin-1) and muscle-specific RING finger protein 1 (MuRF1) are upregulated under atrophic conditions [31, 32] and regulate proteolysis of myofibrillar proteins, muscle-specific enzymes and transcription factors [33-36]. ALP-regulated autophagy clears long-lived proteins and dysfunctional organelles through autophagosome formation, which is initiated or aided by proteins like LC3, Gabarapl1 [37] and BNIP3 [38], whose presence is increased in atrophying muscle [37, 39, 40].

Quiescent satellite cells, located between skeletal muscle basement and sarcolemmal membranes [41], are activated in response to muscle injury or exercise stimulation leading to muscle recovery [42-44]. The loss of myonuclei, and conversely activation and proliferation of satellite cells have been correlated to muscle atrophy and growth, respectively [45-49], implying a role for post-natal myogenesis and myonuclear accretion during muscle regeneration. Satellite cell activation results in increased PAX7 expression [50-52], and cell proliferation characterized by increased expression of Cyclin D1, PCNA or increased protein content of KI-67 [53, 54]. The resulting daughter myoblasts committed to muscle-specific differentiation fuse with existing myofibers [55], which is

in part facilitated through cell–cell interaction sites containing Muscle (M)-cadherin [56-58]. This myogenic differentiation includes muscle-specific gene expression regulated by muscle regulatory factors (MRFs), including Myf5, MyoD and Myogenin [59, 60].

Altered expression of mechano growth factor (MGF), a splice variant of IGF-I [61] accompanies local muscle repair, maintenance and remodeling [62, 63]. Moreover, both muscle protein turnover and myonuclear turnover are subject to regulation by IGF-I signaling. Protein synthesis and muscle-specific gene expression can be stimulated by IGF-I [64-66], whereas muscle proteolysis is suppressed by IGF-I [14, 67]. In addition, muscle-specific expression of IGF-I promotes muscle regeneration [68], and IGF-I signaling stimulates both the proliferative and differentiation potential of myoblasts [69-71]. Akt has been identified as a signaling molecule that coordinates IGF-I-mediated changes in muscle protein synthesis and degradation, but regulation of myonuclear turnover by Akt is less well understood [72-74]. Ample evidence suggests an important role of the Akt substrate GSK-3 in muscle protein turnover, including muscle proteolysis during muscle atrophy [75-77], as well as myogenic differentiation and muscle regeneration [65, 78, 79]. Importantly, whereas GSK-3α and GSK-3β operate in a redundant manner in various cellular processes, previous studies suggest that muscle proteolysis and myogenic differentiation may be under unique control of GSK-3β [75, 79]. We therefore proposed a regulatory role of GSK-3β in skeletal muscle mass maintenance through its involvement in both protein and myonuclear turnover balances [17].



In the current study, we hypothesized that muscle-specific deletion of GSK-3β facilitates disuse-atrophied muscle mass recovery by stimulating signaling associated with net protein accretion and post-natal myogenesis. As inactivity is an important determinant of muscle atrophy associated with chronic disease and cancer [80], a model of reversible, hindlimb suspension (HS)-induced muscle atrophy was deployed to address this hypothesis. Wild-type ctrl (WT) and muscle-specific (M)GSK-3β KO mice were subjected to HS and subsequent reloading (RL), and. indices of muscle mass, protein synthesis, proteolysis signaling and myonuclear accretion were monitored during reloading-induced muscle mass recovery.

# 2 MATERIALS AND METHODS

## 2.1 Animals

The animal study described here was approved by the Institutional Animal Care Committee of Maastricht University (DEC-2009-074). Male skeletal muscle-specific (M) GSK-3β KO animals on a C57/Bl6 background were generated by breeding GSK-3β$^{fl/fl}$ MLC1f-Cre$^{+/-}$ (MGSK-3β KO) with GSK-3β$^{fl/fl}$ MLC1f-Cre$^{-/-}$ (Wild-type ctrl/WT) mice [81]. Double Cre-negative litter mates served as 'wild type' (WT) controls. At the start of the experiment mice were 12.9±1.1 weeks old (young adults) and weighed 27.7±2.4 grams. Animals were housed in a temperature controlled room (21-22°C) with 12:12h light-dark cycle, and received standard chow pellets and water ad libitum. Animals were randomly divided into 6 different groups for each genotype, and were subsequently subjected to no experimental procedures ('baseline'/RL- −14), 14 days of hindlimb suspension ('HS'/RL-0) or HS followed by 1, 2, 3, or 5 days of reloading (RL-1, -2, -3, -5, respectively). Group size was n=8 except for MGSK-3β KO RL-5 (n=7) and baseline for both genotypes (n=9). A modified version of the HS/RL model [82] has previously been described [83]. In brief, a tail harness was placed while mice were lightly anesthetized using isoflurane inhalation. HS was accomplished using a tail suspension device consisting of a plastic-coated iron wire taped around the mouse's tail and connected to a swivel hook to allow circular motility. The latter was attached to a Teflon-coated PVC ring, which slid over an iron rod spanning the length of the cage to allow longitudinal motility. The mice were raised so as to prevent the hindlimbs from touching the cage floor or sides. In this way, four HS mice could be housed in one standard cage. After two weeks of HS, mice were again lightly anesthetized and released from the tail harness and allowed to resume normal cage activity. During the whole treatment period body weight and water consumption were monitored, in addition during the reloading phase chow consumption was assessed. After euthanasia with sodium pentobarbital at the indicated time-points, lower leg muscles were excised using standardized dissection methods, cleaned of excess fat and tendon/connective tissue, pair weighed on an analytical balance, snap frozen in liquid nitrogen, and stored at n80°C for RNA and protein extraction or part of the muscle was embedded in Tissue-Tek O.C.T. (Sakura, Finetek, Zoeterwoude, the Netherlands) for histological analyses. All subsequent analyses were performed in either soleus or gastrocnemius muscle, which despite their slightly differing anatomical positions and locomotive function, both respond to HS and RL alterations in muscle mass.

## 2.2 Western blot analyses

M. gastrocnemius was ground into powder and homogenized in 400 µl of IP lysis buffer (50 mM Tris, 150 mM NaCl, 10% glycerol, 0.5% Nonidet P40, 1 mM EDTA, 1 mM $Na_3VO_4$, 5 mM NaF, 10 mM β-glycerophosphate, 1 mM $Na_4O_7P_2$, 1 mM dithiothreitol, 10 µg/ml leupeptin, 1% aprotenin, 1 mM PMSF, pH 7.4) with a Polytron PT 1600 E (Kinematica). After homogenization, the samples were incubated for 15 min on a rotating wheel at 4°C and spun for 30 min at maximum speed (20,817 × g) in a centrifuge cooled to 4°C. The supernatant was aliquoted, snap-frozen, and stored without sample buffer at −80°C until analysis. A portion of the supernatant was saved for protein determination, prior to the addition with 4x Laemmli sample buffer (0.25M Tris-HCl pH 6.8; 8% (w/v) SDS; 40% (v/v) glycerol; 0.4M DTT and 0.04% (w/v) Bromophenol Blue). The samples

were boiled for 5 minutes at 95°C and stored at −80°C. Total protein was assessed by the Thermo Scientific Pierce BCA Protein Assay kit (Pierce Biotechnology, IL, USA) according to the manufacturers' instructions. The pellet fraction was resuspended in IP lysis buffer and homogenized with a Polytron PT 1600 E (Kinematica), subsequently 4x Laemmli sample buffer was added and boiled for 5 minutes at 95°C and stored at −80°C. For SDS-PAGE 0.2µg-15µg of protein was loaded per lane and separated on a Criterion™ XT Precast 4-12% Bis-Tris gel (Bio-Rad, #3450124), followed by transfer to a 0.45µm Whatman® Protran® Nitrocellulose Transfer membrane (Whatman GmbH, #7324007) by electroblotting (Bio-Rad Criterion Blotter) (Bio-Rad, Hercules, CA, USA). For the pellet fraction containing protein loaded samples the membrane was stained with Ponceau S solution (0.2% Ponceau S in 1% acetic acid; Sigma-Aldrich Chemie) to quantify total protein loading. The membrane was blocked for 1-2h at room temperature in 5% (w/v) NFDM (non-fat dried milk) (ELK, Campina, the Netherlands) diluted in TBS-Tween-20 (0.05%). Nitrocellulose blots were washed in TBS-Tween-20 (0.05%) on a rotating platform, followed by overnight (o/n) incubation at 4°C with specific antibodies directed against: p-mTOR ($Ser^{2448}$) (#2971), mTOR (#2983), p-Akt ($Ser^{473}$) (#9271), Akt (#9272), p-GSK-3β ($Ser^{9}$) (#9336), GSK-3β (#9332), p-p70S6K ($Thr^{389}$) (#9206), p70S6K (#2708), 4E-BP1 (#9452), p-FoXO1 ($Ser^{256}$) (#9461), FoXO1 (#2880), p-FoXO1/3a ($Thr^{24}$) (#9946), and GAPDH (#2118) (all from Cell Signaling Technology, Inc., Danvers, MA,), myosin heavy chain fast (MyHC-f/MHC-2) (#M4276, Sigma-Aldrich) all diluted in TBS/0.05% Tween-20 with or without 5% BSA/NFDM. After 3 washing steps of 10 minutes each, the blots were probed with a peroxidase conjugated secondary antibody (Vector Laboratories, #PI-1000), and visualized by chemiluminesence using Supersignal® WestPico Chemiluminescent Substrate (Pierce Biotechnology, Inc.) according to the manufacturers' instructions and exposed to film (Biomax light film, KODAK) or live imaged (Bio-Rad chemidoc XRS). Western blot images were quantified using the Quantity One analysis software from Bio-Rad. For analyses, gels were loaded with both WT and MGSK-3β KO samples, and samples of the same group were distributed over multiple gels to allow comparisons to RL-0 (HS).



### 2.3 RNA isolation and assessment of mRNA abundance by RT-qPCR

M. gastrocnemius was ground into powder and approximately 10mg of sample was suspended and lysed in RLT solution containing 1% β-mercaptoethanol. Samples were further processed according to manufacturer's instructions of the RNeasy fibrous tissue Mini Kit (Qiagen, Venlo, the Netherlands) including the on-column DNase treatment. RNA was reconstituted in 50µl RNase free water and stored at −80°C. The RNA concentrations were measured spectrophotometrically using a Nanodrop® ND-1000 UV-Vis spectrophotometer. RNA was diluted >5x in $ddH_2O$ and 400 ng of RNA was reverse transcribed to cDNA using the Transcriptor first strand cDNA synthesis kit (Roche Diagnostics GmbH, Mannheim, Germany) with anchored oligo-dT primers according to the manufacturers' conditions for generating cDNA fragment of 4kb in a final reaction volume of 20µl. This cDNA was used for quantification of transcript levels by reverse transcription quantitative PCR (qPCR) analysis. qPCR primers used, were designed using Primer Express 2.0 software (Applied Biosystems, Foster City, CA), and obtained from Sigma Genosys (Haverhill, UK). qPCR reactions (16 µl final volume) contained SensiMix SYBR Hi-ROX Kit (Quantace-Bioline, London, UK) with 300 nM primers and were run in a

384-well MicroAmp Optical 384-Well Reaction Plate (Applied Biosystems, Nieuwerkerk a/d IJssel, the Netherlands) on a 7900HT Fast Real-Time PCR System (Applied Biosystems). Relative cDNA starting quantities for the samples were derived by the standard curve method. Standards curve samples were generated by serial dilution of pooled cDNA samples. Starting quantities were obtained by extrapolating Ct values on the standard curve. The expression of the genes of interest were normalized with a correction factor derived by GeNorm [84]. This factor was determined for each group individually to reduce variation in data resulting from biological spread and analytical imperfections, as reference gene expression between time points responded significantly, in line with observations by others [85]. The GeNorm correction factor was based on the expression levels of GAPDH, RPL13A, ARBP, Calnexin, and β2M as reference genes.

## 2.4 Immunohistochemical analyses

Histological analysis of cell proliferation and myofiber cross sectional area was performed on M. soleus, as fiber type composition and CSA is less heterogeneous compared to M. gastrocnemius [86]. Furthermore compared to the gastrocnemius, the soleus muscle has greater load-sensitivity and thus greater morphological remodeling processes is expected to be undertaken during the reloading phase [87]. OCT-embedded, frozen M. soleus sections were cut (5µm), air dried, treated with 0.5% Triton X-100 in PBS, incubated with primary anti-myosin heavy chain (MyHC)-I [Developmental Studies Hybridoma Bank (DSHB), University of Iowa, Ames, IA], anti-MyHC-IIa (DSHB), and anti-laminin (Sigma, Zwijndrecht, the Netherlands) followed by secondary antibodies labeled with Alexa Fluor 555, Alexa Fluor 488, and Alexa Fluor 350 (Invitrogen, Breda, the Netherlands). Unstained fibers were considered type IIX/B fibers. After staining, all images were digitally captured using fluorescence microscopy (Nikon Instruments Europe). Image processing and quantitative analyses were done using the Lucia 4.81 software package. The mean fiber CSA of each muscle was calculated by analyzing on average >200 fibers per muscle. Additionally frozen M. soleus sections were cut (5µm) were air dried, treated with 0.5% Triton X-100 in PBS, incubated with primary anti-laminin (Sigma, Zwijndrecht, the Netherlands) and Ki-67 (Biocare Medical/Klinipath, Duiven, the Netherlands) followed by secondary antibodies labeled with Alexa Fluor 488, and Alexa Fluor 350 (Invitrogen, Breda, the Netherlands) and propidium iodide (PI) for nuclear staining. After staining, all images were digitally captured using fluorescence microscopy (Nikon Instruments Europe). Image processing and quantitative analyses were done using the Lucia 4.81 software package. Double stained (PI and KI-67) nuclei were counted as proliferating nuclei and divided by the number of fibers per muscle section analyzed to determine the proliferation level for that specific muscle.

## 2.5 Statistical analysis

Statistical data analysis were performed (n=4-9) to allow comparisons between Wild-type littermate ctrl (WT) *vs.* MGSK-3β KO mice at specific time points, and within genotype between a specific time point *vs.* RL-0 (HS). Significant differences were detected with a non-parametric Mann-Whitney U test, $p<0.05$ or smaller as indicated in each figure. Data are expressed as mean ±SEM. Data were analyzed using SPSS/PC+ (Statistical Package for the Social Sciences, Version 22.0 for Windows; SPSS; Chicago, IL).

# 3 RESULTS

## 3.1 Muscle specific deletion of GSK-3β accelerates body weight recovery and muscle MGF expression during muscle reloading

GSK-3β protein content was reduced (~95%) in M. gastrocnemius of MGSK-3β KO compared to WT mice (Figure 1A), whereas GSK-3α protein levels were unaffected. This allowed to address GSK-3β functions that are either non-redundant with GSK-3α, or are dependent on total GSK-3 levels. eIF2Bε ($Ser^{539}$) phosphorylation, a downstream target of GSK-3 enzymatic activity [88], was significantly lower in MGSK-3β KO ($p<0.05$) (Figure 2D). Hindlimb suspension (HS) resulted in approximately 10% body weight (BW) loss (Figure 1B, left panel). The genotypic difference ($p<0.05$) in initial BW loss after two days of HS (Figure 1B, left panel) suggested a short term (initial 24-72hrs of HS) protective effect in MGSK-3β KO mice compared to Wild-type ctrl (WT) mice. During the five day reloading (RL) period BW significantly increased ($p<0.001$) compared to HS (RL-0) for both genotypes (Figure 1B, right panel). However, compared to WT, BW recovery following five days of RL was significantly greater in MGSK-3β KO mice (6.7±1.2% v.s. 11.3±0.8%, $p<0.05$). Fourteen days of HS comparably decreased muscle mass in both WT and MGSK-3β KO mice (16.9±2.7% $p<0.001$ and 16.3±2.2%, $p<0.001$, respectively, Figure 1C, left panel). Importantly, alterations in unloading-sensitive muscle mass were attributable to unloading, as no changes in the unloading-insensitive M. extensor digitorum longus (EDL) were observed (data not shown). Subsequent hindlimb RL showed a comparable but not statistical significant increase in gastrocnemius muscle mass for WT and MGSK-3β KO (6.6±2.6% and 5.8±4.6%, Figure 1C, right panel). Accordingly, evaluation of myofibrillar protein accretion revealed increases in MHC-2 (glycolytic isoform) content in M. gastrocnemius homogenates following 5 days RL (38.7±7.6% $p<0.01$ and 27.8±5.6% $p<0.01$, Figure 1D, right panel), in line with net muscle tissue accretion. Expression of mechano growth factor (MGF), produced during muscle remodeling [89], was clearly induced on RL day 1 (RL-1), suggesting initiation of muscle regeneration in both WT and MGSK-3β KO M. gastrocnemius (Figure 1E, right panel). Although MGF expression levels were not significantly different between genotypes at baseline (RL- −14) or after HS (RL-0) in (Figure 1E, left panel), HS increased MGF expression compared to baseline levels in MGSK-3β KO but not WT (50.9±4.1% $p<0.01$ *vs.* 9.39±7.6% n.s.). During RL, MGF expression was up-regulated (~two-fold, $p<0.01$) in both WT and MGSK-3β KO (Figure 1E, right panel). However, MGF expression induction on RL-1 was more pronounced ($p<0.05$) in MGSK-3β KO compared to WT (3.0±0.3-fold $p<0.001$ *vs.* 2.2±0.2-fold $p<0.001$, Figure 1E right graph). Combined, these data indicate that reloading-induced muscle remodeling may be affected by GSK-3β.

## 3.2 Activation of Akt-mTOR signaling during muscle reloading is not affected by absence of GSK-3β

Hindlimb reloading initiates the muscle remodeling process which involves adjusting the protein turnover balance regulated by protein synthesis and degradation signaling [78, 90-92]. Akt ($Ser^{473}$) phosphorylation is lower in MGSK-3β KO compared to WT at baseline (RL- −14) ($p<0.05$, Figure 2B). Akt-phosphorylation levels were not affected by HS (RL-0) in WT but were increased in MGSK-3β KO (3.6±15.7% *vs.* 49.8±13.6% $p< 0.05$, Figure 2B), and thereby no longer genotypically different from WT. At RL-1 Akt-

phosphorylation increased, compared to HS (RL-0), for WT and MSGK-3β KO (77.8±8.8% p<0.01 and 97.0±15.1% p<0.05, respectively, Figure 2B). During further RL days, Akt phosphorylation decreased again to HS comparable levels for both genotypes. After HS downstream from Akt, inactivating GSK-3β ($Ser^{9}$) phosphorylation was increased by ~50% in WT (Figure 2C). Following HS, GSK-3β phosphorylation did not significantly increase until RL-5 (39.2±8.9% p<0.05, Figure 3C). This coincided with a similar increase in WT Akt phosphorylation (30.5±18.1%, Figure 2B) at RL-5. As expected, eIF2Bε ($Ser^{539}$) phosphorylation, downstream target of GSK-3β phosphorylation [88], was significantly lower in MGSK-3β KO (p<0.05) and did not did not alter throughout HS-RL (Figure 2D). Although in WT muscle eIF2Bε phosphorylation did not significantly change during HS-RL, phospho levels appeared to inversely reflect GSK-3β phosphorylation changes at RL-0 and RL-5 (Figure 2D and 2C).

mTOR ($Ser^{2448}$) phosphorylation levels were unaltered after HS and not different between genotypes (Figure 2E). However, mTOR phosphorylation increased as of RL-1 in both WT and MGSK-3β KO muscle (2.7±0.3-fold, p<0.01, and 3.1±0.5-fold, p<0.001, no genotypic difference), leveling off above HS levels at RL-2 and later time points (>1.5-fold p<0.05), with no genotypic effect of GSK-3β ablation (Figure 2E). S6K ($Thr^{389}$) phosphorylation, a major downstream target of mTOR and effector of mRNA translation capacity, was not detectable at either baseline (RL- −14) or after HS (RL-0) for either genotype (Figure 2F). However, initiation of RL clearly increased S6K phosphorylation (Figure 2F). Following initial increase on RL-1, S6K phosphorylation decreased on RL-2 and RL-3, whilst it increased again at RL-5 for both WT and MGSK-3β KO (1.3±0.3 AU and 2.9±0.7 AU, respectively, Figure 2F) with no statistically significant genotypic effects throughout RL. This biphasic response was not observed in mTOR phosphorylation changes (Fig 2C), but was apparent in Akt phosphorylation changes (Figure 2B). Phosphorylation levels of the suppressor of mRNA translation initiation 4E-BP1 were, in line with S6K and mTOR, unchanged after HS (Figure 2G). However, RL initiation increased 4E-BP1 phosphorylation evidenced by a shift from the relative proportion of un-phosphorylated to hyper-phosphorylated (inactivating) 4E-BP1. Hyper-phosphorylation of 4E-BP1 increased for both WT and MSGK-3β KO (42.2±0.7% p<0.01 and 76.8±10.9% p<0.01, respectively) on RL-1 and remained elevated subsequently until RL-5 for both genotypes (>30% p<0.01, Figure 2G). The inverse was observed for un-phosphorylated 4E-BP1; with no observed effect of GSK-3β ablation on 4E-BP1 phosphorylation distribution during HS-RL (Figure 2G). Thus, apart from its direct target eIF2Bε, deletion of GSK-3β does not appear to affect increased protein synthesis signaling in atrophied muscle in response to RL.

### 3.3 Differential effects of GSK-3β deletion on changes in expression of glucocorticoid sensitive regulators of protein turnover signaling during muscle unloading and reloading

Skeletal muscle protein turnover is affected by glucocorticoid (GC) receptor (GR) signaling [39, 93-96]. GR-mediated transcription of REDD1 has been implicated in inhibition of protein synthesis [20, 97]. HS was accompanied by an up-regulation of REDD1 expression in WT and MGSK-3β KO (3.6±0.6-fold p<0.001 and 2.1±0.3-fold p<0.05, respectively), which was most pronounced in WT muscle (1.7-fold higher than in MGSK-3β KO, p<0.05; Figure 3A left panel). The subsequent decrease in REDD1 expression

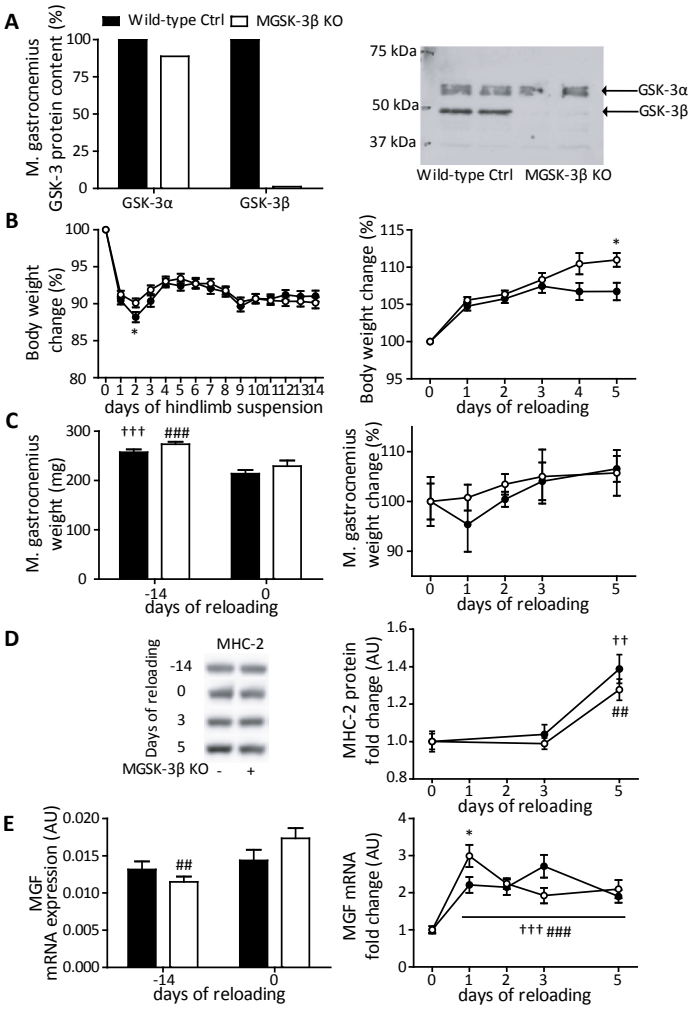


**Figure 1. Muscle specific deletion of GSK-3β accelerates body weight recovery and muscle MGF expression during muscle reloading.** (A) M. gastrocnemius protein lysates were subjected to Western blot and GSK-3β knockdown levels and GSK-3α expression levels were assessed (WT/MGSK-3β KO n=2). Muscle-specific GSK-3β KO or littermate controls (WT) were subjected to 14 days of hind-limb suspension (HS). (B) Body weight (BW) was monitored and expressed as percentage change of starting body weight during HS (left panel, WT n=40, MGSK-3β KO n=39). Following completion of HS and during reloading (RL) BW, expressed as percentage change of BW at the start of RL (RL-0/HS; right panel, both WT/KO decrease with n=8 increments). (C) Paired Gastrocnemius muscle weights were determined at baseline ('RL- −14') and after HS ('RL-0'; left panel). Alternatively, during RL M. gastrocnemius weights were expressed as percentage of HS (RL-0) muscle weight (right panel). (D) Glycolytic myosin heavy chain (MHC-2) protein contents were detected in re-solubilized muscle homogenates pellet fraction, normalized to total protein determined by Ponceau-S staining, and expressed as fold change of HS (RL-0). (E) Mechano growth factor (MGF) mRNA levels were determined during baseline and following HS (left panel), or during RL (right panel). MGF expression levels during RL are presented as fold change compared to RL-0 (HS) to illustrate reloading-induced changes. (C-E group size was n=8 except for MGSK-3β KO RL-5 n=7 and baseline for both genotypes n=9). Averages ± SEM are presented, *: Wild-type

ctrl *vs.* MGSK-3β KO at that specific time point, †: indicates time effect compared to RL-0 (HS) for Wild-type ctrl and #: indicates time effect compared to RL-0 (HS) for MGSK-3β KO; 1 symbol equals p<0.05, 2 symbols equal p<0.01, and 3 symbols equal p<0.001.

after RL-1 observed in WT (1.8-fold, p<0.01) was absent in MGSK-3β KO (Figure 3A right panel). From RL-2 onwards, both WT (p<0.001) and MGSK-3β KO (p<0.01) displayed >70% decrease in REDD1 mRNA expression compared to RL-0 (Figure 3A right panel).

KLF-15 [98] [99], and FoXO1 [98, 100] are two transcriptional regulators of proteolysis affected by GR. KLF-15 was not altered after HS in WT, but increased in MGSK-3β KO (1.0±0.1-fold *vs.* 1.7±0.2-fold p<0.001, respectively; Figure 3B left panel), with levels differing between WT and MGSK-3β KO (1.7-fold p<0.001; Figure 3B left panel). Initiation of RL decreased KLF15 expression until RL-5 for both WT and MGSK-3β KO (46.7±5.8% p<0.001 and 66.3±8.3% p<0.01, respectively), but this decrease started already at RL-2 in MGSK-3β KO muscle (1.6±0.1-fold p<0.01) and was more pronounced compared to WT (RL-2 and RL-3 p<0.01; Figure 3B right panel). Interestingly FoXO1 expression changes throughout HS-RL were relatively similar to KLF15 expression changes (Figure 3C and 3B, respectively). FoXO1 total protein content was increased after HS for both WT and MGSK-3β KO (1.2±0.1-fold and 1.4±0.1 p<0.05, respectively; Figure 3D-E) and remained relatively unchanged until RL-5, when it decreased to RL-0 (HS) comparable levels (1.2±0.1-fold and 1.5±0.1 p<0.05, respectively; Figure 3E). FoXO activity is strongly regulated by its phosphorylation status, which facilitates its nuclear exclusion [101]. HS resulted in increased phosphorylation of FoXO1, which was more pronounced in WT ($Thr^{24}$ and $Ser^{256}$, 2.1±0.2-fold and 1.9±0.3-fold, respectively, p<0.05) compared to MGSK-3β KO ($Thr^{24}$ and $Ser^{256}$, 1.8±0.1-fold p<0.01 and 1.5±0.2-fold, respectively; Figure 3F and 3G). During RL FoXO1 $Thr^{24}$ phosphorylation appeared to decrease for both genotypes, but only significantly in MGSK-3β KO (>25% RL-2 and RL-3 p<0.05; Figure 3F). In contrast, at RL-5 FoXO1 phosphorylation strongly increased compared to RL-0 for both WT ($Thr^{24}$ and $Ser^{256}$, 2.1±0.2-fold p<0.001 and 1.4±0.1-fold, respectively) and MGSK-3β KO ($Thr^{24}$ and $Ser^{256}$, 2.9±0.3-fold p<0.01 and 1.8±0.1-fold p<0.05, respectively; Figure 3F-G). Considering the extensive regulation of FoXO during HS and RL in both genotypes, FoXO transcriptional targets involved in muscle protein degradation were subsequently investigated.

### 3.4 Expression of proteolysis mediators is decreased during muscle reloading and differentially affected by absence of GSK-3β

Atrogin-1 gene expression was lower at baseline (RL- −14) in MGSK-3β KO than WT (~1.5-fold (p<0.01), and increased similarly following HS in both WT and MGSK-3β KO (1.5±0.2-fold p<0.05 and 1.6±0.2-fold, respectively), sustaining the genotypic difference (p<0.05; Figure 4A left panel). With RL, atrogin-1 gene expression levels decreased similarly for WT and MGSK-3β KO until RL-5 (>3.5-fold p<0.01; Figure 4A right panel). Contrary to atrogin-1, MuRF1 gene expression was not different between genotypes at baseline, nor significantly induced after HS in either WT or MGSK-3β KO (Figure 4B, left graph). RL-associated MuRF1 gene expression suppression (>2.5-fold p<0.05; Figure 4B right panel) occurred more rapidly in MGSK 3β KO, similar to REDD1 and FoXO1 (Figure 3A and 3C right panels). Interestingly, MuRF1 expression transiently increased in both WT and MGSK-3β KO muscle at RL-3 (1.4±0.1-fold and 1.8±0.2-fold compared to

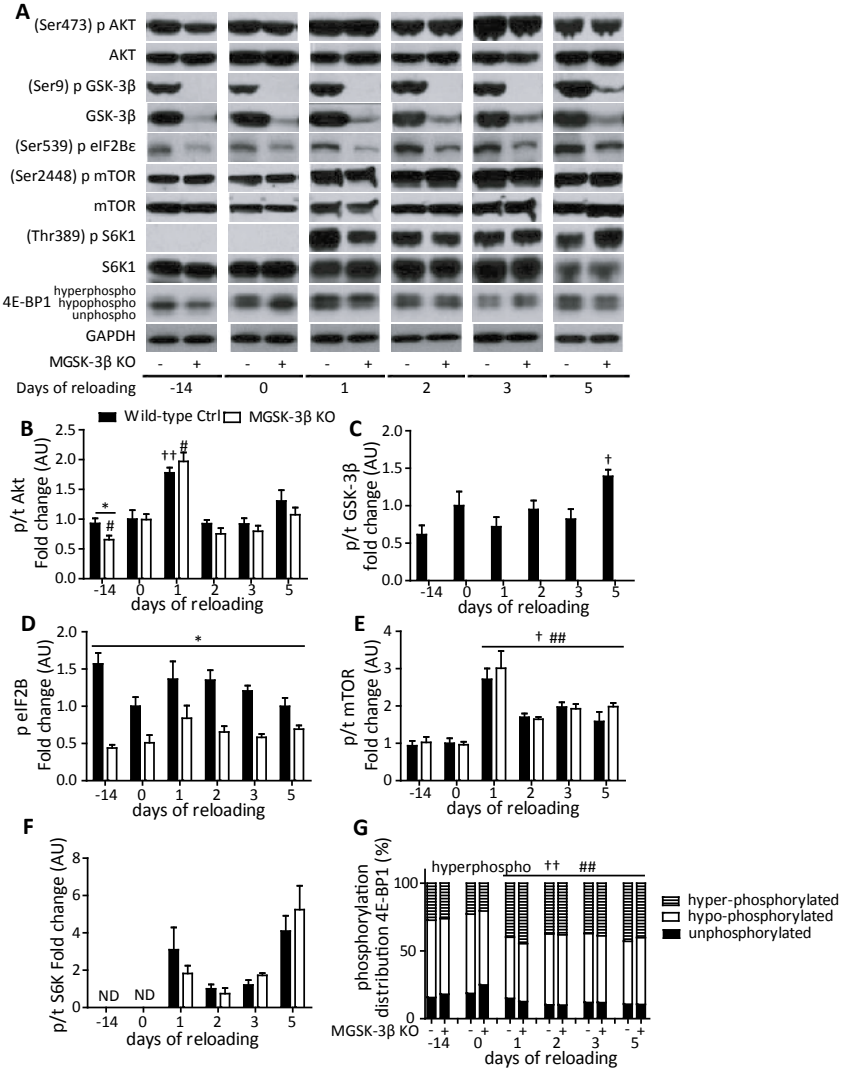


**Figure 2. Activation of Akt-mTOR signaling during muscle reloading is not affected by absence of GSK-3β.** (A) M. Gastrocnemius was prepared for western blot analysis and indicated phosphorylated and total proteins were detected to determine protein synthesis signaling status. Subsequently, (B) AKT phosphorylation (C) GSK-3β phosphorylation (D) eIF2Bε phosphorylation (E) mTOR phosphorylation (F) p70-S6K1 phosphorylation (G) 4E-BP1 phosphorylated-isoform distribution were quantitatively assessed. (B-G group size was n=6-9 for both WT and MGSK-3β KO). Averages ± SEM are presented, *: Wild-type ctrl *vs.* MGSK-3β KO at that specific time point, †: indicates time effect compared to RL-0 (HS) for Wild-type ctrl and #: indicates time effect compared to RL-0 (HS) for MGSK-3β KO; 1 symbol equals p<0.05, 2 symbols equal p<0.01, and 3 symbols equal p<0.001.

RL-2, respectively; Figure 4B right panel), corresponding with the biphasic response of FoXO1 phosphorylation (Figure 3F-G). Autophagy mediator BNIP3 baseline mRNA levels were unaffected by muscle-specific GSK-3β ablation (Figure 4C, left panel). Although HS increased BNIP3 expression in both WT and MGSK-3β KO (13.6±5.7% $p<0.05$ and 64.8±8.8% $p<0.001$, respectively), this resulted in a genotypic significantly different response ($p<0.001$; Figure 4C left panel). BNIP3 expression transiently increased during initial RL for WT and MGSK-3β KO (RL-1: 1.4±0.1-fold $p<0.01$ and 1.2±0.1-fold, respectively), but subsequently decreased until RL-5 for both genotypes (>1.9-fold $p<0.001$). However, BNIP3 expression decreased more rapidly in MGSK-3β KO compared to WT on RL-2 and RL-3 ($p<0.05$ and $p<0.001$, respectively; Figure 4C right panel).

### 3.5 Rapid induction of cell proliferation upon muscle reloading is not affected by GSK-3β ablation

To assess whether HS-RL induced muscle remodeling involves satellite cell proliferative responses which are affected by muscle-specific GSK-3β ablation, Cyclin D1 and PCNA gene expression levels were determined in gastrocnemius muscle. Although at baseline (RL- −14) Cyclin D1 expression was ~1.5-fold ($p<0.05$) lower in MGSK-3β KO compared to WT, HS decreased Cyclin D1 expression for both WT and MGSK-3β KO to comparable levels (2.2±0.2-fold; $p<0.001$ and 1.4±0.1-fold, respectively; Figure 5A left panel). Cyclin D1 levels rapidly increased with RL in both WT and MGSK-3β KO (RL-1: 4.3±0.7-fold $p<0.001$ and 5.0±0.2-fold $p<0.001$, respectively; Figure 5A right panel), and remained elevated throughout RL for both genotypes (~2.5-fold $p<0.01$; Figure 5A right panel). PCNA baseline expression was not significantly altered by HS or genotypically different (Figure 5B, left panel). Similar to Cyclin D1, PCNA gene expression rapidly increased with RL in both WT and MGSK-3β KO (RL-1: 3.5±0.5-fold $p<0.05$ and 3.1±0.6-fold $p<0.05$, respectively; Figure 5B right panel), and displayed a biphasic response. In contrast to Cyclin D1, both WT and MGSK-3β KO PCNA expression levels had returned to RL-0 (HS) comparable levels at RL-5 (Figure 5B right panel). KI-67 protein staining was used to visualize cell proliferation in soleus muscle using immunohistochemistry (Figure 5C). Very few positive nuclei were observed at or baseline (RL- −14) or directly following HS (RL-0). In contrast, striking increases in cellular KI-67 staining appeared on RL-3 and RL-5 (>18-fold; Figure 5D), indicating active cell proliferation in the soleus muscle. However, GSK-3β ablation did not significantly affect the number of proliferating cells.

### 3.6 Absence of GSK-3β enhances myogenesis-associated gene expression upon muscle reloading

To assess whether the increase in cell proliferation was accompanied by a myogenic regenerative response, satellite cell activation and myogenesis were probed. PAX7 expression was not changed after HS for both WT and MGSK-3β KO (Figure 6A left panel). Similar to Cyclin D1 and PCNA, PAX7 transcript levels strongly increased on RL-1 in both WT and MGSK-3β KO (1.6±0.3-fold and 1.9±0.3-fold $p<0.01$, respectively), and decreased at RL-5 (1.5±0.1-fold and 1.9±0.1-fold $p< 0.01$, respectively; Figure 6A right panel) to levels below RL-0 (HS). However, only in WT, PAX7 expression levels progressed with a biphasic response throughout RL (WT *vs.* MGSK-3β KO ~2.5-fold, $p<0.01$ on RL-3; Figure 6A right panel), similar to patterns of Cyclin D1 and PCNA expression. M-cadherin transcript levels were elevated in response to HS only in MGSK-3β KO, resulting in a

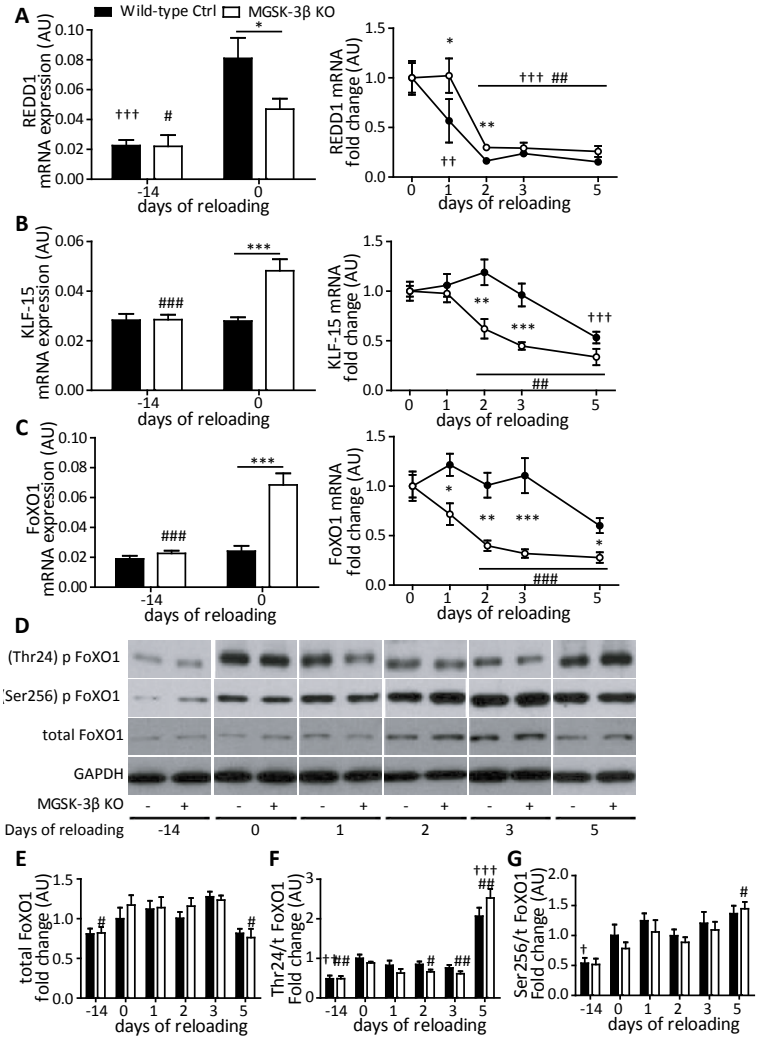


**Figure 3. Differential effects of GSK-3β deletion on changes in expression of glucocorticoid sensitive regulators of protein turnover signaling during muscle unloading and reloading.** M. gastrocnemius was prepared for gene expression analysis and (A) REDD1 (B) KLF-15 and (C) FoXO1 were detected to determine protein degradation signaling status after HS and fold change compared to start reloading (RL-0/HS) gene expression levels during RL. (A-C group size was n=7-9 for both WT and MGSK-3β KO). (D) M. Gastrocnemius was prepared for western blot analysis and indicated total (E) FoXO1, (F) Thr24 and (G) Ser256 FoXO1 phosphorylation were quantitatively assessed to determine protein degradation signaling status. (D-G group size was n=7-8 except for MGSK-3β KO RL-0 n=4 and baseline for both genotypes n=9). Averages ± SEM are presented, *: Wild-type ctrl *vs.* MGSK-3β KO at that specific time point, †: indicates time effect compared to RL-0 (HS) for Wild-type ctrl and #: indicates time effect compared to RL-0 (HS) for MGSK-3β KO; 1 symbol equals p<0.05, 2 symbols equal p<0.01, and 3 symbols equal p<0.001.

difference between WT and GSK-3-KO following HS (RL-0: 1.2±0.1-fold and 1.7±0.1-fold p<0.001, respectively; Figure 6B left panel). RL induced M-cadherin expression changes progressed very similar to PAX7 (Figure 6B and 6A right panels, respectively), including the biphasic increase observed only for WT.

Muscle regulatory factor (MRF) Myf5 increased after HS for both WT and MGSK-3β KO (RL-0: 1.4±0.1-fold p<0.05 and 1.8±0.2-fold p<0.01, respectively; Figure 6C left panel), and did not significantly change during the subsequent RL phase. In contrast to the other two myogenic differentiation associated markers, baseline MyoD expression was lower in MGSK-3β KO compared to WT (RL- −14: 1.6-fold p<0.001; Figure 6D left panel). After HS MyoD expression only significantly increased in MGSK-3β KO (RL-0: 1.8±0.2-fold p<0.001; Figure 6D left panel), abolishing the genotypic difference. MyoD expression transiently increased in WT and MGSK-3β KO on RL-1 (2.3±0.5-fold p< 0.01 and 2.9±0.2-fold p<0.001, respectively), which was slightly more pronounced in MGSK-3β KO compared to WT muscle (1.3-fold p<0.05; Figure 6D right panel). Myogenin expression appeared slightly increased in WT and MGSK-3β KO muscle following HS (RL-0: 1.5±0.2-fold p<0.05 and 1.6±0.3-fold p=0.052, respectively; Figure 6E left panel). RL induced increases in myogenin expression followed a biphasic pattern in both WT and MGSK-3β KO, with marked induction on RL-1 (2.6±0.3-fold p<0.01 and 5.5±0.6-fold p<0.001, respectively; Figure 6E right panel), which was also more pronounced in MGSK-3β KO (2.1-fold p<0.01; Figure 6E right panel). Altogether these data revealed subtle increases in the early myogenic differentiation response during RL in regenerating muscle of MGSK-3β KO compared to WT mice.



### 3.7 GSK-3β deficiency does not prevent disuse-induced atrophy in M. soleus

Considering these consistent subtle effects of GSK-3β ablation on muscle unloading-reloading induced remodeling in M. gastrocnemius, GSK-3β-dependency of loading-induced remodeling was finally evaluated in the small, but very load-sensitive M. soleus. In line with fiber-specific recombination of the floxed GSK-3β alleles, GSK-3β protein content was ~40% decreased in MGSK-3β KO compared to WT mice M. soleus (Figure 7A). After HS, soleus muscle weight decreased comparably in WT and MGSK-3β KO (52.1±3.5% p<0.001 and 58.5±3.2% p<0.001, respectively; Figure 7B), in line with the observations in M. gastrocnemius weight loss (Figure 1C). Correspondingly, compared to baseline, soleus muscle fiber cross sectional area (CSA) decreased after HS in both WT and MGSK-3β KO mice (36.2±3.3% p<0.01 and 51.6±3.1% p<0.001; Figure 7C). The CSA reduction following HS was more evident in M. soleus of MGSK-3β KO compared to WT (RL-0: -14% p<0.05; Figure 7C-D) following HS, which was further reflected in a slightly more leftward shift in fiber size distribution for MGSK-3β KO compared to WT (Figure 7E).

### 3.8 Increased reloading-induced recovery of mass and fiber cross sectional area in GSK-3β deficient soleus muscle

Soleus muscle mass increased during RL for both MGSK-3β KO and WT. However, this increase occurred more rapidly in MGSK-3β KO compared to WT mice (RL-1 *vs.* RL-0: 20.6±3.8% p<0.05, and 2.8±5.2% n.s., respectively; KO *vs.* WT: p<0.05), and was more pronounced in MGSK-3β KO muscle (RL-5: 43.7±6.4% p<0.001, and 28.9±5.2%

p<0.01, respectively; MGSK-3β KO *vs.* WT: p<0.05, Figure 8A). Remarkably, even after five days of RL, soleus muscle mass recovery was not accompanied by a comparable increase in muscle fiber CSA for either genotype (Figure 8B). Nevertheless, during RL there was a trend towards increased fiber CSA compared to HS, but only in MGSK-3β KO *vs.* WT after five days of RL (15.8±3.6% p=0.086 *vs.* -5.9±6.8% p=0.291, respectively; Figure 8B). GSK-3β levels in M. soleus were only reduced by ~40% (Figure 7A). As this may reflect fiber type-specific recombination as a consequence of the MLC-1f promoter driven Cre-recombinase expression, CSA change during HS-RL was determined in analyses of individual fiber types according to slow or fast twitch myosin heavy chain (MHC) composition. Based on MHC-1 or -2A-immunoreactivity, these were divided into MHC-1, MHC-2A, hybrid MHC-1/MHC-2A or negative (MHC-2B and/or MHC-2X) fibers. Fiber type distribution was largely preserved in WT and MGSK-3β KO soleus muscle, and consisted mainly out of MHC-1 and -2A fibers (Figure 8C). MHC-1 fiber CSA did not change during RL in WT muscle, whereas a trend to increase was observed in MGSK-3β KO (RL-5: 0.1±8.5% p=0.808 *vs.* 14.3±5.9% p=0.086, respectively; Figure 8D left panel). Importantly, only in MGSK-3β KO muscle, MHC-2A fiber CSA significantly increased during RL (RL-5: 2.7±7.6% *vs.* 20.6±2.7% p<0.05, respectively; Figure 8D right panel), and



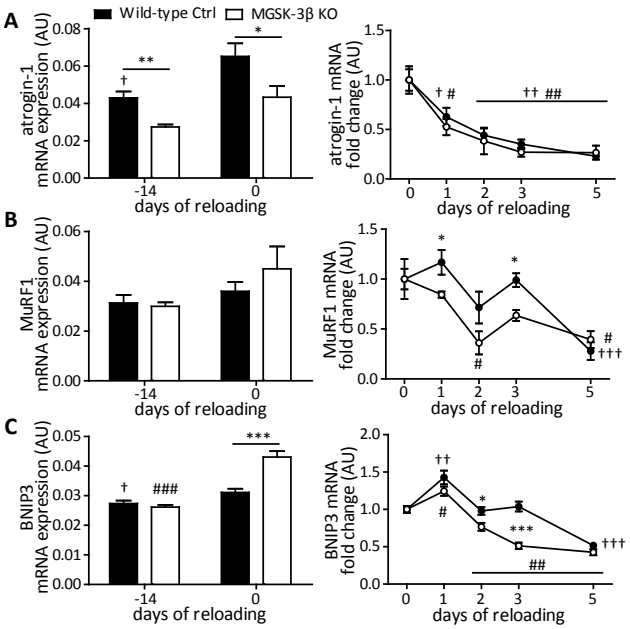


**Figure 4. Expression of proteolysis mediators is decreased during muscle reloading and differentially affected by absence of GSK-3β.** M. gastrocnemius was prepared for gene expression analysis and (A) atrogin-1 (B) MuRF1 and (C) BNIP3 were detected to determine possible effects of protein degradation signaling status after FoXO1 phosphorylation changes due to HS and fold change compared to start reloading (RL-0) gene expression levels during RL. (A-C group size was n=7-9 for both WT and MGSK-3β KO). Averages±SEM are presented, *: Wild-type ctrl *vs.* MGSK-3β KO at that specific time point, †: indicates time effect compared to RL-0 (HS) for Wild-type ctrl and #: indicates time effect compared to RL-0 (HS) for MGSK-3β KO; 1 symbol equals p<0.05, 2 symbols equal p<0.01, and 3 symbols equal p<0.001.

MHC-2A fiber CSA at this timepoint was significantly ($p<0.05$) increased in MGSK-3β KO compared to WT muscle. This difference in recovery of MHC-2A fiber CSA during RL is further illustrated by the clear rightward shift in fiber size distribution between RL day 0-3-5 in MGSK-3β KO, but not for WT soleus muscle (Figure 8E). These data underscore the subtle, but consistent effects of GSK-3β deficiency indicative of accelerated muscle regeneration during recovery from disuse-induced muscle atrophy.



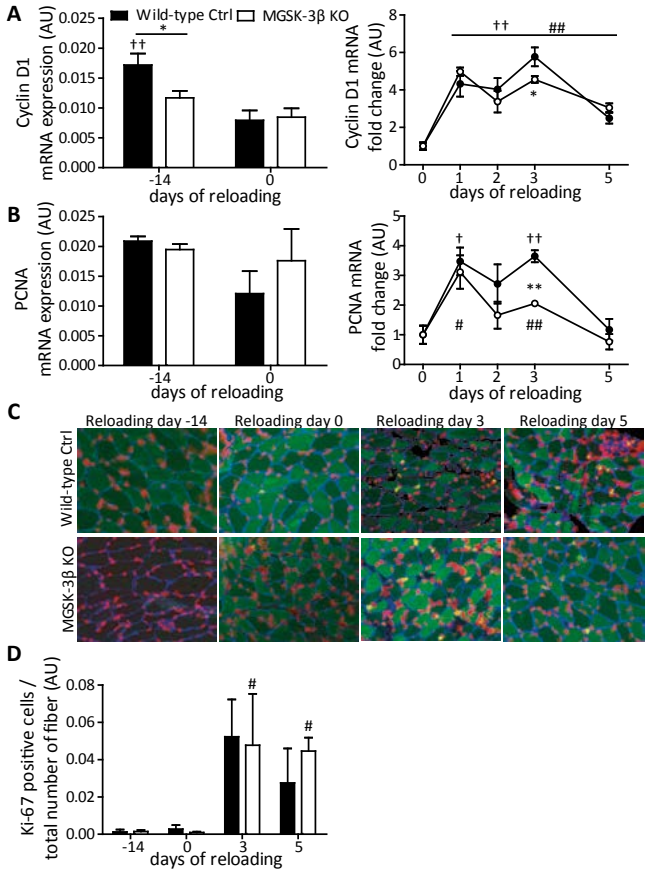


**Figure 5. Rapid induction of cell proliferation upon muscle reloading is not affected by GSK-3β ablation.** M. gastrocnemius was prepared for gene expression analysis and (A) Cyclin D1 and (B) PCNA were detected to determine cell proliferation status due to HS and fold change compared to start reloading (RL-0) gene expression levels during RL. (A-B group size was n=7-9 for both WT and MGSK-3β KO). Histologically Ki-67 protein positive nuclei were determined in (C) M. soleus RL- −14 (baseline), RL-0 (HS), RL-3 and RL-5 and (D) quantified as number of positive nuclei over total number of fibers thereby identifying proliferating nuclei per section. (C group size was n=2-4 for both WT and MGSK-3β KO). Averages ± SEM are presented, *: Wild-type ctrl *vs.* MGSK-3β KO at that specific time point, †: indicates time effect compared to RL-0 (HS) for Wild-type ctrl and #: indicates time effect compared to RL-0 (HS) for MGSK-3β KO; 1 symbol equals $p<0.05$, 2 symbols equal $p<0.01$, and 3 symbols equal $p<0.001$.

# 4 DISCUSSION

In this study muscle specific deletion of GSK-3β (MGSK-3β KO) was used to uncover functions in muscle mass modulation that are either non-redundant with GSK-3α, or depend on total GSK-3 levels. The main hypothesis that muscle mass recovery of atrophied muscle is accelerated in absence of GSK-3β was tested in a reversible disuse-induced muscle atrophy model. Reloading-associated changes in muscle protein turnover were not affected by the absence of GSK-3β. However, the extent and kinetics of satellite cell activation, proliferation and myogenic differentiation during reloading revealed coherent effects of GSK-3β absence, suggestive of accelerated myonuclear accretion. This was accompanied by a subtle but consistent increase in reloading-induced muscle mass accretion in GSK-3β KO muscle. Combined, these data reveal a role for GSK-3 in muscle regeneration-associated myogenesis and myonuclear accretion, independent of regulation of muscle protein turnover, during recovery of disuse-atrophied muscle.

Although the main hypothesis addressed in this work concerned the role of GSK-3 in muscle regeneration following unloading-induced muscle atrophy, previous work by others [102] demonstrated myotube hypertrophy in response to GSK-3 inhibition. In line, muscle-specific over-expression of IGF-I [64] correlates with GSK-3β inactivation and muscle hypertrophy [103], suggesting that abrogation of GSK-3β may induce muscle mass and myofiber hypertrophy. Therefore, the effect of genetic ablation of muscle GSK-3β was first characterized under basal conditions.



Except for the anticipated decreased phosphorylation of eIF2Bε, no overt differences were detected in markers of protein turnover or cell proliferation and myogenic differentiation at baseline in absence of GSK-3β. Nonetheless, MGF expression was slightly reduced in MGSK-3β KO muscle. Little information is available on the regulation of MGF transcript levels, and no previous report on effects of GSK-3 modulation on MGF expression is available. This decrease correlated with reduced Akt phosphorylation, which may suggest attenuated *in vivo* auto- paracrine signaling by this growth factor. Conversely, in a report by Ochi *et al.* increased MGF mRNA expression was accompanied by elevated Akt phosphorylation in response to exercise [104]. Although Akt signaling is inversely related to atrogene expression [105], atrogin-1 levels were lower despite reduced Akt phosphorylation in MGSK-3β KO mice. This is in line with *in vitro* studies revealing that in absence of GSK-3β, the induction of protein degradation signaling resulting from Akt inhibition is markedly attenuated [75], and implicates atrogin-1 expression regulation by GSK-3β downstream of Akt.

As reduced Akt phosphorylation and atrogin-1 expression were accompanied by decreased Cyclin D1 and MyoD levels in MGSK-3β KO muscle, it is tempting to speculate that basal protein turnover and myonuclear turnover are lowered in GSK-3β ablated muscle. As atrogin-1 targets MyoD for degradation [106], lowered atrogin-1 expression may result in decreased turnover rates of MyoD protein, requiring lower MyoD mRNA expression levels maintain MyoD protein levels. Although skeletal muscle weights did not significantly differ, there was a subtle but consistent tendency towards increased muscle mass and myofiber CSA in the reloading soleus muscle, which was more outspoken in the MHC-2A than MHC-1 fibers. Such increase in fiber CSA in the GSK-3β KO muscle could be the consequence of increased sacromere formation, as IGF signaling and GSK-3β inactivation stimulate this process [107]. However, it remains to be determined whether these changes translate into improved recovery of muscle function:.in myostatin-

depleted muscle, hypertrophy was evident but this was not accompanied by increased muscle force generation [108]. Overall, these findings are in line with a previous study in these mice in which no overt phenotypical differences on muscle and whole body mass were observed under baseline conditions [81].

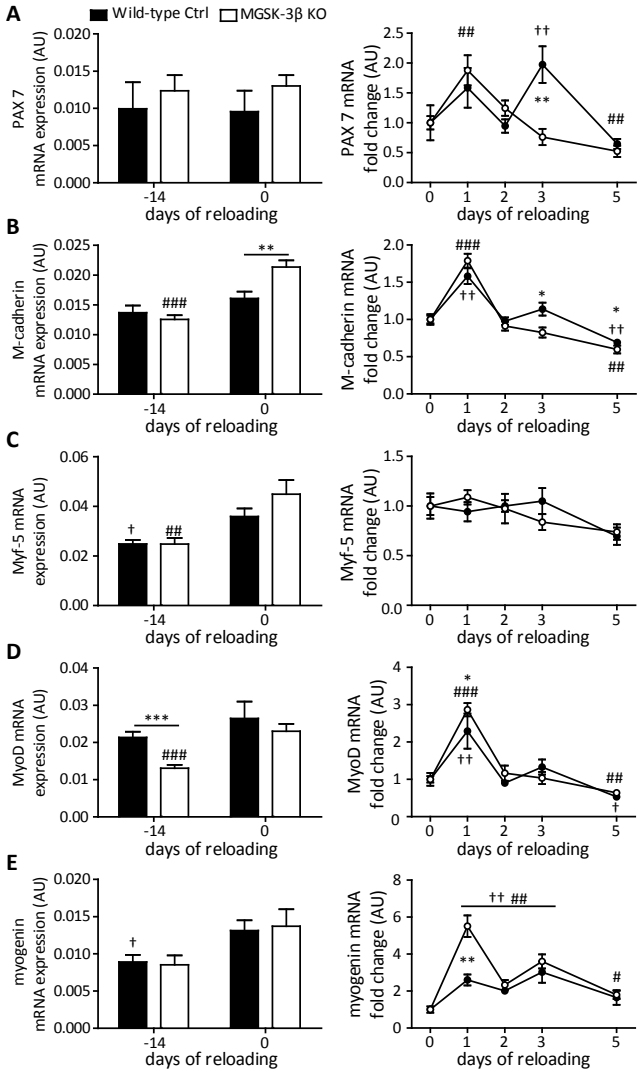


**Figure 6. Absence of GSK-3β enhances myogenesis-associated gene expression upon muscle reloading.** M. gastrocnemius was prepared for gene expression analysis and (A) PAX7, (B) M-cadherin, (C) Myf5, (D) MyoD and (E) myogenin were detected to indicate satellite cell proliferation and myogenic differentiation due to HS and fold change compared to start reloading (RL-0) gene expression levels during RL. (A-E group size was n=8 except for MGSK-3β KO RL-5 n=7 and baseline for both genotypes n=9). Averages ± SEM are presented, *: Wild-type ctrl *vs.* MGSK-3β KO at that specific time point, †: indicates time effect compared to RL-0 (HS) for Wild-type ctrl and #: indicates time effect compared to RL-0 (HS) for MGSK-3β KO; 1 symbol equals $p<0.05$, 2 symbols equal $p<0.01$, and 3 symbols equal $p<0.001$.

Inhibition of enzymatic activity or expression of GSK-3(β) conveyed resistance to glucocorticoid-induced myotube atrophy in previous studies [75, 109]. In contrast, unloading-induced gastrocnemius or soleus muscle mass loss was not prevented nor alleviated in MGSK-3β KO mice after 14 days of HS. In fact, myofiber atrophy, based on CSA analysis appeared even slightly greater in GSK-3β deficient muscle. Discrepancies between muscle weight- and fiber CSA loss have also been observed by others [110], and although these have not convincingly been addressed, differences in interstitial fluid accumulation may contribute to this phenomenon [111, 112]. That GSK-3β absence did not prevent disuse-induced muscle (fiber) atrophy was contrary to our expectations. Redundancy between GSK-3α and -β may account for these results as GSK-3α levels were unaffected in MGSK-3β KO. However, Pierno *et al.* showed that muscle-specific IGF-I over-expression, despite resulting in muscle hypertrophy, did neither protect nor alleviated disuse-induced muscle atrophy from HS, compared to control [113], indicating that inactivation of GSK-3, subsequent to increased IGF-I signaling, is not sufficient to prevent disuse-induced muscle atrophy. Overall, no consistent alterations in regulation of protein or myonuclear turnover were observed in muscles of MGSK-3β KO compared to WT mice following HS; in fact only minor changes –including modestly increased atrogin-1, BNIP3 and REDD1 levels were observed in HS WT muscle compared to baseline conditions, likely reflecting a new balance in aforementioned processes associated with stabilized muscle mass following HS [114].



Nonetheless, at day two of HS, BW loss was significantly less in MGSK-3β KO compared to WT mice. During the initial phase of HS, a strong reduction in food consumption likely contributed to decrease in BW. This semi-starvation, in combination with subjection to HS itself, likely increases stress hormone release, leading to increased muscle glucocorticoid signaling [115] and subsequent muscle atrophy [116]. Interestingly, as glucocorticoid-induced muscle atrophy can be abrogated by inhibition [109] or ablation of GSK-3β in muscles (unpublished data), the attenuated loss of BW observed in MGSK-3β KO mice, may reflect prevention of GC-induced muscle atrophy during the initial stages of HS. However, expression of GC-sensitive genes KLF-15 and FoXO1 [98, 100] were not affected at the end of HS in WT muscle, indicating an unlikely contribution of GR signaling to disuse-induced muscle atrophy throughout HS. In line with this, denervation-induced muscle atrophy does not require GR-signaling [117]. Interestingly, this suggests overlapping GSK-3β and GR-dependency for distinct atrophy-inducing cues.

Based on previous *in vitro* studies by our group and others, we hypothesized that GSK-3β absence would stimulate muscle mass recovery from atrophy. In line with our hypothesis, soleus muscle mass and fiber CSA regain of MGSK-3β KO mice were enhanced compared to WT after five days of reloading. Conversely, the modest changes in gastrocnemius muscle weight were similar for both genotypes. This may be the consequence of the difference in sensitivity of soleus versus gastrocnemius to unloading-induced muscle atrophy leading to a greater decrease in muscle mass in soleus; therefore allowing detection of significant increase only in soleus muscle mass as a consequence of a more robust response to reloading. However, regeneration-associated changes reflecting reloading-induced myonuclear accretion were detected in gastrocnemius muscle that are more pronounced in MGSK-3β KO compared to WT.

Unexpectedly, no discernable increase in soleus fiber CSA was observed in WT and only a slight increase for MGSK-3β KO was observed after five days of reloading, despite clear increasing muscle weights for both genotypes. Similar discrepancies between muscle mass and fiber CSA changes have been observed in rats [118] and mice [110]

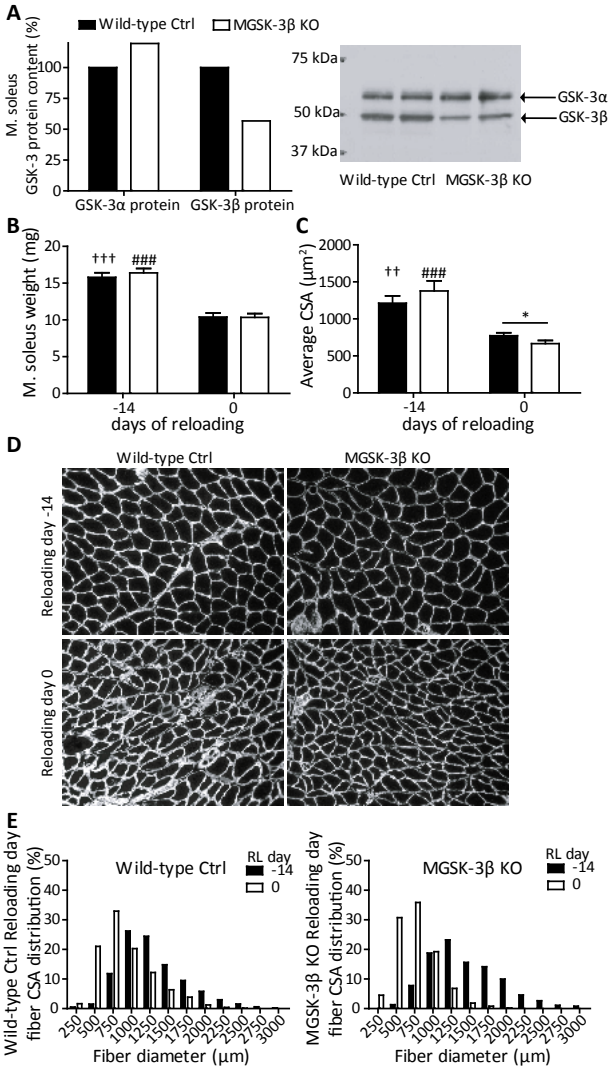


**Figure 7. GSK-3β deficiency does not prevent disuse-induced atrophy in M. soleus.** (A) M. soleus protein lysates were subjected to Western blot and GSK-3β knock down levels and GSK-3α expression levels were assessed (group size for WT/MGSK-3β KO n=2). (B) Paired M. soleus weight loss muscle and (C) CSA change after HS. (D) Depicted CSA changes are (E) specified for fiber size distribution change for each genotype. (B-E group size for WT and MGSK-3β KO time points RL--14 and RL-0 n=8 and 5 and n=8 and 7, respectively). Averages ± SEM are presented, *: Wild-type ctrl *vs.* MGSK-3β KO at that specific time point, †: indicates time effect compared to RL-0 (HS) for Wild-type ctrl and #: indicates time effect compared to RL-0 (HS) for MGSK-3β KO; 1 symbol equals $p<0.05$, 2 symbols equal $p<0.01$, and 3 symbols equal $p<0.001$.

subjected to hindlimb unloading and reloading and could be contributed to interstitial fluid accumulation during muscle regeneration [111, 112]. This may in particular have contributed to very early changes in muscle mass observed after reloading, as increases in myofibrillar protein content were not detectable prior to 5 days of reloading. In line, separate analysis of MHC-1 and MHC-2A muscle fibers revealed a moderate but

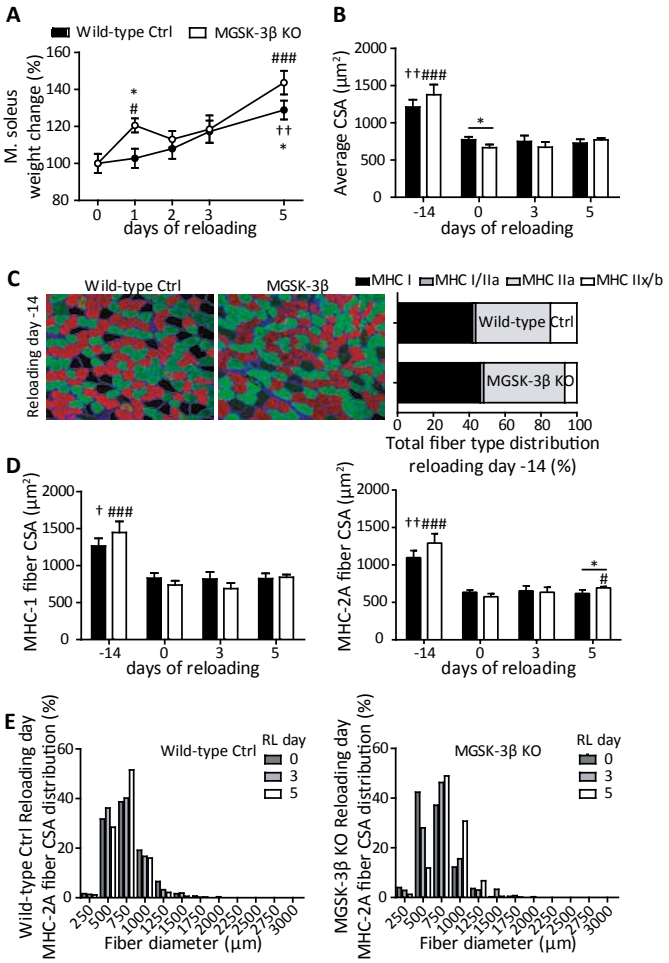


**Figure 8. Increased reloading-induced recovery of mass and fiber cross sectional area in GSK-3β deficient soleus muscle.** (A) Paired M. soleus weight as a percentage increase compared to start reloading weight (RL-0) (group size was n=8 except for MGSK-3β KO RL-5 n=7). (B) CSA change after HS and during RL. (C) M. soleus CSA histological fiber type distribution with MHC-1 (red) and MHC-2A (green), and MHC-2X/B (black) and Laminin (blue), and represented as percentile distribution per genotype. (D) CSA change determination dependent on fiber type MHC-1 (left) and MHC-2A (right). Herein a (E) specification in MHC-2A fiber size distribution change for each genotype. (B-E group size for WT and MGSK-3β KO time points RL- -14, 0, 3 and 5 n=8, 5, 5, 7 and n=8, 7, 7, 6). Averages ± SEM are presented, *: Wild-type ctrl *vs.* MGSK-3β KO at that specific time point, †: indicates time effect compared to RL-0 (HS) for Wild-type ctrl and #: indicates time effect compared to RL-0 (HS) for MGSK-3β KO; 1 symbol equals p<0.05, 2 symbols equal p<0.01, and 3 symbols equal p<0.001.

significant increase in MHC-2A fiber CSA within MGSK-3β KO soleus muscle only after 5 days of reloading. As Cre-mediated recombination for GSK-3β ablation is directed by the MyLC-1f promoter, which is most prominently expressed in MHC-2 type fibers, this may explain the selective increase in MHC-2A but not MHC-1 in MGSK-3β KO muscle [119]. Overall these fiber CSA data are in line with the observed accelerated muscle mass gain during RL in absence of GSK-3β.

MGF was up-regulated in response to RL, which is in line with its postulated role in local muscle repair, maintenance and remodeling by association with activation of satellite cell and IGF signaling [62, 63]. This initial increase in MGF expression is enhanced in MGSK-3β KO muscle. Signaling by IGF-I influences protein synthesis [15, 20-22]. Initiation of reloading (RL-1) showed a transient increase Akt phosphorylation in WT muscle, in line with literature [78, 120]. However, in contrast to our previous findings in soleus muscle [78], a significant increase in GSK-3β phosphorylation was only observed in the later phase of RL (RL-5) in the gastrocnemius muscle. The latter is consistent with the attenuated responsiveness of GSK-3β phosphorylation in plantaris muscle [78], and could be related to the extent of atrophy a muscle is recovering from during RL. GSK-3β phosphorylation changes did not correlate with Akt phosphorylation, except for RL-5, but was inversely associated with eIF2Bε phosphorylation in WT muscle, according to literature [88]. Despite the absence of consistent changes in gastrocnemius GSK-3β or eIF2Bε phosphorylation during RL in WT muscle, both were clearly decreased in MGSK-3β KO muscle, which may have yielded a more permissive state for muscle regeneration-associated changes in protein turnover and myonuclear accretion. In addition, this also suggests a major contribution of GSK-3β relative to GSK-3α in the phosphorylation of this GSK-3 substrate, which is in line with previous estimations of the relative expression of GSK-3α and -3β [121].

In line with Akt, mTOR phosphorylation markedly increased with RL initiation, which was expected [120]. Phospho-mTOR levels remained moderately up-regulated during the course of RL, in line with the importance of mTOR related signaling for muscle recovery from disuse induced muscle atrophy previously shown by Lang *et al.* [122]. Similarly following literature, increased 4E-BP1 and S6K phosphorylation reflected mTOR activity and persisted throughout RL [120, 122, 123]. Correspondingly, REDD1 expression, a negative regulator of mTORC1 signaling [15, 20, 97, 124, 125] decreased rapidly during RL. REDD1 expression decreased more sharply in WT compared to MGSK-3β KO muscle. This likely resulted from the elevated REDD1 mRNA levels in WT at the end of HS, as mTOR-, 4E-BP1- and S6K phosphorylation were not affected by GSK-3β absence. This suggests GSK-3β presence is not a limiting factor of translation initiation or capacity during RL-induced muscle regeneration [15, 20-22]. In line with this, when GSK-3β expression was silenced *in vitro* using a previously described approach [75], basal or IGF-I stimulated puromycine incorporation as a measure of protein synthetic rate was not affected (data not shown). In addition, this also indicates that regulation of S6K phosphorylation by GSK-3β, as described *in vitro* [126], does not apply to reloading muscle, or may point at redundant functions of GSK-3α. Of note, S6K phosphorylation revealed a clear biphasic induction, which did not correlate with changes in mTOR, but rather with Akt phosphorylation. Speculatively, this may signify the initiation of a second phase of protein synthesis, as at RL-5 muscle weights had not recovered to baseline levels. Altogether, these data do not support a non-redundant role of GSK-3β or dose-

limiting effects of GSK-3 in the regulation of protein synthesis signaling during reloading-induced muscle regeneration, although it cannot be ruled out that translation initiation is facilitated in MGSK-3β KO muscle in case eIF2Bε activity becomes rate-limiting under conditions of markedly elevated protein synthesis.

In contrast to protein synthesis, protein degradation is expected to be decreased during RL [122, 127]. FoXO is a major transcriptional regulator of muscle proteolysis [38, 39, 128]. Nevertheless, FoXO protein abundance only decreased after five days of reloading, which may be attributable to decreased transcription of FoXO1, as marked decreases in its mRNA transcript levels were observed; in particular in MGSK-3β KO muscle. As KLF-15 closely corresponded with FoXO1 expression levels, it is tempting to implicate KLF-15 in the transcriptional regulation of FoXO1 during HS and RL. In addition, over-expression of KLF-15 in Tibialis muscle increases FoXO1 expression [98], but no studies previously addressed regulation of KLF-15 or FoXO expression by GSK-3β. Increased $Thr^{24}$ or $Ser^{256}$ phosphorylation levels of FoXO1 corresponded with the second induction of Akt phosphorylation, suggesting initiation of a second phase of protein synthesis with coordinated suppression of FoXO activity. Although these data do not support regulation of FoXO phosphorylation by GSK-3β, it has been implicated in the regulation of FoXO1 transcriptional activity [129]. Genes involved in protein degradation that are transcriptionally regulated by FoXO 9[38, 39, 128] include effectors of UPS- and ALP-mediated proteolysis, i.e. atrogin-1 and MuRF1 [31, 32, 113], and BNIP3 [37, 39, 40], respectively, which are known to increase under muscle atrophying conditions [122]. As expected [122, 127] both atrogin-1 and MuRF1 mRNA expression decreased during RL. Conversely, BNIP3 expression transiently increased, in line with literature [127], and subsequently decreased throughout further RL. Compared to the UPS markers, this differential response of BNIP3 to reloading is corresponding to the notion that autophagy is involved in remodeling and maintenance of skeletal muscle [130]. Although the reduction of MuRF1 and BNIP3 levels were slightly accelerated in MGSK-3β KO muscle, overall no defining role of GSK-3β absence on reloading-induced suppression of effectors of proteolysis was apparent.

These data show for the first time that muscle mass gain in response to disuse-induced muscle atrophy reloading not only involves increases in protein synthesis signaling, but that reloading is also accompanied by marked down-regulation of protein degradation signaling. Overall, these changes in protein turnover regulation were unaffected by absence of GSK-3β.

In parallel to the balance between muscle protein synthesis and degradation, myonuclear turnover may also contribute to muscle mass regulation [10]. Myonuclear accretion during muscle regeneration involves satellite cell activation, proliferation, and differentiation and fusion. Quiescent satellite cells are activated in response to muscle injury and/or exercise stimulation leading to muscle recovery [42-44]. In line with literature, PAX7 expression increased in response to exercise/muscle loading [50-52]. Activated satellite cells, once committed to myogenic differentiation become myoblasts, which fuse with existing myofibers [55] through cell-cell interaction involving M-cadherin [56-58]. Indeed, RL was accompanied by increased expression of M-cadherin. Remarkably, the biphasic induction of PAX7 and M-cadherin required GSK-3β, as only a single but more pronounced increase in their expression was observed in MGSK-3β KO muscle.

Consistent with literature, RL rapidly induced cell proliferation markers Cyclin D1, PCNA and KI-67 in reloading muscle [110, 131-133], including a second increase on RL-3, which may signify a secondary round of myogenic cell proliferation during muscle regeneration. This matches previous reports on myogenic differentiation changes with a biphasic or greater pattern [134, 135]. Although stimulating effects of GSK-3 inhibition on myogenic differentiation and myotube formation have been documented [79, 136], it remained to be established whether GSK-3β ablation affects myoblast proliferation. GSK-3β suppresses Cyclin D1 expression as reviewed by Takahashi-Yanaga and Sasaguri in cancerous cells [137], whereas it is required for PCNA content in vascular smooth muscle cells [138]. This disparity in regulation may reflect a GSK-3β dependency that is determined by cellular context. In the reloading muscle, expression patterns of Cyclin D1 and PCNA changed coordinately, and were impacted similarly by absence of GSK-3β: GSK-3β ablation blunted the secondary expression increase of both proliferation markers. Importantly, the identical GSK-3β-dependency on biphasic increases in satellite cell activation and proliferation markers during RL supports the notion that the latter reflects dividing myoblasts and no other resident cell-types. Moreover, since GSK-3β suppression did not directly affect expression of proliferation markers but rather promoted myogenic differentiation of cultured myoblasts [136], the relative suppression of PCNA and Cyclin D1 expression at day 3 may point at accelerated myogenesis in absence of GSK-3β.



Subsequent myogenic differentiation of myoblasts includes increased muscle-specific gene expression, regulated by the MRFs Myf-5, MyoD and myogenin [59, 60]. Myf-5 expression, suggested to be involved in satellite cell proliferation [139], was unaffected by RL or GSK-3β absence. MyoD and myogenin increase during RL, which is in line with literature [120, 132]. Interestingly, early up-regulation of MyoD and especially myogenin was markedly increased in MGSK-3β KO compared to WT regenerating muscle, which is consistent with stimulation of myogenic differentiation in response to GSK-3 inhibition observed in a previous *in vitro* report [65].

Of interest, satellite cell activation and myoblast proliferation revealed a biphasic response, which preceded the secondary rise in protein synthesis signaling, i.e. increased phosphorylation of Akt, and S6K on RL-5, suggesting coordinated control of muscle protein synthesis and proliferation signaling. This possible second rise of satellite cell activation and proliferation markers was absent in MGSK-3β KO muscle, whereas the initial rise in MGF expression and myogenic differentiation markers were clearly more pronounced in MGSK-3β KO muscle. This may suggest that a second round of satellite cell activation and proliferation may not be required in MGSK-3β KO muscle as a consequence of more extensive myogenic differentiation of the initially activated and recruited satellite cells. In agreement with this notion, muscle mass and myofiber CSA increases were not blunted, but even slightly enhanced in MGSK-3β KO muscle. However, this could have potential implications for replenishment of the quiescent satellite cell pool, considering the requirement for asymmetrical cell division postulated to confer sustained myogenic potential of skeletal muscle in muscle mass maintenance [140]. In congruence with this idea, the absence of GSK-3α was recently postulated to accelerate aging of multiple tissues including skeletal muscle [141].

All the potentiating effects of GSK-3β deletion on initial stages of molecular myogenic differentiation, reloading-induced muscle mass and myofiber CSA recovery were subtle. However, this does not rule out further attention for GSK-3 as a nexus in

skeletal muscle mass plasticity. We previously reported that reloading of disuse-induced atrophied soleus muscle mass recovery is accompanied by increased inactivating GSK-3β phosphorylation [78]. Under physiological healthy conditions reloading may suppress GSK-3 activity levels sufficiently to accommodate muscle recovery. However, whether such reduction in GSK-3 activity is required for efficient recovery remains to be addressed; especially as aberrant GSK-3 regulation has been associated with impaired muscle mass plasticity [142-144]. Moreover, putative beneficial effects of modulating GSK-3 activity may vary between different triggers of muscle atrophy, and subsequent recovery conditions. To illustrate this, the systemic delivery of a GSK-3β inhibitor was recently found to attenuate muscle atrophy in a model of chronic pulmonary inflammation, and improve cytokine- or glucocorticoid-induced inhibition of myogenic differentiation [145].

In conclusion, GSK-3β deletion potentiates initiation of the molecular response underlying myonuclear accretion, resulting in enhanced muscle mass and fiber CSA gain. This study therefore suggests that GSK-3β specific, non-redundant functions with GSK-3α, or total GSK-3 (activity) levels suppress muscle regeneration during recovery of disuse-atrophy.

6

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# Chapter 7

## General discussion

## PROLOGUE AND MAIN FINDINGS

In the 'Western world' average life expectancy is increasing and birth rates decreasing, leading to an aging population. Concurrently more people suffer from one or more chronic diseases [1, 2], and the prevalence of cancer is also rising [3-5], causing an explosive increase in healthcare costs [6-9]. This economic burden is likely unsustainable if there are no advancements in improving both prevention and treatment strategies. Chronic diseases and cancer are diverse in aetiology but in both conditions patients are at risk of developing muscle weakness and wasting, which increases with disease severity and aggravates disease burden [10-14]. Muscle wasting decreases physical performance, quality of life, reduces efficacy of medical interventions and increases morbidity and mortality [15]. Prevention or reversal of muscle wasting is therefore seen as an unmet medical need [16].

To date, multimodal intervention programs including supervised (strength) training and nutritional support are the only well-documented strategies to partially or temporarily reverse muscle atrophy in certain populations [17-19]. Anabolic agents, such as growth hormone, insulin-like growth factor (IGF) and testosterone or analogues thereof [17, 20-22] have previously been investigated to enhance muscle mass gain by stimulating the IGF-I/Akt signalling pathway. Increased IGF-I/Akt signalling reduces proteolysis and increases protein synthesis, proliferation and muscle-specific gene expression [23-25]. However, variable efficacy and pharmaceutical side effects [21, 26, 27] clearly indicate that there is room and need for improvement of our understanding of the processes involved in muscle mass plasticity regulation as well as for more effective pharmacological target identification. Creutzberg *et al.* showed that patients receiving concurrent systemic glucocorticoid treatment were more responsive to the anabolic treatment compared to the group that were not. This intriguing difference in responsiveness to the anabolic stimulation of IGF/Akt signalling was further investigated in **CHAPTER 2** by *in vitro* research focusing on cellular signalling mechanisms that could be involved. We found that *in vitro* stimulation of myogenesis with IGF-I was enhanced in the presence of glucocorticoids, possibly reflecting the putatively synergistic interaction between the glucocorticoid treatment and anabolic treatment regimen of the patients. Considering the opposing effects of glucocorticoids and IGF-I on muscle protein turnover, this is a surprising finding and further showcases the necessity of not only focussing on the primary effects of utilized stimuli but, also looking at their generated side effects like in the case of glucocorticoids increasing muscle-specific gene expression [28], which could be translated into protein through IGF-I stimulated protein synthesis.

Muscle mass plasticity results from a balance between muscle protein synthesis and breakdown, and a balance in myonuclear accretion and loss. Elucidating master regulatory molecules of these processes is essential in identifying preferential targets to modulate muscle mass. Past (J. van der Velden thesis 2008) and current research results from our research group point towards GSK-3 as a potential target for pharmaceutical intervention to 1) prevent or abrogate muscle mass wasting (K. Verhees thesis 2013) and 2) improve muscle mass regeneration and recovery (this thesis).

Our *in vitro* work and recently also our *in vivo* work shows that modulation of GSK-3 activity affects skeletal muscle mass plasticity. In cultured differentiated muscle cells, myotube atrophy resulting from atrophic stimuli like glucocorticoids or interfering with IGF-I/Akt signalling can be mitigated by modulation of GSK-3 activity [29]. We

furthermore have shown that inactivation of GSK-3 results in improved myogenic differentiation from myoblasts to myotubes which involves two different signalling pathways. IGF-I mediated inactivation of GSK-3 [30] causes de-repression of NFATc3 transcriptional activity [31] and stimulates myogenic differentiation. Alongside, canonical Wnt-3a-mediated inactivation of GSK-3, which by phosphorylation marks β-catenin for degrdation, also results in enhanced myotube formation during myogenic differentiation (**CHAPTER 4**). Interestingly, although both IGF-I/Akt and Wnt/β-catenin signalling involve inactivation of GSK-3, their effects on myogenic differentiation differ. Stimulation with IGF-I mainly resulted in enhanced biochemical myogenic differentiation (muscle protein content), whilst stimulation with canonical Wnt resulted in enhanced morphological differentiation (myotube formation) without affecting biochemical myogenic differentiation (**CHAPTER 4**). Pharmacological inhibition of GSK-3 activity results in enhancing both biochemical and morphological aspects of myogenic differentiation [30, 32], suggesting GSK-3 inhibition is sufficient to stimulate *in vitro* myogenesis.

In recent work by our group the effects of GSK-3 inactivation on triggers of muscle atrophy were investigated *in vivo* (K. Verhees thesis) and this showed that it led to an attenuation of muscle proteolysis leading to reduced muscle mass atrophy. However, a functional role for GSK-3 in muscle mass plasticity regulation during muscle mass recovery has scarcely been investigated. Therefore, a major part of the experimental work conducted in thesis was aimed at elucidating the role of GSK-3 in muscle recovery from atrophy *in vivo*. Our group has shown that following disuse-induced muscle atrophy, muscle mass recovery upon reloading is associated with GSK-3β inactivation [33]. To address whether GSK-3 inactivation is required for recovery from unloading-induced muscle atrophy, genetically modified mice ubiquitously expressing constitutively active, instead of endogenous, GSK-3 [34] were employed. Based on evaluation of muscle mass, protein turnover signalling and myogenesis regulatory pathways, constitutively active GSK-3 does not appear to impede the process of reloading-induced muscle mass recovery of disuse-induced atrophied soleus muscle as reported in **CHAPTER 5**. Conversely, to address whether inactivation of GSK-3β stimulates muscle recovery following atrophy, muscle-specific GSK-3β knock-out mice [35] were subjected to the same model of unloading-induced muscle atrophy and reloading-induced muscle mass recovery. Based on our findings we conclude that GSK-3β deficiency is sufficient to stimulate the process of early muscle mass recovery of disuse-induced atrophied muscle as reported in **CHAPTER 6**. The implications of these findings as presented in this thesis (**CHAPTER 2-6**) are integratively discussed below in light of the current literature.



## 1. GSK-3 IN CELLULAR SIGNALLING PROCESSES IMPLICATED IN MUSCLE MASS REGULATION

### 1.1. Cellular processes controlling muscle mass

Skeletal muscle mass plasticity is dependent on alterations in muscle protein and myonuclear turnover. Muscle protein turnover is determined by the balance between protein synthesis and degradation. A change in the balance resulting in net protein accretion is involved in muscle growth (hypertrophy) and muscle mass recovery (re-growth). Regulatory pathways involved in protein synthesis in response to physiological stimuli of muscle (re)growth include the IGF-I/Akt/mTOR and IGF-I/Akt/GSK-3(β) signalling pathways that control the rate-limiting step in protein synthesis, i.e.

mRNA translation [36, 37]. Conversely, when the balance tilts towards net protein loss, muscle atrophy occurs. Muscle proteolysis involves controlled activation of three main cellular proteolytic systems: the ubiquitin 26S-proteasome system (UPS), the autophagy-lysosomal pathway (ALP) and the calcium-dependent calpains [38-42]. These cellular regulatory processes involved in controlling both synthesis and degradation of muscle protein content have been shown to cross talk at multiple levels.

Myonuclear turnover is determined by the balance of loss and accretion of muscle fibre myonuclei. Changes in the rate of myonuclear loss or accretion have been implicated in muscle plasticity by virtue of the myonuclear domain theory [43]. The myonuclear domain is the region of the sarcoplasm a myonucleus is capable of providing with sufficient mRNA to properly function. When reaching the lower limit of the myonuclear domain, myonuclei may disappear from the muscle fibre as their transcriptional capacity becomes redundant. In contrast, when reaching the upper limit of the myonuclear domain, new nuclei provided by muscle precursor cells are incorporated to maintain a functional myonuclear domain [43, 44]. Although the need for myonuclei accretion to achieve muscle mass growth was recently disputed [45-47], myonuclear accretion has been observed during recovery of atrophied muscle [48, 49]. The mechanisms of controlled loss of myonuclei are far from being elucidated, although apoptosis has been proposed as a supporting mechanism. Myonuclear loss has been reported to involve apoptosis signalling and has indeed been observed with both muscle disuse and disease-related muscle atrophy [50-53]. Conversely, the accretion of new myonuclei has been researched more in depth and found to rely on the activation of resident muscle precursor cells, the satellite stem cells located in between the muscle fibres [54, 55]. Upon activation, these stem cells go through cell proliferation and the resulting daughter cell population either return to a quiescent state as satellite cells or continue as myoblasts to differentiate and fuse with muscle fibres [44]. Myonuclear accretion derived from satellite cells has been described during post-natal muscle growth, muscle regeneration and muscle hypertrophy [56-58]

The research in this thesis focused on the effects of GSK-3 activity modulation on *in vitro* myogenesis and *in vivo* muscle mass recovery. In vitro, GSK-3 activity modulation through the IGF-I/Akt and Wnt/β-catenin signalling pathways and their respective effects on myogenic differentiation were investigated. The consequences of *in vivo* genetic modulation of GSK-3(β), activity or expression, on the regulation of protein turnover and myogenic differentiation signalling were addressed in skeletal muscle during conditions in which muscle mass was stable, atrophied or recovering. The involvement of GSK-3 in the cellular processes that determine muscle mass is discussed first, followed by the involvement of GSK-3 in muscle mass maintenance, muscle mass atrophy and muscle mass recovery from disuse-induced muscle atrophy.

## 1.2. GSK-3 in (muscle) protein synthesis signalling

Our group has previously shown that pharmacological inactivation of GSK-3 leads to increased muscle-specific protein content during myogenic differentiation [59]. This was in part attributable to increased protein-synthesis signalling based on the involvement of GSK-3 in the above mentioned cellular signalling cascades. IGF-I can increase muscle protein synthesis [60, 61]. IGF-I-induced protein synthesis signalling involves Akt, and GSK-3 may affect upstream synthesis signalling as activated GSK-3

destabilizes the insulin receptor substrate 1 (IRS-1) thereby reducing Akt signalling [62-64]. However most of this data is the result of *in vitro* studies into the effects of modulation of GSK-3(β) activity in non-muscle cells. In line with these findings, the early stage of muscle reloading, which is accompanied by IGF-I signalling, slightly increased Akt phosphorylation is observed in absence of GSK-3β, while in presence of CA GSK-3 reloading-induced Akt phosphorylation is slightly attenuated.

Further downstream, IGF-I/Akt signalling involves mTORC1 [60, 65] of eIF4E binding protein 1 (4E-BP1) and p70-S6K1 [66] and Akt-mediated phosphorylation of GSK-3 [67], which results in de-repression of GSK-3-mediated inhibitory phosphorylation of eIF2Bε [68, 69]. Combined, the subsequent increased activity of eIF2, eIF4 and S6 promotes mRNA translation. The *in vivo* analyses assessing eIF2Bε confirmed its phosphorylation is subject to GSK-3(β) regulation, but whether its reduced inhibition in absence of GSK-3β was sufficient to stimulate protein synthesis remains to be determined. On one hand, such an effect could be anticipated: GSK-3 is not only directly inactivated by Akt, but also indirectly via a mechanism involving p70S6K and mTORC1 [70]. This overlapping inactivation mechanism suggests tight control as GSK-3 activity is essential for a permissive state to accommodate protein synthesis signalling. Conversely, protein synthesis appears to be controlled by GSK-3 on multiple levels in addition to regulation of eIF2 activity: an 'auto regulatory loop' has been described by which activated GSK-3 in cooperation with adenosine monophosphate-activated protein kinase (AMPK) activates Tuberous sclerosis protein 2 (TSC2) and subsequently inactivates mTORC1 [71],

REDD1 is important in TSC2-dependent inactivation of mTORC1 and REDD1 mRNA expression in reloading muscle inversely correlated with muscle protein synthesis signalling, in line with its postulated suppressive effects on this process [72, 73]. REDD1 mRNA expression is increased in response to GC/GR mediated signalling [74], and GSK-3(β) can affect GC/GR-mediated cellular signalling [75, 76]. In vivo the absence of GSK-3(β) slightly altered REDD1 mRNA expression patterns in a manner that may facilitate protein synthesis. However, inverse regulation was not consistently observed in the presence of CA GSK-3, and the changes in REDD1 mRNA expression were not accompanied by alterations suggesting increased mTOR activity in absence of GSK-3β. Therefore, GSK-3 regulation of REDD1 may be more relevant in conditions of muscle mass plasticity involving GC signalling.

In conclusion, in contrast to eIF2B, the parallel signalling pathways driving eIF4 and S6 activity and abundance were unaltered by absence of GSK-3β or constitutively active GSK-3, and as eIF2 activity is considered permissive but not rate-limiting for mRNA translation, this suggests that the control of protein synthetic rate is not altered by modulation of GSK-3 activity in skeletal muscle *in vivo*.

## 1.3. GSK-3 in (muscle) protein degradation signalling

GSK-3β inhibition leads to increases in myotube size *in vitro* and therefore GSK-3β is labeled as a negative regulator of skeletal muscle mass [77]. Fang *et al.* showed that inactivation of GSK-3 not only allows for increased protein synthesis but also decreased protein degradation [78, 79] thereby implicating GSK-3 activity in proteolysis regulation. Similar observations have been reported by Li *et al.* [66], and findings revealing GSK-3 activity increases with increased catabolism [80, 81] further substantiate its potential regulatory role in proteolysis.

Muscle proteolysis involves controlled activation of the three main cellular proteolytic systems UPS, ALP and the calcium-dependent calpains [38-42] as described in more detail in **CHAPTER 3**. Calpains are calcium-dependent [42, 82] cysteine proteases which are upregulated during catabolic conditions [83, 84] and involved in myofibrillar proteolysis [42, 85, 86]. In muscle, increased calpain activity was associated with decreased Akt activity [87]. Furthermore, calpain-cleaved products undergo UPS-mediated degradation [88], which associates calpain with UPS-mediated proteolysis.

UPS rate-limiting enzymes muscle-specific E3 ligases atrogin-1 (/MAFbx) and muscle RING finger 1 (MuRF1) are upregulated during catabolism [89, 90] and regulate the proteolysis of muscle-specific transcription factors [91], enzymes [92] and myofibrillar proteins [93, 94]. The glucocorticoid Dexamethasone increases UPS activity [95] by a large extent through activation of glucocorticoid receptor (GR) signalling [96] thereby causing muscle atrophy [29, 97]. This GC/GR-mediated increase in expression of atrogin-1 and MuRF1 can be prevented by inhibiting GSK-3 activity [29, 81]. It has also been shown that overexpression of constitutively active GSK-3β alone is sufficient to increase atrogin-1 expression [98], directly linking GSK-3(β) activity with skeletal muscle proteolysis regulation.

Autophagy Lysosome Pathway (ALP)-mediated proteolysis consists of a highly conserved bulk degradation process removing long-lived proteins and dysfunctional organelles [99, 100], and is constitutively active to maintain normal muscle physiology [101]. During catabolic conditions ALP activity increases [102-104]. Genetic ablation or pharmacological inhibition of GSK-3β prevents or attenuates the ALP response, whilst increased expression of GSK-3β further potentiates the ALP response to catabolic stimuli [105]. The involvement of GSK-3(β) in the ALP signalling may come from regulatory involvement related to its ability to phosphorylate TIP60 on $Ser^{86}$, which leads to increased acetylation and kinase activity of ULK1, an important autophagy gene [106].

One main finding of the work described in the *in vivo* studies of this thesis concerns the coordinated decrease in UPS and ALP signalling during muscle mass recovery following disuse atrophy. This decrease appeared transient in nature and may further facilitate protein accretion driven by the increased protein synthesis signalling occurring in the same time frame. Although GSK-3 is temporarily phosphorylated during reloading, this does not appear to be required as a similar transient decrease in proteolytic signalling is present in reloading muscle of CA GSK3 mice. In contrast, in absence of GSK-3β, UPS/ALP gene expression is even further reduced in recovering muscle. This indicates that the GSK-3-dependent transcriptional program responsible for UPS/ALP gene expression in conditions of muscle mass loss [29] may involve the same regulators during muscle mass regain. Therefore, in addition to studies showing that GSK-3 is required for the induction of these proteolytic gene expression following an atrophy response it also indicates that GSK-3 is involved in sustaining proteolytic effector expression levels during muscle mass recovery.

FoXO3a has been shown to regulate transcription of both UPS-related E3 ligases atrogin-1 and MuRF1 and ALP-related genes LC3 and Bnip3 during muscle catabolism [98, 107, 108]. However, FoXO regulation during muscle growth has mostly been postulated to be facilitated through inhibitory phosphorylation by Akt resulting in its nuclear exclusion. In recovering muscle we now demonstrate enhanced suppression of FoXO1 expression levels in absence of GSK-3β, whereas an attenuated decrease at

initiation of reloading and a possible earlier rise after two weeks of reloading is indicated in the presence of CA GSK-3. Although GSK-3 has been shown to interact with FoXO3a resulting in increased FoXO-3a-dependent transcriptional activity [109], regulation of FoXO expression by GSK-3(β) was not described previously. Of interest, KLF-15 [110, 111] which operates as a transcriptional regulator of FoXO and atrogenes, displays a similar expression pattern during muscle recovery. In absence of GSK-3β, FoXO expression is also further reduced, suggesting that proteolytic gene expression during muscle mass remodeling is controlled by KLF15 expression levels, and implicating GSK-3β in its transcriptional regulation.

Cross-talk between UPS and ALP-mediated proteolysis [112] and compensatory redundancy of these pathways have been described [113-115]. Furthermore, as mentioned KLF-15 and FoXO1 are GR-sensitive transcriptional regulators of proteolysis [110, 111]. Glucocorticoid-mediated activation of glucocorticoid receptor (GR) signalling [95, 96] causing muscle atrophy [29, 97] can be prevented by inhibition of GSK-3 activity [29, 81], whilst CA GSK-3β can increase it *in vitro* [98]. GSK-3(β) can directly phosphorylate the human GR and thereby significantly alter the repertoire of GR-regulated gene expression due to this modification [116]. This further indicates a regulatory role of GSK-3(β) in muscle proteolytic signalling.

## 1.4. GSK-3 in myonuclear accretion

As loss of muscle fibre nuclei was not studied in this thesis, only the accretion of new myonuclei is discussed as part of myonuclear turnover here. Muscle fibre myonuclei accretion has been observed during atrophied muscle recovery [48, 49] and involves activation of resident muscle precursor cells, the 'satellite' stem cells located between the muscle fibres [54, 55]. These stem cells, upon activation go through a few cycles of cell proliferation after which part of the daughter-cell population either returns to a quiescent state or differentiate into muscle precursor cells (myoblasts) that then fuse with existing muscle fibres [44, 117]. IGF-I/Akt [60, 118] and Wnt/β-catenin [119-121] signalling have been implicated in all three stages that satellite cells progress through during this myonuclear accretion process. Therefore, a possible key role of GSK-3, as a constituent in both of these cellular signalling pathways, may be anticipated in those processes.

### *1.4.1. Proliferation*

Activated proliferating satellite cells have been shown to express Wnts [122] which can induce Wnt/β-catenin signalling, and thereby preventing GSK-3(β) from marking β-catenin [123, 124] for UPS-mediated degradation [125]. Instead GSK-3 is recruited to the Wnt cellular receptor complex improving signal transduction [126-128]. Accumulated cytoplasmic β-catenin is transported to the cell membrane where it can form a complex with cadherins for cell-cell interactions [129, 130] or to the nucleus where β-catenin can function as a transcriptional co-activator inducing the expression of proliferation-associated genes such as c-Myc and cyclin D1 [131].

In **CHAPTER 4** we have shown that pharmacological inhibition of GSK-3 (using LiCl) results in increased β-catenin-dependent gene transcription in differentiating myoblasts. However, ectopic exposure of myoblasts to Wnt-3a is far more potent in activating β-catenin-assisted transcriptional activation compared to LiCl. A possible explanation

for this difference in responsiveness to Wnt-3a-mediated inactivation of GSK-3 might be the dual role that GSK-3 appears to have in induction of Wnt/β-catenin signalling: although pharmacological inactivation of GSK-3 will reduce β-catenin phosphorylation and subsequent UPS-mediated degradation, it also potentially reduces Wnt-induced signalling, because of the reduction of GSK-3-mediated phosphorylation of the LRP5/6 protein that is involved in the signal transduction process.

Wnt/β-catenin signalling is a potent trigger of proliferative responses [122, 132]. Despite the potent signalling response elicited by Wnt-3a or LiCl, in **CHAPTER 4** no increase in myoblast proliferation was observed. This is possibly related to initiation of myogenic differentiation leading to a general down regulation of cell proliferation markers Cyclin D1 and PCNA expression. This suggests that cell cycle exit preceding the initiation of differentiation is irreversible, and that Wnt/β-catenin signalling feeds into the mitogenic or myogenic program dependent on cell cycle status.

In addition to Wnt/β-catenin signalling, IGF-I is known to stimulate both myoblast proliferation and differentiation [60]. Whereas Wnt/β-catenin signalling was not assessed, IGF-I expression and signalling are increased during early phase of muscle mass recovery, and is accompanied by raised GSK-3 phosphorylation levels (**CHAPTER 5 + 6**), which suggest inactivation of GSK-3 during reloading as observed previously [33].Based on evaluation of Cyclin D1 and PCNA expression levels and KI-67-positive mononuclear cells, reloading-induced muscle mass recovery in our studies (**CHAPTER 5 + 6**) is accompanied by cell proliferation, in line with literature [133-136]. Neither the presence of CA GSK-3 (**CHAPTER 5**) nor the absence of GSK-3β (**CHAPTER 6**) significantly affected this increase in overall cell proliferation. However, histological detection to determine Cyclin D1 or PCNA, as performed for Ki-67, in combination with PAX 7 staining is required to distinguish proliferating satellite cells [137, 138] from other cell types to specify any effects of GSK-3 towards dividing myogenic cells. Interestingly, PAX 7 mRNA levels increase during reloading, and the kinetics by which this occurs is inversely affected by the absence of GSK-3β and presence of CA GSK-3, respectively. As this implies a role for GSK-3 in the regulation of satellite cell activity regulation, further investigation of satellite cell activation and proliferation in recovering muscle is warranted..



### *1.4.2. Myogenic differentiation and fusion*

Myogenic differentiation and fusion of myoblasts with myoblasts or muscle fibres *in vivo* is an essential part of muscle remodeling following recovery from damage evoked by myotoxins [139], and also accompanies muscle mass regeneration responses [140]. At a transcriptional level, myogenic differentiation and myoblast fusion are directed by the expression and activity of the muscle regulatory factors (MRFs), including MyoD, Myf5 and myogenin [30, 141-143]. LiCl, a GSK-3 inhibitor, increases MRF expression in differentiating myoblasts [30] and MRF-mediated transcriptional activity (**CHAPTER 4**), which implicates GSK-3 in the subsequently observed increases in muscle-specific gene expression and myoblast fusion. The unrelated ligands IGF-I and Wnt-3a have in common that part of their signalling pathways involve inactivation of GSK-3. Increasing IGF-I signalling with IGF-I improves myogenic differentiation, which not only involves increasing protein synthesis signalling but also muscle-specific gene expression [30, 144, 145]. We have shown in **CHAPTER 4** that Wnt-3a improves myogenic differentiation, although only on a morphological level, i.e. accelerated and enhanced myotube

formation.

Despite the partial overlapping effects of IGF-I and Wnt-3a on myogenic differentiation, no cross talk in the their signalling pathways involving GSK-3 appears to be present. In previous work our group showed that pharmacological inhibition or genetic ablation of GSK-3β is sufficient to enhance myogenesis [30, 31], and that improved muscle-specific gene expression involves reduced phoshorylation of transcriptional regulator nuclear factor of activated T cells (NFAT) [31, 146, 147] by GSK-3 [148, 149]. NFAT activation is exclusively observed in response to IGF-I and not Wnt-3a (**CHAPTER 4**). Conversely, increased β-catenin-associated TCF/LEF-mediated gene expression is observed in response to pharmacological or genetic inactivation of GSK-3 or Wnt-3a, but not IGF-I.

During reloading of disuse-atrophied muscle, GSK-3β phosphorylation is transiently increased [33]. This likely reflects decreased GSK-3β activity elicited by increased IGF signalling, which coincides with increased MRF expression levels (**CHAPTER 5 + 6**). No information on Wnt-mediated inactivation of GSK-3 was obtained in these studies. However, as Wnt is essential during embryonic muscle development, and involved in muscle growth and regeneration [121, 150-152], Wnt-signalling-induced GSK-3 inactivation may be anticipated during reloading. This could be assessed in future studies by evaluation of the dynamic composition of the 'degradation complex' [126, 153, 154] during muscle mass recovery, or alternatively, by studying β-catenin accumulation which would ensue canonical Wnt signalling-induced GSK-3 suppression (**CHAPTER 4**).



Most of the data that report beneficial effects of decreased GSK-3(β) activity during myogenic differentiation are based on *in vitro* studies, or *in vivo* transient modification of upstream regulators of GSK-3. Here we report the effect of genetic modulation of GSK-3(β) on regulators of MRFs *in vivo*. Muscle recovery from disuse atrophy is accompanied by increased myogenic differentiation gene expression (**CHAPTER 5 + 6**). As this induction of MRF mRNA transcript levels is not significantly attenuated in the presence of CA GSK-3, this indicates that IGF-mediated inhibition of GSK-3 is not required during the early stage of myogenic differentiation. However, whether MRF protein levels or transcriptional function are preserved in presence of high GSK-3 activity remains to be determined; the slightly prolonged increase in MRF mRNA expression may point to a reduced efficacy of the myogenic differentiation process, a notion that is supported by the delayed muscle fibre CSA recovery observed during reloading of CA GSK-3 expression in the soleus muscle. Wnt/β-catenin signalling has been implicated in myotube structural integrity [155]. This involves the Wnt-dependent formation of β-catenin-M-cadherin complexes which are essential for cellular adherence [156-158]. Interestingly, the increased M-cadherin expression levels observed with initiation of muscle reloading suggest the involvement of M-cadherin in muscle remodeling during muscle mass recovery (**CHAPTER 5 + 6**). Considering the dependency on GSK-3 inactivation of Wnt-induced β-catenin accumulation in these catenin-cadherin complexes [129, 130], impaired β-catenin accumulation in the presence of CA GSK-3 may have caused the delay in myofibre recovery observed in CA GSK-3-expressing mice during reloading. In opposition, absence of GSK-3β appears to improve myofibre CSA recovery, which corresponds with shortening of the transient increase in MRF gene expression in the recovering muscle (**CHAPTER 6**). However, to further address the idea that GSK-3 activity is involved in myofibre CSA recovery through Wnt signalling, it is critical that

β-catenin levels and localization are determined in follow-up studies that evaluate *in vivo* muscle mass recovery. This to determine and distinguish its potential role in cell-cell interactions and transcriptional activity that contribute to myofiber recovery. This notion is supported by the *in vitro* data presented in **CHAPTER 4** showing that genetic or pharmacological inactivation of GSK-3(β) not only promotes myotube formation but is accompanied by increased β-catenin signalling [159, 160].

## 2. EFFECT OF GSK-3 ACTIVITY MODULATION ON *IN VIVO* MUSCLE MASS REGULATION

In vivo, protein and myonuclear turnover occur in parallel and their coordinated activities determine whether muscle mass is stable, or decreases, or increases. Skeletal muscle hypertrophy is an increase in skeletal muscle size, which has primarily been attributed to increased muscle protein synthesis [161, 162] with little attention paid to proteolysis regulation in response to hypertrophy stimuli. In addition, increased satellite cell activation and myogenic differentiation have been observed in hypertrophy responses [57, 163, 164]. Conversely, in skeletal muscle atrophy, both decreases in protein synthesis signalling and increases in protein degradation-related signalling pathways have been reported [36, 165]. Finally, as described above, muscle mass recovery following a period of atrophy is also accompanied by extensive changes in these processes. Considering the evidence as reviewed in **CHAPTER 3** that suggests a role for GSK-3 in the regulation of protein turnover and myonuclear turnover, a large portion of the experimental work conducted in this thesis was aimed at elucidating the effect of GSK-3 activity modulation on muscle mass *in vivo*. A mouse model of reversible disuse-induced muscle atrophy was employed to create conditions of altered protein and myonuclear turnover to evaluate the contribution of GSK-3(β) to muscle mass maintenance, muscle mass atrophy, and muscle mass recovery. To investigate whether GSK-3 inactivation is required for muscle mass recovery, transgenic mice with CA GSK-3α/β and their wild-type counterparts (ctrl) [34] were used in the hindlimb suspension-reloading model (**CHAPTER 5**). To address whether reducing GSK-3β activity is sufficient to improve muscle mass recovery, mice with a muscle-specific deletion of GSK-3β [35] were used (**CHAPTER 6**).



### 2.1. Effect of GSK-3 activity modulation on baseline muscle mass

Although this thesis primarily addresses the role of GSK-3(β) in the regulation of muscle recovery from muscle disuse atrophy, genetic modulation of GSK-3 may affect muscle weight in absence of muscle plasticity cues. We and others have shown that decreased GSK-3(β) activity results in improved myogenic differentiation and myotube hypertrophy *in vitro* [30, 77, 143]. In vivo, muscle-specific over-expression of IGF-I, which is accompanied by GSK-3 inactivation [118], results in muscle hypertrophy [60]. Similarly, local overexpression of constitutively active Akt results in muscle fibre hypertrophy, while over-expression of dominant negative GSK-3β induces a modest muscle fibre hypertrophy [97, 166]. On the other hand, conditional ablation of muscle insulin receptor expression in 'MIRKO' mice results in muscle atrophy [167]. As the complete lack of (basal) insulin receptor activation caused suppressed Akt signaling, the resulting increase in GSK-3 activity may have contributed to muscle atrophy. However, under baseline conditions IGF-1/Akt signalling is expected to be relatively low [97, 168]. Therefore, GSK-3 activity levels are anticipated to already be high in muscle of normal mice. This may explain the

similar muscle masses between wild-type and CA GSK-3-expressing mice as observed in our study. Moreover, the decreased muscle mass that is observed in MIRKO mice [167] may reflect a deficit in the rate of muscle development rather than atrophy. In line with this notion, delayed whole-body and muscle weight development as observed in transgenic mice with reduced IGF-I receptor activity is ameliorated over time [169-171]. Whether whole-body expression of CA GSK-3 also evokes such a delay in muscle mass development could be assessed by evaluation of muscle mass at younger ages. Apart from time-dependent differences, adaptive mechanisms to compensate for the effects of CA GSK-3 presence on muscle mass may also explain the absence of any overt phenotype of CA GSK-3 expression. For instance, alternative pathways to adaptively control GSK-3 activity may be activated, such as the phosphorylation of amino acid residues of GSK-3 involved in stabilisation of its protein structure, reduced protein-protein interactions between GSK-3 and other proteins (like Wnt/β-catenin signaling 'degradation complex') as well as reduced priming phosphorylation of GSK-3 phospho-substrates, as described in **CHAPTER 3**. This could be further assessed by detailed analyses of phospho targets of GSK-3 or GSK-3-activity assays in the muscle of these CA GSK-3-expressing mice.

Remarkably, CA GSK-3-expressing mice display increased body weight compared to their wild-type counterparts, which was also reported by McManus *et al.* [34]. As this increase in body weight is not accompanied by a proportional increase in skeletal muscle weight, this suggests an altered body composition towards increased extracellular water or fat mass. GSK-3 inactivates Glycogen Synthase (GS), a key enzyme that promotes glycogen assimilation [172, 173]. Stimulation with insulin failed to increase muscle GS activity in the presence of CA GSK-3β [34] thereby preventing glycogen synthesis and blood glucose uptake by the muscle. As a consequence, excess glucose will have to be stored in other tissues than muscle, like in (pre-)adipocytes which are reported more receptive to glucose uptake in the presence of CA-GSK-3 [174]. This would then result in expansion of adipose tissue mass and contribute to the increased body weight in the CA GSK-3-expressing mice. This is in line with an increased adipose compartment in the absence of muscle insulin signalling as reported by Kim *et al.* [175]. Additionally, Pearce *et al.* showed that over-expression of GSK-3β in muscle leads to an increase in whole-body fat mass [176] and a slight decrease in lean mass.

A greater body weight normally is associated with a proportional increase in muscle mass to sustain locomotive ability [177], and this correlation between muscle mass and body weight is observed in the control wild-type mice (Figure 1).

Such a compensatory increase in muscle mass is not observed in CA GSK-3-expressing mice (Figure 1). The absence of increased muscle mass due to increasing body weight bearing may result from impaired IGF-I signalling at the level of GSK-3. With increased loading of the muscles, IGF-I or MGF gene expression increases. Previously it has been shown that CA GSK-3β can abrogate IGF-I-NFAT signalling-induced muscle hypertrophy [77]. This indicates that CA inactivation-resistant CA GSK-3 may impair compensatory muscle mass increases. Interestingly, in those same experiments it was also shown that CA GSK-3β expression did not decrease baseline NFAT signalling, which indicates that certain levels of anabolic signalling could be maintained even in the presence of CA GSK-3. Therefore, under baseline conditions negative effects of CA GSK-3 on muscle mass regulation may not become evident. To address putative interference

of inactivation-resistant GSK-3 with physiological and genetic hypertrophy cues, the CA GSK-3-expressing mice could be included in physiological, pharmacological or genetic models of hypertrophy involving IGF-signalling, such as, respectively, synergist-ablation [178, 179], testosterone treatment [180] or muscle-specific expression of mIGF- or CA-Akt [118, 181].

Based predominantly on *in vitro* literature, muscle hypertrophy could be anticipated in case of muscle-specific deletion of GSK-3β (MGSK-3β KO). In MGSK-3β KO mice no overt phenotypical differences on muscle or whole body mass are observed. However, a non-significant but consistently higher muscle mass is present at baseline conditions in MGSK-3β KO mice compared to control (**CHAPTER 6**). Limitations of the genetic model as applied here may contribute to the absence of robust hypertrophy. The Cre-transgene to induce recombination and deletion of the GSK-3β floxed allele is controlled by the MLC-1f promoter, which is primarily expressed in myosin heavy chain (MHC) type II muscle fibres [182]. Potential consequences of GSK-3β ablation will primarily appear in MHC type-II expressing muscle fibers. As such we observed that muscles consisting of mix of MHC type I and type II fibres have different levels of GSK-3β ablation. Interestingly, GSK-3β-ablated muscle showed a subtle but consistent tendency towards increased myofibre CSA, which was more outspoken in the MHC-IIA than MHC-I myofibres. Obviously, application of the MLC-1f-Cre transgenic mouse is extremely useful in studying the role of GSK-3β or other genes of interest in conditions with a fibre type-specific pathology, e.g. the type-2 fibre atrophy observed with aging [183]. To affect total muscle, promoter sequences of muscle-specific genes that are not fibre-type dependent, such as muscle creatine kinase [184], could be used to drive transgenic Cre expression to accomplish pan-fibre type GSK-3β ablation, although this could lead to cardiac hypertrophy as well [185-187]. Alternatively, the application of promoters of genes involved in satellite cell proliferation, e.g. PAX3, PAX7, or regulators of myogenic differentiation, e.g. MyoD, [188-190] to control Cre expression would result in an earlier and more complete ablation of GSK-β in muscle. Consequently, this could result in a more evident muscle hypertrophic phenotype similar to that observed *in vitro*, where inhibition of GSK-3(β) at initiation of myogenic differentiation results in improved myogenic differentiation and muscle protein content [30, 31].

An alternative explanation for the absence of a hypertrophic phenotype in the GSK-3β KO mouse model is that despite an acceptable recombination efficiency, low levels of residual GSK-3β levels may still provide sufficient GSK-3β kinase activity to phosphorylate and suppress regulatory molecules of muscle hypertrophy signalling. To illustrate this, Vyas *et al.* reported that a decrease in GSK-3β phosphorylation by 800% only resulted in a reduction of GSK-3β kinase activity of 25% [77]. In MGSK-3β KO gastrocnemius muscle GSK-3β ablation reached 90%, but based on aformentioned work the residual 10% GSK-3β protein activity may still be very high, possibly sufficient toabrogate muscle hypertrophy development.

Finally, targeting of GSK-3β in our approach did not affect GSK-3α expression. In *in vitro* work, GSK-3β ablation and pharmacological inhibition of GSK-3α/β gave comparable results on improved myotube formation [30]. Moreover, GSK-3β protein content in muscle is three to four times higher than that of GSK-3α [34], and previous studies implicate distinct control of both muscle proteolysis and myogenic differentiation by GSK-3β [29, 31]. Therefore we opted to selectively target GSK-3β using muscle-

specific GSK-3β ablation. However, this does not exclude an influence of GSK-3α under conditions of reduced GSK-3β presence, because ablation of GSK-3β leaves room for possible compensatory GSK-3α activity due to partial redundancy between GSK-3α and GSK-3β protein functions. Per example in Wnt/β-catenin signalling pathway GSK-3α and GSK-3β are functionally exchangeable [124, 191]. The generation of a GSK-3α and GSK-3β muscle-specific knock out animal would allow us to determine if alongside GSK-3β, GSK-3α ablation is required to induce muscle hypertrophy by GSK-3 inhibition. Conversely, to determine if GSK -3α/β inactivation is required for the development of muscle hypertrophy, mice of the CA GSK-3α/β knock-in strain could be crossed with mIGF or CA-Akt expressing mice [118, 181] to address whether strong hypertrophic phenotype in these mice resulting from increased IGF-I/Akt signalling is dependent on GSK-3 inactivation.

## 2.2. Effect of GSK-3 activity modulation on inactivity-related muscle mass loss

Muscle atrophy can be triggered by numerous factors, including hypoxemia, malnutrition, oxidative stress, systemic inflammation, systemically or pharmacologically increased glucocorticoid levels, and disuse or inactivity [192-196]. Previous work by our and other groups have implicated inhibition of GSK-3 in attenuation of muscle atrophy [59, 97, 197]. However, to date the effects of modulating GSK-3 activity on disuse-induced muscle atrophy have scarcely received attention. Therefore, we addressed this question using genetic modulation of GSK-3 activity and evaluated whether this affected disuse-induced muscle atrophy as investigated in **CHAPTER 5** and **CHAPTER 6**.



Disuse-induced muscle atrophy was achieved by utilization of the animal hindlimb suspension model for 14 days after which the effects on muscle mass and cellular signalling state were investigated. Although the hindlimb suspension model intends to study muscle unloading/disuse atrophy, the initial adjustment phase, approximately 5-7 days, involves additional physiological adaptations that may contribute to body and muscle weight loss. This initial adjustment period is characterized by a short period of reduced nutritional intake and water consumption (48-72hrs), which may evoke increased stress hormone release. This likely involves release of corticosterone, the endogenously expressed murine glucocorticoid, that will in turn increase muscle glucocorticoid signalling [198] which could subsequently increase muscle atrophy [199].

Both glucocorticoid-induced and nutritional deprivation-induced muscle atrophy can be abrogated by inhibition [97] or ablation of GSK-3β in muscle (K. Verhees thesis). Though per example the abrogative effect is more evident in the gastrocnemius muscle (52%) than the soleus muscle (34%) following 48 hours of nutritional deprivation (K. Verhees thesis). The difference in muscle fibre-type composition of these muscles may have contributed to this observed differential level of muscle atrophy in relation to the type of atrophic triggers due to the level of GSK-3β ablation (**CHAPTER 6**). Interestingly, the loss of body weight during the initial stages of hindlimb suspension is attenuated in MGSK-3β KO mice, which may reflect prevention of muscle atrophy in response to non-disuse related atrophy triggers such as decreased food intake or elevated corticosterone levels. This putatively attenuating effect of GSK-3β ablation on muscle mass loss during the initial adjustment phase, is no longer present at the end of the hindlimb suspension period, which indicates a ‘purely’ disuse-driven muscle atrophy at the end

of the hindlimb suspension protocol that is not spared by GSK-3β ablation. Nonetheless, increased glucocorticoid signaling still needs to be verified, for instance by determining native corticosterone levels in the blood or glucocorticoid receptor nuclear translocation in myofibers. In addition, subsequent evaluation whether muscle-specific GSK-3(β) or GR ablation, respectively, attenuates skeletal muscle mass loss during the initial phases of hindlimb suspension will address the dependency of early muscle mass loss in this models of the unloading-independent factors and the role of GSK-3-related GR signaling therein.

In **CHAPTER 5** and **CHAPTER 6** we have shown that neither CA GSK-3 expression nor muscle-specific GSK-3(β) ablation aggravates or attenuates soleus muscle mass loss, respectively. Several studies have shown that stimulation with anabolic factors like IGF-I or inhibition of GSK-3β can abrogate or even prevent a range of muscle atrophic stimuli. However, our study suggests that this is not the case for disuse-induced muscle atrophy. While the level of GSK-3β reduction in our study might not have been sufficient to attenuate disuse-induced soleus muscle atrophy, other literature shows that muscle-specific IGF-I over-expression neither protected nor alleviated disuse-induced muscle atrophy from hindlimb suspension [200]. Our findings in the CA GSK-3-expressing mice extend this observation as they reveal that lack of IGF-mediated inhibition of GSK-3 does not aggravate soleus disuse atrophy. In a related type of disuse muscle atrophy, spinal cord injury-induced muscle atrophy has been shown to involve a temporal increase in IGF-I expression [201, 202]. Although this may reflect a muscle-intrinsic response to preserve function, it appears futile as muscle atrophy still ensues. Combined, this suggests that IGF-I-mediated signalling is not sufficient, and increased GSK-3 activity is not involved in disuse atrophy of the postural-type soleus muscle.



However, CA GSK-3 aggravates muscle atrophy of the partly unloading-sensitive gastrocnemius, but also and induces muscle mass loss in the tibialis muscle, which is not targeted by unloading and is unaffected in the control wild-type mice (Figure 2). Thus, the question can be raised whether the presence of CA GSK-3 sensitizes these muscles to other, not disuse related, atrophic triggers that are present during the execution of the hindlimb suspension model, e.g. nutritional deprivation and increased glucocorticoid signaling. In **CHAPTER 5** we reported that CA GSK-3-expressing mice lost almost two-fold more body weight compared to their wild-type control counterparts during the initial adjustment phase of the hindlimb suspension period. This greater decrease in body weight is not reflected in similarly greater soleus muscle mass loss, nor can be explained by the other muscles losing more mass than control (±28% more). This indicates that

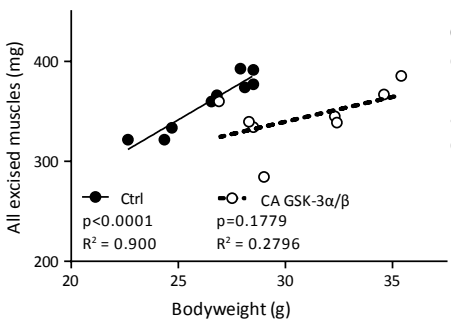


**Figure 1. Correlation between body- and muscle weight is altered by CA GSK-3.** Relationship of starting body weight and all excised lower leg muscles (soleus, plantaris, gastrocnemius, tibialis and EDL) weights for both ctrl/ Wild-type and CA GSK-3α/β-expressing mice.

besides the skeletal muscular system, a significant weight reduction occurs due to the whole body expression of CA GSK-3. Because it appears that the CA GSK-3-expressing mice might have a higher adipose tissue content it is very likely that the initial phase of hindlimb suspension, which involves both stress-hormone release and nutritional deprivation, might have resulted in significant reduction of the adipose compartment mass [203-206].

Due to depletion of their supposedly larger adipose tissue ´energy´ reserve, their lean/muscle compartment should have been spared throughout the nutritional insufficiency phase occurring during the adjustment phase to hindlimb suspension [207-209]. However, at the end of two weeks of hindlimb suspension, soleus muscle mass is comparable between CA GSK-3 and control mice. This could mean that dispite the potential sparing due to larger 'energy' reserves CA GSK-3 did aggravate soleus muscle mass loss to a now comparable level of soleus muscle mass loss. However, know from **CHAPTER 6** that ablation of GSK-3β will not spare soleus muscle mass loss possibly excluding involvement of GSK-3(β) activity in disuse-mediated muscle mass loss. Leading to the current postulation that CA GSK-3 expression does not aggravate soleus muscle mass loss.

As mentioned, CA GSK-3 expression did cause a further decrease in muscle mass due to the hindlimb suspension process in gastrocnemius muscle compared to control (Figure 2). The difference in muscle fibre-type composition and function may have contributed to this observed differential level of muscle atrophy in relation to CA GSK-3 expression. Soleus muscle is a postural muscle consisting of mainly type I and IIA fibres, whilst gastrocnemius and plantaris are primarily phasic muscles of predominantly MHC IIB/X fibres, which are known to be less affected by the hindlimb suspension model [210]. A possible explanation for this observed differential effect could be related to glucocorticoid signalling as a consequence of a HS-model-induced stress response discussed above. Glucocorticoid administration affects glycolytic muscles more than oxidative muscles (K. Verhees thesis), and CA GSK-3 may have aggravated plantaris and gastrocnemius muscle mass loss by sensitizing glycolytic MHC type-II fibers to an interaction between disuse- and glucocorticoid-atrophy cues. In fact, CA GSK-3 may have sensitized other muscles to non-disuse-related atrophy triggers as the tibialis muscle, which is not target by hindlimb unloading, only shows a slight decrease in muscle mass in control but a significant decrease in the presence of CA GSK-3 (Figure 2).

In conclusion, soleus muscle mass atrophy due to hindlimb suspension is neither affected by the presence of CA GSK-3, nor is it alleviated by GSK-3β ablation. However, CA GSK-3 did aggravate muscle mass loss of the gastrocnemius muscle but also the, non-targeted by hindlimb unloading, tibialis muscle. To evaluate if indeed either nutritional deprivation or increased glucocorticoid signalling could explain this aggravated muscle mass loss in the presence of CA GSK-3, CA GSK-3α/β knock-in mice could be subjected to these aforementioned atrophic triggers to evaluate their individual contribution to the observed aggravated loss of gastrocnemius and tibialis muscle mass after hindlimb suspension.

## 2.3. The role of GSK-3(β) in muscle mass recovery

Recovery from disuse-induced muscle atrophy coincides with GSK-3β inactivation [33] and reduced GSK-3 activity promotes myogenic differentiation (**CHAPTER 4**) and myotube hypertrophy [30, 77, 143]. In **CHAPTER 5** we show that reloading-induced muscle mass recovery does not appear to require GSK-3 inactivation. However, in **CHAPTER 6** we show that muscle-specific ablation of GSK-3β stimulates initiation of reloading-induced muscle mass recovery.

A surprising observation is that in the presence of CA GSK-3, soleus muscle mass recovery actually exceeds that of control. This is in opposition of what is expected based on literature where expression of CA GSK-3β interferes with muscle growth [77]. Detailed analysis of muscle fibre CSA suggests that the rapid increase in CA GSK-3-expressing soleus muscle mass is not reflected by a concomitant muscle fibre CSA recovery, and compared to control even a transiently decreased myofibre CSA is observed in the CA GSK-3-expressing mice. Increases in muscle mass can grossly be attributable to either increased muscle fibre CSA size (hypertrophy) or total muscle fibre number (hyperplasia) [211]. As such, one possible explanation for the lack in concomitant increase in myofibre CSA could be muscle fibre hyperplasia. Although whole muscle histological analyses would be required to address this hypothesis, the additional proliferative response in CA GSK-3 soleus muscle compared to control is unlikely to accommodate such an extent of muscle plasticity response (**CHAPTER 5**) that could explain the observed difference between soleus muscle mass and myofibre CSA gains. Such a discrepancy between changes in muscle mass and fibre CSA has been observed previously [136, 212] by Zhang *et al.* who showed a 'delay' in myofibre CSA recovery compared to muscle mass [213]. This discrepancy could be contributed to interstitial fluid accumulation during muscle recovery/regeneration [214-216], in which CA GSK-3 may sensitize myofibres to the mild damage that accompanies (untrained) muscle reloading, resulting in a greater level of oedema formation. Muscle swelling due to post-exercise inflammation has been postulated to involve infiltration of immune cells [217, 218], as well as NF-kB signalling activation [219-221]. GSK-3 is involved in regulating NF-kB signalling [191], and in the presence of CA GSK-3 aberrant NF-kB activity may have altered inflammatory cell recruitment, resulting in abnormal damage and oedema formation. However, from the obtained soleus muscle fibre CSA we were not able to discern increased interstitial fluid accumulation.

The accelerated increase in soleus muscle mass in the absence of GSK-3β after five days of reloading was as expected. In contrast to the CA GSK-3-expressing soleus muscle, the improved muscle mass gain in absence of GSK-3β was accompanied by a relative increase of muscle fibre CSA recovery, most notably in type IIA fibres. This fibre-type specificity is likely attributable to the preferential GSK-3β ablation in the cells expressing MyLC-1f [35], which is most prominently expressed in MHC-II type expressing muscle fibres [182]. These data certainly warrant follow up studies that should employ pharmacological approaches or conditional genetic models that will result in reduced activity or depletion of both GSK-3β and GSK-3α in all myofibres to address the full potential of GSK-3 modulation to promote muscle mass recovery.

Only few studies have addressed the mechanisms of muscle mass recovery to identify targets for intervention to stimulate muscle mass regain in clinical settings. To our knowledge none exist focusing primarily on inactivation of GSK-3(β) and the effects

thereof on muscle mass recovery or muscle regeneration, but in some studies cellular signaling pathways involving GSK-3 were modulated. Conditional ablation of the IGF receptor at adulthood resulted in perturbed muscle regeneration, although this did not concern a mass recovery model [222]. Furthermore, hyperglycemia, often associated with decreased insulin/Akt-mediated signalling, has also been shown to impair atrophied muscle recovery [223]. On the other hand, transient or genetic over-expression of IGF-I has been shown to improve disuse-induced muscle mass recovery [224-226]. Modulation of mTOR-related protein synthesis signalling has also been shown to affect muscle mass recovery following disuse atrophy [227, 228], partly through increased expression of IGF-I. Although this clearly indicates that activation of the IGF-I signalling pathway is beneficial in aiding muscle mass recovery, crossing these genetically modified mice with the CA GSK-3-expressing mouse model is required to investigate the extent of dependency on GSK-3 inactivation. Beyond stimulation with IGF-I to improve muscle recovery, resveratrol has also been shown to have a positive effect on muscle mass recovery [229], in part through reduction of Caspase-3 activity and BAX gene expression, which both are factors involved in apoptosis signalling that are affected by GSK-3 activity modulation [230, 231]. This implies that during muscle mass recovery an integrative view of all processes contributing to muscle protein and nuclear turnover should also include evaluation of these putative negative effectors of muscle mass.

Combined, our data as reported in **CHAPTER 5** and **CHAPTER 6** imply a role for GSK-3 inhibition during recovery from atrophy. However, despite the potential for improving the robustness of GSK-3 inhibition, the observed results on muscle mass recovery are rather subtle. Muscle mass recovery following disuse atrophy did not require GSK-3 inactivation and the potentiating effects of GSK-3β ablation are restricted to the initial stage of reloading-induced muscle mass and myofibre CSA recovery. However, this does not discard GSK-3 as a nexus in skeletal muscle mass plasticity, specifically as reports have already shown that muscle-specific GSK-3β ablation except for disuse-induced atrophy (**CHAPTER 6**), will abrogate diverse conditions leading to muscle atrophy (K. Verhees thesis).



## 3. FUTURE PROSPECTIVE AND CONCLUSION

Based on the results presented in this thesis (**CHAPTER 5 + 6**) the question can be raised whether the role of GSK-3 in regulation of skeletal muscle mass recovery is overestimated. We and others have shown in *in vitro* studies that reducing GSK-3 activity improves myogenic differentiation **CHAPTER 4**, and results in myotube hypertrophy [30, 77, 143]. However, this work mainly reports improved myotube formation and increased muscle protein content due to GSK-3 inactivation when starting from the myoblast stage, a stage that more closely resembles embryonal and peri-natal myogenesis than post-natal muscle mass recovery or regeneration. The current investigation in **CHAPTER 6** used a method of muscle-specific GSK-3β ablation which will only effectively have depleted GSK-3β in differentiated muscle fibers, but not satellite cells and presumably myoblasts, as the conditional Cre-mediated deletion of GSK-3β is dependent on Cre-expression upon MLC-1f promoter activation. On one hand this could therefore preclude some of the positive effects of GSK-3(β) inhibition as observed with *in vitro* myogenic differentiation, but it also indicates that GSK-3 inhibition in the myofiber facilitates myogenesis and possibly myonuclear accretion, suggesting an impact of GSK-3 on

myofiber-myoblast cellular communication. To further determine the role of GSK-3β in myofibers, and proliferating and differentiating satellite cells/myoblasts, it would be possible to deploy an *in vitro* culture system that could better model post-natal myogenesis, which consists of co-culturing myoblasts and myotubes. This would allow to simulate *in vivo* muscle recovery from atrophy in the presence or absence of GSK-3β (**CHAPTER 6**), myotubes exposed to an atrophic trigger are subsequently supplemented with myoblasts for myonuclear accretion, while GSK-3β expression is selectively knocked down in myotubes or myoblasts, respectively. This will provide a more in-depth insight into the satellite cell- or myofiber-specific role of GSK-3β in myogenesis and myonuclear accretion.

As non-muscular cells are involved in muscle mass recovery [217, 218, 232] which could potentially activate or inhibit and cellular signaling pathways, *in vitro* systems that mimic the *in vivo* situation will have their limitations. Therefore, the approach of GSK-3 muscle-specific ablation should be further adapted as well. As mentioned above, limitations of the manner of genetic modulation of GSK-3β hamper accurate determination of the degree of muscle mass hypertrophy. We therefore suggest the use of promoters of muscle-specific genes that are not fibre-type dependent to drive Cre expression, such as muscle creatine kinase [184]. Moreover, promoters of genes involved in satellite cell proliferation like PAX3 and PAX7 or genes involved in driving myogenic differentiation such as MyoD [188-190] would be preferred, because this would allow removal of GSK-3(β) earlier in the muscle cell lineage, and would allow comparison to the *in vitro* results reported in literature where inhibition of GSK-3(β) improves myogenic differentiation. This genetic modulation of GSK-3 activity should be made inducible to avoid potential long-term adaptations to the absence of GSK-3(β) in cellular signalling pathways, like what may have been apparent in the GSK-3β-depleted muscle described in **CHAPTER 6**. We have previously shown that starvation, glucocorticoid treatment and systemic inflammation induced by intra-tracheal instillation of LPS (K. Verhees thesis), but not disuse (**CHAPTER 5 + 6**), trigger muscle mass atrophy which can be attenuated by GSK-3β ablation. Therefore, to study the role of GSK-3 in muscle mass recovery after an atrophic trigger, GSK-3 ablation should not be induced before the initiation of muscle mass recovery. Furthermore the utilisation of an inducible method of genetic GSK-3 modulation will more closely predict the efficacy of administration of pharmacological inhibitors to maintain or stimulate muscle mass recovery. The more profound advantage of using tissue-specific inducible GSK-3 modulation compared to the use of administered drugs is the prevention of off-target effects involving other kinases or tissues. Furthermore, total ablation of the target will allow for an assessment of the maximally achievable results of GSK-3 inhibition.

When critically evaluating a potential role of GSK-3's involvement in muscle mass recovery, one automatically has to review the evidence of the involvement of myonuclear accretion and post-natal myogenesis. Relatively few *in vivo* studies have been performed to address the molecular mechanisms of muscle mass regain compared to muscle atrophy and the attenuation thereof. Of the investigations performed addressing muscle recovery, muscle regeneration [233] has been explored most extensively. However, such studies typically involve induction of severe muscle damage and myofiber necrosis followed by a strong regenerative response executed by satellite cells in interaction with immune cells involving de novo generation of muscle fibers (hyperplasia). Moreover,

these studies focus on myofiber structure rather than muscle mass changes.

Although there are medical conditions where muscle is severely damaged to the extent that muscle mass restoration requires hyperplasia-associated regeneration, e.g. snake venom induced myotoxicity [234-236], the majority involves far less severely damaged skeletal muscle , and will rely on recovery of muscle mass involving regeneration to facilitate myofiber 'repair' rather than complete replacement with new myofibers. Although reloading of the atrophied muscle induces muscle fiber damage to some extent, the resulting regenerative response is expected to approximate physiological repair mechanisms that also operate in muscle mass recovery in human subjects. Regarding the involvement of post-natal myogenesis in muscle mass recovery, some literature has reported that satellite cell involvement does not appear to be required for muscle mass growth [45, 136, 237, 238]. Nonetheless, considering the consistent observations of satellite cell activation and induction of post-natal myogenesis in studies of physiological stimuli of muscle mass gain [58, 239, 240] as well as experimental *in vivo* models of muscle mass growth [57, 138, 163], this strongly suggests an important contribution of post-natal myogenesis to muscle mass growth. In the absence of GSK-3β there is a subtle but consistent stimulation of essential components of myogenic differentiation and improved muscle mass recovery (**CHAPTER 6**), which is in support of an important contribution of post-natal myogenesis to muscle mass recovery.

Importantly, this thesis postulates that during muscle mass recovery the coordinated changes in at least three processes are of importance, namely increased protein synthesis, suppressed proteolysis, and activation of post-natal myogenesis and myonuclear accretion. Protein synthesis signalling during recovery is neither affected by the presence of CA GSK-3 nor the absence of GSK-3β (**CHAPTER 5 + 6**). However, suppression of proteolysis signalling in recovering muscle is slightly more prominent in the absence of GSK-3β (**CHAPTER 6**). This may contribute to the subtle (transient) improvement in muscle mass gain and suggests that influencing the proteolysis part of protein turnover during recovery –via GSK-3 or other targets– may also be a strategy to improve muscle mass recovery. This is in line with literature that shows that deletion or



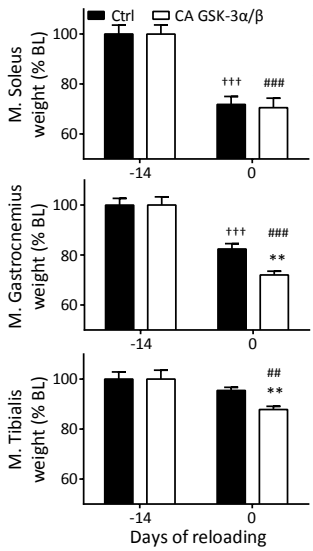


**Figure 2. Constitutively active GSK-3 sensitises unloading resistant tibialis muscle to muscle mass loss during hindlimb suspension.** Soleus, gastrocnemius and tibialis muscle weight of ctrl/ Wild-type and CA GSK-3α/β expressing mice at baseline (BL) (-14) and following completion of hindlimb suspension (0) were determined. BL muscle weight was set at 100% and muscle mass post hindlimb suspension displayed as percentage thereof for each mouse genotype, e.g. ctrl/ Wild-type and CA GSK-3α/β expressing mice. Averages ± SEM are presented, *: Ctrl vs. CA GSK-3α/β at indicated time point, †: indicates treatment effect of hindlimb suspension for Ctrl and #: indicates treatment effect of hindlimb suspension CA GSK-3α/β; 1 symbol equals p<0.05, 2 symbols equal p<0.01, and 3 symbols equal p<0.001.

inhibition of myostatin, a negative regulator of skeletal muscle mass [241], can increase muscle mass [237, 242] and thereby could also aid with muscle mass recovery.

Although not studied in this thesis, inactivity is also accompanied by a loss of muscle oxidative phenotype (OXPHEN), which governs muscle endurance and therefore together with muscle strength determines muscle function. During recovery from disuse atrophy the transcriptional program in control of OXPHEN is upregulated [243]. Considering that key regulatory molecules of OXPHEN e.g. NFAT [147, 244] and PGC-1 [245, 246] can be affected by GSK-3 [148, 149, 247] it would be of interest to evaluate whether recovery of OXPHEN during reloading is influenced by GSK-3 modulation.

The results from the *in vivo* studies presented in this thesis, though subtle, suggest that there is a potential for GSK-3 modulation to facilitate muscle mass recovery. Despite its inhibitory phosphorylation during reloading, suppression of GSK-3 activity is not required for muscle mass recovery in healthy mammals. However, this does not preclude putative unfavorable effects of aberrant GSK-3 activity during disease limiting muscle mass maintenance or recovery. Disease conditions such as chronic inflammation and/or glucocorticoid medication, processes that are both associated with muscle catabolism and which can both be present in patients with COPD [248]. Especially under conditions where it is known that inhibition of GSK-3 can abrogate muscle mass loss of these atrophic triggers (K. Verhees thesis), muscle mass recovery could potentially also be stimulated through inhibition of GSK-3. Therefore, the data presented in this thesis warrants further exploration of the beneficial effects of GSK-3 activity modulation on muscle mass recovery and plasticity in health and disease.

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# Summary

A common feature in chronic diseases irrespective of the primary organ impairment is the development of limb muscle weakness and wasting. This results in decreased physical performance independent of disease severity and contributes to a reduced quality of life, reduced efficacy of medical interventions and increased mortality. Therefore treating muscle wasting should be considered a cornerstone in integrated management of chronic diseases. Developing successful intervention strategies to recover muscle mass does not only require better understanding of the factors and mechanisms involved in muscle mass loss (atrophy). We also need to know how different factors and mechanisms contribute to or interfere with muscle mass gain (recovery).

Resistance exercise is a powerful stimulus to gain muscle mass in health and to combat ageing-induced muscle mass loss, but it is less effective as primary or single treatment intervention in acute or chronic disease with prevailing muscle wasting. This is partly related to diminished feasibility of high-intensity exercise by these patients due to their disease state, but also related to decreased efficacy due to disease and treatment-induced catabolic stimuli. This indicates that patients, who are incapable of performing sufficient levels of exercise to achieve muscle mass recovery, could benefit from pharmacological support in gaining muscle mass.

This thesis focuses on better understanding of the mechanisms of muscle mass recovery and more specifically the role of *glycogen synthase kinase* (GSK)-3, a negative regulator of muscle mass, therein. GSK-3 is explored as a target for pharmacological modulation to prevent muscle wasting and stimulate muscle mass recovery.

Pharmacological intervention trials to improve muscle mass recovery have previously been performed. Anabolic agents that were used therein included growth hormone, insulin-like growth factor (IGF)-I, and testosterone or analogues thereof. These anabolic agents can activate the IGF-I/Akt signalling pathway, which leads to abrogation of proteolysis and increased protein synthesis, proliferation and muscle-specific gene expression. However, side effects and varying efficacy indicate that a good therapeutic window of pharmacological anabolic stimulation remains to be determined. Furthermore, the extent to which interactions of anabolic agents with concurrent pharmacological treatment such as systemic glucocorticoids (GCs) occur and affect physiological state and response is currently unclear.

This is exemplified by the reanalysis of a clinical trial that investigated the efficacy of anabolic steroid supplementation in patients with advanced COPD during participation in a pulmonary rehabilitation program. This reanalysis, described in **CHAPTER 2**, showed overall that there was a positive effect of the testosterone analogue nandrolone decanoate (ND) on muscle mass gain; however the increase in muscle mass gain was clearly greater and significant in patients who were concurrently taking maintenance dose glucocorticoids compared to those who did not. Therefore, a potential interaction between GC and IGF-I/Akt signalling was investigated, as the latter is postulated to convey the anabolic effects of testosterone analogues in skeletal muscle. We used a translational research approach in **CHAPTER 2** by reanalysing the clinical data and investigating the interaction between GC and IGF-I stimulation on muscle cells during myogenic differentiation *in vitro*. GCs alone strongly impaired protein synthesis signalling, myotube formation and muscle-specific protein expression. In contrast, combined with IGF-I, a synergistic stimulation occurred leading to enhanced myotube formation and myofibrillar protein expression. This corresponded with restored protein synthesis

signalling by IGF-I and increased transcriptional activation of muscle-specific genes by GCs, which is possibly part of the basis for the observed synergistic enhancement of myogenic differentiation. In patients with COPD, the clinical trial also revealed an enhanced effect of anabolic steroids on muscle mass and respiratory muscle strength in the presence of glucocorticoid treatment. Synergistic effects of anabolic steroids and GCs on muscle recovery may be caused by effective translation of glucocorticoid-induced accumulated muscle-specific gene transcripts, resulting from anabolic steroid treatment-induced IGF-mediated relief of the glucocorticoid-imposed blockade on protein synthesis signalling.

Besides controlling protein synthesis and proteolysis (protein turnover), numerous studies also implicate IGF-I signalling in regulation of myonuclear turnover. In particular myogenic differentiation associated with muscle growth and regeneration has been investigated. GSK-3 is a signal transduction protein whose phosphorylating capacity is suppressed by activation of the IGF-I/Akt pathway, which suggests it may serve as a target for pharmacological inhibition to modulate muscle mass. GSK-3 consists of two isoforms from two separate genes namely GSK-3α and GSK-3β, which can operate in a redundant manner in various cellular processes. In human and mouse muscle, protein content of GSK-3β is three to four times higher than that of GSK-3α, which indicates that GSK-3β is the predominant GSK-3 isoform in muscle. Therefore, many studies, not only in muscle, have only focussed on genetic modulation of GSK-3β instead of both isoforms. Inhibition of GSK-3(β) has been shown to abrogate proteolysis and improve myogenic differentiation. We therefore reviewed the literature on GSK-3 as a potential master regulator of muscle mass plasticity and hence a suitable pharmacological target in **CHAPTER 3**.

Targeting GSK-3(β) for treating skeletal muscle wasting requires better understanding of the effects of modulation of GSK-3β activity on the molecular mechanisms governing muscle mass plasticity. The **MAIN HYPOTHESIS** of this thesis is that *GSK-3(β) inactivation is required and sufficient to stimulate myogenic differentiation and muscle mass recovery of atrophied muscle*. This hypothesis was tested in **CHAPTER 4-6** using a combination of *in vitro* and *in vivo* experimental models of muscle cell differentiation and recovery of muscle mass following atrophy.

Previous work by our group has shown that inactivation of GSK-3(β) both through activation of the IGF-I/Akt pathway and pharmacological inhibition with lithium chloride salt (LiCl) promotes myogenic differentiation *in vitro*. In that work alleviation of GSK-3(β)-mediated inactivation of nuclear factor of activated T-cells (NFAT)c3 was postulated as the basis for increased myogenic gene expression in response to GSK-3 inhibition. Noteworthy however was that inhibition of GSK-3 by LiCl had a remarkably greater effect on myoblast fusion and myotube formation than stimulation with IGF-I. LiCl is a known Wnt/β-catenin signalling mimetic leading to transcriptional co-activator β-catenin accumulation, which is a GSK-3 phospho-substrate. Wnt/β-catenin signalling has been associated with muscle hypertrophy and regeneration. β-catenin regulation via GSK-3 has been well described in response to signalling in response to the Wnt ligand rather than IGF-I. In **CHAPTER 4** we therefore assessed the role of GSK-3(β)–NFAT and GSK-3(β)–β-catenin signalling in relation to myogenic differentiation after stimulation with ectopic IGF-I, Wnt-3a or pharmacological inactivation of GSK-3. Herein we demonstrate that Wnt-3a and LiCl but not IGF-I-dependent GSK-3 inhibition induced β-catenin signalling

during myogenic differentiation. Furthermore, Wnt-3a significantly promoted myoblast fusion and myotube formation without enhancing muscle-specific gene expression, whilst LiCl stimulated both and IGF-I mainly the latter. These results point to two distinct signalling routes that control GSK-3 activity and independently affect myoblast fusion and muscle-specific gene expression during myogenic differentiation.

A vast majority of the studies implying a role for GSK-3 in muscle mass plasticity and specifically muscle regeneration were performed using *in vitro* models. We therefore focused on the effect of genetic GSK-3 activity modulation on muscle mass recovery *in vivo*. In addition to disease-specific and medical treatment induced triggers of muscle wasting, an important common denominator of muscle wasting in chronic diseases is decreased muscle usage. This is due to an adaptive sedentary lifestyle due to aging and concomitant chronic disease, and frequent periods of bed rest related to acute hospitalizations due to disease exacerbations. Specifically the aspect of disuse-induced muscle wasting is investigated, and the effects of modulation of GSK-3 activity therein. To accomplish this, a mouse model of reversible disuse-induced muscle atrophy was employed: mouse hind limbs were unloaded for two weeks resulting in atrophy of the unloading-sensitive calf muscles, *i.e.* the soleus and gastrocnemius muscles. After two weeks of hindlimb unloading the mice are allowed to resume free movement, which leads to loading and use of the calf muscles that then start to recover their muscle mass.

Previous work by our group has shown that in this mouse model of disuse-induced muscle atrophy, the mass recovery of the soleus muscle after hindlimb reloading was associated with significantly increased GSK-3β phosphorylation, which is an indicator of GSK-3 inactivation. First we investigated whether inactivation of GSK-3 is indeed required for reloading-induced muscle mass growth. This was done by using a mouse strain that carries a serine to alanine mutation for both GSK-3α (S21A) and GSK-3β (S9A) isoforms. These mutant forms of GSK-3 are insensitive to IGF-I/Akt-mediated inactivation of GSK-3, and are referred to as constitutively active (CA). Mice expressing whole-body (CA) GSK-3 or control mice were subjected to hindlimb unloading and allowed to recover their lost muscle mass. The results described in **CHAPTER 5** indicate that CA GSK-3 does not affect protein turnover regulation during muscle reloading. In contrast, myogenic differentiation, proliferation and muscle-specific gene expression appeared to be increased, which was unexpected. Furthermore, soleus muscle mass recovery was greater in the presence of CA GSK-3 than in that of wild-type GSK-3, but this was not accompanied by a similar increase in muscle fiber cross-sectional area (CSA) during reloading. Overall, this indicates that inactivation of GSK-3 is not required for disuse-induced atrophied-muscle mass recovery.

Ample evidence suggests that inhibition of GSK-3(β) suppresses muscle proteolysis. Furthermore, *in vitro* inhibition of GSK-3(β) is sufficient to enhance myogenic differentiation and induce muscle hypertrophy. However, *in vivo* the effects of GSK-3(β) inactivation on muscle mass recovery have not been investigated. To explore if additional inactivation of GSK-3β during hindlimb reloading could improve muscle mass recovery, a muscle-specific GSK-3β knock-out mouse was used. These mice lacking GSK-3β expression in their muscles were similarly subjected to the aforementioned mouse model of hindlimb unloading and reloading from which the obtained results are described in **CHAPTER 6**. We found that protein turnover signalling was grossly unaffected in the absence of GSK-3β in the muscle. However, coherent effects in the extent and

kinetics of satellite cell activation, proliferation and post-natal myogenic differentiation observed during reloading suggested that myonuclear accretion in recovering skeletal muscle lacking GSK-3β was enhanced. Furthermore, skeletal muscle lacking GSK-3β had a greater increase in muscle mass and muscle fiber CSA after five days of muscle reloading. This indicates that reducing GSK-3β activity accelerates the early phases of muscle mass recovery following atrophy.

Lastly, the insights and implications resulting from the original research data obtained in this thesis (**CHAPTER 4-6**) were critically evaluated and integratively discussed with respect to the most recent literature in **CHAPTER 7**. There appears to be no significant role for GSK-3 in protein synthesis signalling. However protein degradation signalling is attenuated in the absence of GSK-3β. Furthermore, post-natal myogenic signalling appears to be stimulated in the absence of GSK-3β. Expression of genetically modified GSK-3(β) or lack of GSK-3(β) expression appears to adjust baseline conditions of investigated markers of both protein and myonuclear turnover regulators, however this does not appear to significantly affect skeletal muscle mass compared to control conditions. We observed that disuse-induced muscle mass loss is not affected by modulation of GSK-3 activity, which contrasts to studies that show that inhibition of GSK-3β prevents muscle mass loss due to other atrophic factors e.g. GC administration, nutritional deprivation and increased inflammatory signalling. We therefore postulate that GSK-3 could be considered as a putative target for prevention of muscle atrophy in response to multiple triggers but not muscle disuse. Finally, although muscle mass recovery did not require GSK-3 inactivation, the findings that GSK-3β ablation enhances the initial stage of reloading-induced muscle mass and myofiber CSA recovery are considered as an incentive to propose further evaluation of GSK-3 as a potential pharmacological target to affect skeletal muscle mass plasticity, with potential to aid muscle mass recovery in a therapeutic multimodal treatment approach.

# Valorisation proposal

## SOCIO-ECONOMIC IMPACT OF MUSCLE WASTING

Globally the average life expectancy has increased over the last century and this is even more pronounced in the 'Western' world. Furthermore, the population age composition in the Netherlands is clearly indicating progressive ageing of the population (WHO data 2002-2012 on the Netherlands). Relatively few people actually die from injuries and accidents compared to disease-related causes. Of these disease-related deaths 88% can be attributed to the non-communicable category, meaning diseases such as chronic heart failure (CHF), chronic obstructive pulmonary disease (COPD), chronic renal failure (CRF), rheumatoid arthritis (RA) and cancer. The incidence of these chronic morbidities rises with age and disease progression increasingly reduces patients' quality of life. Chronic diseases are diverse in aetiology. However, many patients suffer from muscle weakness and wasting. This muscle wasting results in decreased physical performance and quality of life, reduced efficacy of medical interventions and increased mortality independent of disease severity. Therefore, addressing muscle wasting can be seen as a cornerstone in the treatment strategy development to improve quality of life and reduce morbidity and mortality by maintenance of mobility and daily physical activity in chronic diseases that are accompanied by this debilitating condition. However, the treatment of these diseases imposes a significant economic burden on society. In the Netherlands for the last sixteen years health costs per person have increased by $200 (a 6.8% increase) per year to $5122 in 2011. With 16.7 million people this adds up to an $85.4 billion expenditure, which is approximately 14% of our gross domestic product (GDP; $607 billion in 2012). If nothing changes, even when adjusting for an average annual inflation rate of 2%, the economic burden for those medical costs could double every 15-16 years. This is economically unsustainable and means that the burden to support older generations in their need for medical support would become enormous for the younger generations. Eventually, optimal treatment of the medical-support needing older generations might prove impossible with progressively insufficient financial means for all. This in turn would put further social and economic stress on both society and the individual, thereby imposing also a very significant emotional burden on the individual. Improvement of medical treatment/intervention strategies is needed to proactively ease both burdens.



## CONSIDERATIONS FOR IMPLEMENTATION OF GSK-3 INHIBITION IN MUSCLE WEIGHT MANAGEMENT

### Patient population and window of opportunity

Patients with chronic morbidities, though diverse in aetiology, suffer from muscle weakness and wasting that aggravate the disease and reduce intervention efficacy. Treatment of muscle wasting can be seen as a cornerstone in the treatment strategy to improve quality of life. Muscle mass wasting can result from 1) short-term catabolic insults as mentioned in this thesis, e.g. glucocorticoid treatment or physical inactivity, to combinations of such catabolic stimuli and 2) chronic exposure to catabolic stimuli, such as low-grade chronic inflammation, that results in a progressive but slower muscle mass loss.

Resistance exercise is a powerful stimulus to gain muscle mass that works well in health and can even to some extent combat ageing-associated loss of muscle mass (sarcopenia). However, research has shown that it is less effective as primary or single

treatment, or as intervention in acute or chronic disease conditions with prevailing muscle wasting. This is partly related to diminished feasibility of high-intensity exercise by these patients under these conditions due to limitations associated with disease severity, but also related to decreased efficacy resultant from disease- or treatment-induced catabolic stimuli. Therefore patients who are incapable of performing sufficient levels of exercise to achieve muscle mass recovery could benefit from pharmacological support in gaining muscle mass. As discussed in this thesis, the effects of GSK-3 activity modulation on muscle mass gain during recovery from disuse atrophy are subtle under healthy conditions. However, considering the potential impact of concurrent disease-related catabolic factors on muscle recovery, the potential of GSK-3 inhibition to advance muscle mass recovery under conditions where catabolic causes remains, such as after acute inflammatory disease exacerbations, remains to be established. With the use of GSK-3 inhibitors we would aim to improve the natural response to exercise, possibly in combination with nutritional and nutraceutical support to achieve muscle mass gains that would otherwise be harder to achieve for such patients. This would then form a basis for the development of a multimodal treatment protocol.

A point of attention is to determine the optimal treatment window. Intervention usually is started at a stage when muscle mass loss has already occurred and clearly been established. Alternatively, intervention could be initiated at a much earlier time point to prevent muscle mass loss during disease exacerbations. In this intervention strategy, treatment for the specific chronic disease would be accompanied by an effort to prevent muscle mass loss. For example, when a patient with COPD is hospitalised with an acute exacerbation and is consequently treated with glucocorticoids, the muscle is exposed to two known catabolic factors and the patient will likely suffer from muscle wasting. We have shown in animal models that reduced GSK-3β content attenuates muscle mass loss due to both inflammation and glucocorticoid treatment. Therefore, it could be an option to complement the treatment of the exacerbation with a GSK-3 inhibitor to reduce or prevent muscle mass loss. This intervention could be further improved upon by including other strategies to prevent muscle wasting during exacerbations and recovery thereof, e.g. electrical muscle stimulation and physiotherapy. Although it is yet unclear how a GSK-3 inhibitor will interact with other pharmacological agents that are administered simultaneously, we believe that trials should certainly be initiated in case pharmacological inhibition in appropriate experimental models prove beneficial. Eventually we hope that the use of GSK-3 inhibitors will reduce exacerbation incidence, enhance efficacy of rehabilitation therapy and improve patient's quality of life by preventing muscle mass loss or improving muscle recovery.



### Benefits of kinase inhibition over growth factor-mediated stimulation of muscle mass growth

The therapeutic interventions are aimed at preventing or recovering from muscle wasting by either targeting impaired cellular signalling that underlies disturbed muscle mass homeostasis, or aiming at modulation of master regulators of the processes that govern muscle mass plasticity. In the past several randomized controlled trials have investigated the efficacy of exercise with nutritional and pharmacological (anabolic steroids, testosterone, IGF-I) supplementation to stimulate muscle mass recovery and/or increase muscle mass in muscle-wasted patients. In these studies a significant though

variable increase in muscle mass was reported, thereby indicating that increasing muscle mass is possible but not consistent. The pharmacological approach used in those studies is utilisation of a stimulating agent e.g. IGF-I. Upon stimulation of the cell, IGF-I can activate a plethora of cellular signalling cascades. To reduce the incidence of off-target effects, often a choice is made to aim for proteins downstream of IGF-I in the signalling pathways that are the intended target. As mentioned, GSK-3 is a downstream target of the IGF-I signalling pathway and has been shown to impede protein synthesis and myogenesis and to be required for regulation of protein degradation. Thus by opting for GSK-3, multiple cellular signalling processes involved in muscle mass regulation can be targeted simultaneously. However, most importantly and in contrast to many other kinases in the cell, phosphorylation of GSK-3 by IGF-I signalling leads to inactivation rather than activation. An important feature of GSK-3 in that physiologic stimuli, such as IGF-I, reduce GSK-3 activity by 50 to 80% in muscle. This indicates that complete inhibition may not be needed, or desirable, to achieve results, which could prove to be a very beneficial factor for choosing GSK-3 as a target for modulation of muscle mass. Furthermore, over-activity of the IGF-I signalling pathway has often been associated with cancer development, whilst aberrant GSK-3 activity to date has not. However, over-inhibition of GSK-3 under normal circumstances might have detrimental effects like cardiac hypertrophy, induction of neurodegenerative markers and could nonetheless lead to cancer development. Thus, because of the pleiotropic actions of GSK-3 in addition to its involvement in muscle mass regulation, effects of inhibition of GSK-3 need to be carefully monitored.

**Existing clinical information on GSK-3 inhibition**

Already for more than a couple of decades pharmacological modulation of GSK-3 has been applied in clinical practice, namely in the form of lithium, an FDA-approved drug. It has been used for the treatment of psychiatric conditions including bipolar disorder and manic depression, and thus far there have been no reports associated with increased incidence of cancer or cancer-related deaths due to long-term lithium usage. Regrettably, those reports on the utilisation and efficacy of lithium salts in these disorders do not typically include any parameters related to muscle mass or function.



There are currently many different GSK-3 inhibitors being tested in laboratories, with more under development, because of the reported positive effect of GSK-3 activity regulation in relation to the aforementioned mental illnesses. Therefore, this implies possibilities to apply an already developed and FDA approved, or soon to be approved, GSK-3 inhibitor in a clinical setting to address its usefulness in improving muscle mass gain or preventing loss thereof. In skeletal muscle of COPD patients, some level of aberrant GSK-3 activity has already been established and in patients with chronic complete spinal cord injury, who have muscle atrophy,GSK-3β phosphorylation levels have been shown to be strongly decreased, which is indicative of increased GSK-3 activity. Arguably, these reports do not establish sufficient evidence to imply the involvement of impaired or aberrant GSK-3 regulation and/or activity that are associated with chronic diseases in muscle wasting. Nonetheless, there is a considerable amount of information available that shows that decreased GSK-3 activity has a positive effect on the maintenance of muscle mass in the presence of catabolic factors and improves myogenic differentiation. Therefore, there is sufficient basis to proceed into a proof-of-concept clinical trial with

GSK-3 inhibitors to determine their effect on either or both muscle mass recovery and reduction or prevention of loss.

**Application of a GSK-3 inhibitor in a clinical trial**

As mentioned the economic burden of the increased medical costs is in part related to the increased incidence of chronic diseases with age and subsequent treatment of these diseases. The amount of money involved would provide enough incentive for the pharmaceutical industry to pursue an avenue of research for development of pharmaceuticals aimed at muscle mass recovery and attenuation of loss. Therefore, opting for a GSK-3 inhibitor that is already FDA approved for another disease or in the final stages before approval, would reduce the costs for setting up and performing trials to confirm the inhibitor's functionality related to muscle mass maintenance and recovery. Because drug safety and application information would already have been thoroughly investigated, the application of the GSK-3 inhibitor for other purposes would be expedited.

Currently there are some clinical trials reported that are focusing on the role of GSK-3 in brain disorders like Alzheimer's disease and bipolar disorder. However, because these clinical trials do not focus on skeletal muscle, there have been no measurements of muscle mass or muscle function to supply useful data on the effects of systemic GSK-3 inhibitor administration on muscle. Therefore, the effects that those GSK-3 inhibitors have on skeletal muscle may be overlooked and potentially underappreciated. Consequently, such expected effects on skeletal muscle may readily become available by including additional functional outcome measurements that are relatively simple in ongoing clinical trials or to include them in upcoming ones.

As research is required to determine the effect of systemic pharmacological GSK-3 inhibition on skeletal muscle mass, an outline for a proof-of-concept clinical trial involving current patients suffering from a chronic disease who are at risk of or suffering from muscle mass loss is proposed. As mentioned above there are two catabolic situations, one being short-term and the other chronic, that result in either immediate large or long-term but slower muscle mass loss. Treatment here would generally consist of 1) recovery of muscle mass via rehabilitation therapy and 2) prevention or reduction of muscle mass loss upon admittance to a hospital. When aiming for muscle mass recovery using the GSK-3 inhibitor, the clinical trial would be best set-up within a multimodal approach as mentioned above. With the involvement of exercise, systemically administered doses of the GSK-3 inhibitor may potentiate the exercise-induced decrease of GSK-3 activity in the skeletal muscle. The exercise would also enhance localised drug absorption due to increased blood flow in the exercising muscles. The efficiency of this may depend on timing of drug administration as currently there are no suitable or sufficient means to achieve targeted drug delivery in the body. But beyond timing, and specificity in delivery there is specificity of the inhibitor itself. As mentioned on GSK-3 inhibitors in Chapter 3 of this thesis, specifically substrate-competitive inhibitors should deserve more development, which should focus on inhibitory specificity towards targets downstream of GSK-3 signalling involved in muscle mass plasticity.

Alongside improving muscle mass recovery, there is prevention of muscle mass loss from either short-term or chronic catabolic states. Both, though dose dependently, could benefit from chronic administration of a GSK-3 inhibitor to prevent muscle wasting.

Though we have mentioned that there is already some data available on the prolonged usage of lithium salt for GSK-3 inhibition that do not indicate any overt problems, other types of inhibitors could affect a different range of proteins besides GSK-3. Especially with patients that are on medication for longer term disease management, possible drug-drug interactions need to be determined ahead of time as much as possible. Nonetheless, by utilization of already approved and clinically available GSK-3 inhibitors possible patient trials could be implemented much earlier. Therefore, pharmaceutical companies stand to benefit from an expansion of the spectrum of medical conditions for which their specific GSK-3 inhibitor is currently being developed. This may encourage them to participate in such an endeavour and thereby a source of funding will be opened up for these clinical trials in the future.

### Broader impact of GSK-3 inhibition on muscle dysfunction

The role of GSK-3 is not limited to skeletal muscle mass regulation. There are also the intrinsic metabolic properties of the muscle to consider. In parallel with, but also independently of muscle mass loss, a reduction in muscle endurance may occur resulting from a shift in the biochemical properties of the muscle fibres. Muscle mass recovery from disuse atrophy is accompanied by a concomitant increased expression of genes involved in the regulation of muscle energy metabolism. The processes regulating skeletal muscle mass and metabolism are in part interlinked. However, the exact level of this remains poorly understood.

A well-known 'master' regulator of energy metabolism is the transcriptional coactivator peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) that regulates amongst others oxidative metabolism and mitochondrial content in the muscle. Expression of PGC-1α increases in response to exercise. In animal models overexpression of PGC-1α leads to a substantial increase in muscle mitochondrial content and decreased fatigability of the muscles. Furthermore, it reduces muscle catabolism to some extent, leading to prolonged preservation of muscle mass. This indicates the interlinked nature of mass and metabolism in muscle. There are however reports that in chronic diseases such as COPD PGC-1α expression regulation is perturbed indicating that there could be an option for targeting PGC-1α to affect muscle metabolism.

Reduced GSK-3 activity has been reported to increase muscle oxidative phenotype and mitochondrial biogenesis. This could possibly be resultant from GSK-3 repressing PGC-1α activity via increased degradation of PGC-1α. Although the exact mechanisms need to be further investigated, by inhibiting GSK-3 PGC-1α activity can be increased, thereby GSK-3 could potentially influence not only muscle mass but also muscle metabolism. Therefore, it becomes tempting to put GSK-3 as an interconnecting molecular regulator of both muscle mass and muscle metabolism. Therefore targeting GSK-3 pharmacologically could potentially have bigger therapeutic potential on skeletal muscle function.

## COMMUNICATION OF THE RESULTS

The normal spread of most of the scientific information produced from research performed at universities goes via publication in peer-reviewed scientific journals. Already, the information described in Chapters 2, 3, 4 and 6 of this thesis have been published, read and cited, which indicates the spread of the generated knowledge.

Abstracts and poster presentations at conferences allow for direct social interaction between various scientists. Of the current research presented in this thesis, data has already been shown at conferences in Canada, Germany and the Netherlands and a poster presentation of the data from Chapter 5 'has been scheduled for the European Respiratory Society Congress 2015 in Amsterdam.

## BEYOND THE THESIS

This thesis concerns the role of GSK-3 in myogenesis and recovery of atrophied muscle. The acquired new insights can be valorised by a continuation of the current research focusing on the role of GSK-3 in the regulation of skeletal muscle mass. Although muscle mass recovery from disuse atrophy does not require GSK-3 inactivation, as shown in this thesis, the findings that GSK-3β ablation improves the initial stage of reloading-induced muscle mass and myofiber cross-sectional area recovery indicates that a GSK-3 inhibitor could potentially aid in improving muscle mass recovery as part of a multimodal treatment approach. In addition to this, muscle function is not only determined by mass but also by intrinsic metabolic properties. The sensitivity of these intrinsic properties to GSK-3 modulation has not been addressed in the current thesis but is currently pursued in our research group. Although some aspects of the cell biology of skeletal muscle plasticity affected by GSK-3 modulation remain to be addressed in further fundamental research, the availability of clinically proven safe inhibitors of GSK-3 for other medical conditions may accelerate the utilisation of these GSK-3 inhibitors to assess their potential to modulate muscle wasting in clinical trials.

# Samenvatting

## INLEIDING

Hoewel de wereldbevolking nog steeds groeit, is deze toename in Nederland aan het krimpen. Aan de andere kant verlaat de babyboomgeneratie (1946 - 1955) sinds 2011 (65 jaar) de arbeidsmarkt. Hierdoor vindt er een versnelde vergrijzing van de Nederlandse bevolking plaats. Een bijkomende factor is dat de gemiddelde levensverwachting door verbeterde leefomstandigheden en medische kennis is toegenomen. Echter de toegenomen kans op het bereiken van een hogere leeftijd gaat gepaard met een toename van chronische ziekten, zoals chronisch hartfalen, COPD, nierfalen, reumatische artritis en kanker. Dit kan leiden tot een lagere kwaliteit van leven voor de patiënt en vervroegt overlijden. De behandeling van deze chronische ziekten levert een toenemende economische druk op de maatschappij. Die is op dit moment ongeveer 14% van het bruto binnenlands product (BBP; €607miljard in 2012). Als de trend in toename van deze hoger wordende kosten voor gezondheidszorg (zoals de afgelopen 15 jaar) doorzet, dan zal de economische druk op de maatschappij elke 15-16 jaar verdubbelen. Deze toenemende last is zowel voor het individu als maatschappelijk niet aanvaardbaar en geeft aan dat er iets moet gebeuren. Dit houdt in dat medische behandelingen en rehabilitatietherapieën moeten verbeteren om zo de leefomstandigheden te verbeteren en de stijgende kosten het hoofd te bieden. Het toepassen van rehabilitatietherapieën lijkt te helpen in het verbeteren van zowel de kwaliteit van leven voor de patiënten als het reduceren van medische kosten, maar er is zeker ruimte voor verbetering.

Bovengenoemde chronische ziekten hebben een diversiteit aan symptomen en oorzaken. Ze worden echter allemaal gekenmerkt door verzwakking en verlies van spiermassa. Dit gaat vaak gepaard met verminderde lichamelijke activiteit, kwaliteit van leven en medisch behandelingsresultaat, waardoor de kans bestaat op verergering van de ziekte of zelfs voortijdig overlijden. Daarom wordt het behandelen van spieratrofie gezien als een belangrijke pilaar in de rehabilitatietherapieën. Voor het ontwikkelen van goede rehabilitatietherapieën is meer inzichtelijke kennis nodig over 1) wat veroorzaakt spieratrofie en 2) hoe verloopt spierherstel waardoor het mogelijk zou moeten worden om, respectievelijk dit te verminderen en te verbeteren.

Daarom wordt in dit proefschrift de rol van het eiwit *glycogeen synthase kinase* (GSK-)3, een negatieve regulator van spiermassa, bij spierceldifferentiatie en spierherstel bestudeerd. Voor achtergrondinformatie wordt even kort de spiermassaregulatie in brede termen uitgelegd.



## SPIERMASSAREGULATIE

In de skeletspier vindt continu, op een laag niveau, een gebalanceerd proces van afbraak en opbouw plaats. Dit proces bestaat onder andere uit verandering in eiwitafbraak en –synthese en spiercel-kernverlies en -aanwas. Het verschuiven van die balansen kan leiden tot spierhypertrofie, -atrofie of herstel.

Voor spierherstel of hypertrofie is een verschuiving in de balans nodig wat resulteert in netto meer eiwitsynthese dan -afbraak. Dit kan gestimuleerd worden door versterking van de IGF-I/Akt- signalering die onder andere leidt tot inhibitie van GSK-3-activiteit. Echter vindt tijdens spieratrofie juist meer spiereiwitafbraak dan -synthese plaats. Dit kan veroorzaakt worden door verhoogde ontstekingsreacties en/ of glucocorticoïdsignalering, waarbij GSK-3 een rol speelt. Het proces van eiwitomloop berust op samenspraak van zowel afbraak- als synthese-signalering dat op meerdere

niveaus in deze signaleringsroutes plaatsvindt voor een efficiënte regulatie van die balans.

Spiervezels zijn, in tegenstelling tot andere celtypes, gedifferentieerde cellen die meerdere celkernen per cel bevatten. In de cel wordt de celkern omgeven door cytoplasma. In de spiercel voorziet iedere celkern een bepaalde regio van cytoplasma met genexpressieinformatie die onder andere dient om aan te geven welke eiwitten er aangemaakt kunnen worden. De aanname dat een celkern een dergelijke regio van informatie voorziet wordt de myonucleair domein-theorie genoemd. Deze theorie beschrijft dat tijdens spieratrofie het cytoplasmatisch volume afneemt. Als dit een bepaalde ondergrens voorbij gaat, ziet de spier dit als een overschot van celkernen. Op dat moment worden één of meerdere celkernen uit de spiervezel verwijderd door middel van celkernapoptose, zodat weer voldaan wordt aan het myonucleair domein-concept. Anderzijds gaat spierherstel of spierhypertrofie als gevolg van intense krachttraining gepaard met een toename van het cytoplasmatisch volume. Als deze cytoplasmatische toename de capaciteit van spiercelkernen om in die bepaalde spiervezel genexpressie te verzorgen te boven gaat, wordt de bovengrens van het myonucleair domein overschreden. Als gevolg moeten er celkernen toegevoegd worden aan de spiervezel om weer te voldoen aan de myonucleair domein-theorie. Deze nieuwe celkernen stammen af van de satellietcellen, die de 'stamcel-nis' vormen. Deze cellen liggen op de spiervezels en zijn normaliter inactief. Als deze cellen, bijvoorbeeld door spieroefeningen, het signaal krijgen dat er nieuwe celkernen nodig zijn voor de spiervezels in hun omgeving, beginnen zij zich te delen. Een deel van de dochtercellen zal de inactieve status weer aannemen terwijl het andere deel, genaamd myoblasten, zich verder klaarmaakt om te gaan fuseren met de spiervezels die extra kernen nodig hebben. Dit klaarmaken voor fusie houdt in dat die cellen spier-specifieke genen tot expressie gaan brengen onder leiding van 'muscle regulatory factors (MRFs)' zoals MyoD en myogenine.

## EFFECT VAN ANABOLE EN KATABOLE MEDICATIE TIJDENS EEN REHABILITATIETHERAPIE-STUDIE

Als je gezond bent, is krachttraining een goede stimulans om spiermassatoename te verkrijgen en zodoende verlies van spiermassa door veroudering, oftewel sarcopenie, tegen te gaan. Deze stimulus is echter minder effectief als primaire of incidentele behandeling bij chronisch zieken, vooral na acute exacerbatie van de ziekte bij die patiënten. Een reden hiervoor is onder andere een verminderde capaciteit voor het volbrengen van voldoende hoge intensiteit-trainingen die leiden tot spiermassatoenames. Dit hangt samen met de ernst van de chronische ziekte en de daaraan verbonden fysieke beperkingen bij de patiënt, maar ook met het ziekteproces zelf en bepaalde voorgeschreven medicatie die zorgen voor een katabole toestand bij de patiënt waarbij spiermassaverlies door blijft gaan. Juist deze patiënten, die beperkt zijn in het uitvoeren van de oefeningen met de voor spiermassawinst benodigde intensiteit, zouden baat kunnen hebben bij farmaceutische ondersteuning in hun streven naar spiermassatoename.

Onderzoeken naar farmaceutische interventie ter bevordering van spiermassaregeneratie zijn al eerder uitgevoerd. Hiervoor zijn anabole stimuli gebruikt zoals groeihormoon, insulin-like growth factor 1 (IGF-I), testosteron of derivaten hiervan. Deze anabool stimulerende factoren leiden in de cel tot activatie van de IGF-I/Akt-

signaleringsroute, welke zorgt voor het afremmen van de eiwitafbraak, stimulatie van eiwitsynthese, celproliferatie en verhoging van spier-specifieke genexpressie. Echter, gebruik van deze anabolen is ni*et alt*ijd effectief in het stimuleren van spiermassatoename en leidt vaak tot ongewenste neveneffecten. Hieruit is af te leiden dat een effectief therapeutisch middel nog niet is gevonden, of dat het gebruik van anabole middelen nog niet voldoende is geoptimaliseerd. De variabele resultaten van de gebruikte anabolen zijn mogelijk te wijten aan gelijktijdig gebruik met andere medicatie, zoals systemisch gebruik van glucocorticoïden bij patiënten met COPD om hun ziekte te behandelen.

Als voorbeeld hiervan heronderzochten wij een klinische trial aangaande het effect van anabole supplementatie voor spiermassaherstel tijdens een pulmonair rehabilitatieprogramma van patiënten met COPD. Er was gemiddeld een toename te zien in spiermassa na behandeling met het testosteronderivaat Nandrolone Decanoate (ND). Echter in tegenstelling tot onze verwachting, was het effect groter in de subpopulatie van met ND behandelde patiënten die tegelijkertijd een onderhoudsdosis glucocorticoïden (GC) gebruikten. Dit heeft geleid tot onderzoek naar een mogelijke interactie tussen GC-geïnduceerde eiwitafbraaksignalering en anabolen stimuli-geïnduceerde IGF-I/Akt-signaleringsroutes. Hierbij is gekozen voor een translationele aanpak, welke is beschreven in **HOOFDSTUK 2**. Wij hebben de klinische data van de ND supplementatie-studie opnieuw onderzocht waarbij de patiënten zijn opgedeeld op basis van glucocorticoïdengebruik en ND-toediening. Toen we keken naar spiermassatoename was duidelijk te zien dat juist de groep die beide medicaties gebruikte de meeste spiermassatoename had. De groep die alleen een onderhoudsdosis glucocorticoïden kreeg vertoonde zelfs een lichte afname van spiermassa dat een bekend neveneffect van dat medicijn op spiermassa is. Omdat de effecten van GC op spiermassaregeneratie nog weinig aandacht hebben gehad en omdat er mogelijk een interactie optreedt van GC met gelijktijdige activatie van de IGF-I/Akt-signaleringsroute, hebben wij dit met behulp van celkweekexperimenten verder onderzocht. Hierbij hebben wij gevonden dat GC spierceldifferentiatie verslechterde door middel van remming van eiwitsynthese en spiercelfusie. Echter, gelijktijdige toediening van GC en IGF-I zorgde juist voor een synergistische versterking van het spierceldifferentiatieproces. Er was meer spier-specifiek eiwit gevormd en een betere spiercelfusie opgetreden. Het synergistische effect is mogelijk deels te verklaren doordat GCs onverwacht zorgen voor verhoogde spier-specifieke genexpressie. Echter omdat de GC's eiwitsynthese remmen wordt dit nooit vertaald naar eiwit. Door toevoeging van IGF-I wordt juist die blokkade op eiwitsynthese weggenomen. Hierdoor is het synergistische effect van anabole steroïden en glucocorticoïden op spiermassaherstel in de patiënten met COPD mogelijk te verklaren.



## GSK-3: REGULATOR VAN SPIERMASSAPLASTICITEIT

Zoals aangegeven richt dit proefschrift zich op de activiteitmodulatie van GSK-3, een negatieve regulator van spiermassa, bij spiermassaregeneratie. GSK-3 is een eiwit betrokken bij zowel IGF-I/Akt-signalering als GC-geïnduceerde signalering. Echter GSK-3 is ook nog betrokken bij andere signaleringsroutes die een rol spelen in spiermassaregulatie. De huidige kennis over spiermassa regulatie bestaat uit de hierboven beschreven balansen van eiwit- en spierkernomloop die worden gereguleerd door verschillende signaleringsroutes in de spiercel. Bepaalde signalerings-eiwitten dienen als een soort hoofdregulator in de reactie op bepaalde stimuli, waardoor in een soort cascade-effect

veel processen tegelijkertijd aan- of uitgezet kunnen worden. Dergelijke eiwitten lenen zich vaak uitstekend als doelwit voor het ontwikkelen van farmaceutische middelen om cellulaire processen aan te sturen. Voor bijvoorbeeld het tegen gaan van spieratrofie maar ook voor het stimuleren en verbeteren van spierregeneratie/-herstel. GSK-3 is een signaaltransductie-eiwit dat uitgeschakeld wordt door de IGF-I/Akt-signaleringroute, waardoor het mogelijk een geschikt doelwit is voor farmacologisch ingrijpen in de sturing van spiermassaregulatie. Er zijn twee vormen van GSK-3, namelijk GSK-3α en GSK-3β, die elk gecodeerd worden door een apart gen in het genoom dat zich in de celkern bevindt. Deze twee varianten van GSK-3 vervullen veel overlappende functies maar hebben ook eigen specifieke functies binnen de cel. In zowel mens als muis is meer GSK-3β- dan GSK-3α-eiwit aanwezig in de spiercel. Daarom is in de literatuur veel onderzoek te vinden met vooral de focus of GSK-3β. Hierin is onder andere aangetoond dat inhibitie van GSK-3(β) leidt tot verminderde (spier)eiwitafbraak en verbeterde myogene differentiatie. Wij hebben een beschouwing geschreven op basis van de bestaande literatuur met de focus op GSK-3 als een hoofdregulator van spiermassaplasticiteit in **HOOFDSTUK 3**. Hierin wordt aandacht besteed aan hoe GSK-3(β)-activiteit wordt gereguleerd in de cel en wat voor farmaceutische modulaties er mogelijk zijn. Daarna wordt de focus verlegd naar de rol van GSK-3 in spiereiwitsynthese en -afbraak evenals zijn betrokkenheid bij spiercelkernverlies en -aanwas. De beschouwing wordt afgesloten met een kort betoog over hoe modulatie van GSK-3-activiteit een rol kan spelen bij het optimaliseren van zijn rol als hoofdregulator van spiermassa-adaptatie voor het behandelen van spieratrofie in toevoeging tot spiertraining, dieet en andere ondersteunende therapieën als een multimodale aanpak van het probleem.

## EFFECT VAN REGULATIE VAN GSK-3-ACTIVITEIT OP SPIERMASSA

Effectieve toepassing van farmaceutisch ingrijpen op GSK-3 om spieratrofie te verminderen en/of te voorkomen en spierherstel te bevorderen vereist betere kennis over wat de moleculaire, cellulaire, spier-specifieke en lichamelijke gevolgen hiervan zijn. Daarom is als **HOOFDHYPOTHESE** geformuleerd dat *inhibitie van GSK-3(β)-activiteit noodzakelijk en voldoende is om spierregeneratie te stimuleren*. Deze hypothese wordt getest in **HOOFDSTUKKEN 4-6** door zowel celkweek- als dierexperimentele modellen te gebruiken om de effecten van GSK-3activiteitmodulatie tijdens spier(cel)differentiatie/-regeneratie en -herstel te onderzoeken.

Voorgaand werk van onze groep heeft aangetoond dat via activatie van de IGF-I/Akt-signaleringsroute met behulp van IGF-I of de GSK-3-inhibitor lithium chloride (LiCl) de myogene differentiatie van spiercellen bevorderd kan worden in celkweekexperimenten. Dit effect ligt deels ten grondslag aan de voorkoming van GSK-3-inhibitie van het NFATc3 eiwit dat onder andere spier-specifieke genexpressie stimuleert. Opvallend was echter dat met het gebruik van LiCl de spiercelfusie zichtbaar beter was dan bij het gebruik van IGF-I. LiCl is ook bekend als een mimeticum voor de Wnt/β-catenin-signaleringsroute waarin GSK-3 betrokken is als deelregulator van β-catenin. Voor de Wnt/β-catenin-signaleringsroute is al eerder aangetoond dat het betrokken is bij spierhypertrofie en -regeneratie. Regulatie van β-catenin door GSK-3 is duidelijk beschreven door stimulatie met Wnt-eiwit maar niet door IGF-I. We hebben daarom in **HOOFDSTUK 4** onderzocht wat de rol van GSK-3 is in NFAT- en β-catenin-signalering in reactie op stimulatie met

IGF-I, Wnt, en farmacologische inhibitie van GSK-3 tijdens spiercelkweek. Onze resultaten laten zien dat tijdens spierceldifferentiatie stimulatie met Wnt-3a of LiCl, maar niet met IGF-I, β-catenin-geïnduceerde genexpressie is verhoogd. Verder was duidelijk te zien dat stimulatie met Wnt-3a spiercelfusie en spiervezelformatie verbeterde echter zonder verhoging van spier-specifieke genexpressie. LiCl was in staat zowel morfologische spiercelfusie als spier-specifieke genexpressie te verbeteren ten opzichte van controles terwijl IGF-I voornamelijk spier-specifieke genexpressie verbetert. Dit geeft aan dat er twee afzonderlijke signaleringroutes verlopen via GSK-3 die vrijwel onafhankelijk van elkaar spiercelfusie en spiervezelvorming, en spier-specifieke genexpressie reguleren tijdens spierceldifferentiatie.

Het overgrote aandeel van de literatuur aangaande de rol van GSK-3 in spiermassaregulatie en specifieker de rol bij regeneratie is vooral afkomstig uit spiercel-celkweekexperimenten '*in vitro*'. Daarom hebben wij ons in dit proefschrift gericht op het effect van GSK-3-activiteitmodulatie tijdens spierregeneratie/spierherstel '*in vivo*'.

Patiënten met chronische ziekten of kanker lijden aan spieratrofie die verergert naarmate hun ziekte ook verergert. Verder zijn deze patiënten ook minder in staat om voldoende krachttraining te volbrengen om effectieve spiermassa-instandhouding en/ of -regeneratie te bevorderen. Dit heeft vaak ni*et all*een te maken met de primaire symptomen van hun ziekte maar ook met de systemische effecten die de ziekte met zich meebrengt, zoals ontstekingsstoffen die zich door het lichaam verspreiden, of de behandeling ervan die zorgen voor een katabole toestand van het lichaam. Een andere factor die spieratrofie veroorzaakt is een verhoogde sedentaire levensstijl of verhoogde bedlegerigheid. Met andere woorden, niet voldoende gebruik maken van de spieren leidt tot spiermassaverlies.

Om deze component van spieratrofie te onderzoeken hebben wij gebruik gemaakt van een diermodel waarin wij omkeerbaar spieratrofie veroorzaken door de belasting van de achterpoten van muizen te reduceren wat resulteert in atrofie van de kuitspieren. Na twee weken van niet belasten van die spieren resulterende in spieratrofie worden deze spieren weer belast. Dit zou gezien kunnen worden als een trainingsprikkel die leidt tot spierregeneratie/ -herstel, omdat de geatrofieerde spieren in de kuit door het tijdelijke niet belasten van deze een verminderd vermogen hebben om het volledige lichaamsgewicht te dragen. De kuit bestaat hoofdzakelijk uit de gastrocnemius, plantaris en soleus spieren. Vooral deze laatste spier is zeer gevoelig voor deze manier van geïnduceerde spieratrofie.

Uit voorgaand werk van onze vakgroep is gebleken dat tijdens het her-belasten van de kuitspieren bij muizen in de soleus spier GSK-3β werd geïnactiveerd. Het is echter niet bekend of het deactiveren van GSK-3β een noodzakelijke actie is om spierregeneratie toe te staan. Om juist dat punt te onderzoeken hebben wij gebruik gemaakt van muizen die genetisch gemodificeerd zijn waardoor GSK-3 niet geïnactiveerd kan worden via de IGF-I/Akt-signaleringroute. Dit is mogelijk door een mutatie aan te brengen in de genen van zowel GSK-3α en GSK-3β , wat ervoor zorgt dat in plaats van het aminozuur serine, het animozuur alanine wordt ingebouwd bij de eiwitsynthese van GSK-3 (S21A voor GSK-3α en S9A voor GSK-3β). Deze verandering zorgt ervoor dvat die serine, nu een alanine, niet meer gefosforyleerd kan worden. De fosforylatie is het normale proces waardoor het eiwit geïnactiveerd kan worden na activatie van de IGF-I/Akt-signaleringroute. Deze gemodificeerde muizen, samen met controle-muizen zonder de beschreven mutatie,

zijn gebruikt in het spierontlasting-model om spierregeneratie te onderzoeken, waarvan de resultaten in **HOOFDSTUK 5** zijn beschreven. We vonden dat constitutief actief (CA) S21/9A GSK-3 geen effect heeft op eiwitomloopsignalering tijdens spierregeneratie. Echter lijkt spiercel-gerelateerde genexpressie voor proliferatie en differentiatie verhoogd. Verder is soleus spiermassatoename in de mutante muizen hoger na vijf dagen belasting dan in de controle-muizen. Echter de gemiddelde oppervlakte van spiervezeldwarsdoorsnedes in die spier is niet evenredig verhoogd en gelijk aan dat in de controle-muizen. Dit wijst mogelijk op meer oedeemvorming in plaats van werkelijk spiermassa en -functieherstel. Samenvattend lijkt echter dat het inactiveren van GSK-3 niet nodig is voor spiermassaherstel onder gezonde omstandigheden.

Er is meer dan genoeg overtuigend bewijs dat GSK-3β-inhibitie leidt tot vermindering van eiwitafbraak. Ook is er op spiercelkweekniveau voldoende evidentie dat GSK-3-inhibitie leidt tot verbeterde spierceldifferentiatie en -hypertrofie. Of dit ook standhoudt *in vivo* is nog onbekend. Om dit te onderzoeken hebben we GSK-3β gedeactiveerd in muizen door gebruik te maken van een systeem waarbij spier-specifiek het GSK-3β-gen verwijderd is. Deze mutanten en controle-muizen zijn eveneens volgens bovengenoemd diermodel –spierafbraak door het niet belasten van de spieren en -opbouw door belasting van diezelfde spieren– behandeld en de resultaten hiervan staan in **HOOFDSTUK 6** beschreven. Wij vonden dat eiwitomloopsignalering in grote lijnen niet was aangedaan door GSK-3β-afwezigheid. Er waren echter wel effecten te zien op satellietcelactiviteit, celproliferatie en genexpressie van myogene regulatoren. Deze waren verhoogd door afwezigheid van GSK-3β. Ook was spiermassa-herstel na vijf dagen belasting verder gevorderd dan bij controle-muizen. Dit geldt eveneens voor de toename van oppervlakte van de spiervezeldwarsdoorsnedes. Dit geeft aanleiding tot de aanname dat ook *in vivo* GSK-3-inactivatie kan leiden tot verbeterd spiermassaherstel na door ontlasting veroorzaakte spieratrofie.

## CONCLUSIE

Afrondend zijn alle inzichten en implicaties voortvloeiend uit de in dit proefschrift beschreven resultaten kritisch geëvalueerd en integraal besproken aan de hand van de meest recente literatuur in **HOOFDSTUK 7**, waarbij ook toekomstige onderzoeksopties zijn aangestipt met betrekking tot spiermassaregulatiemodulatie en de rol van GSK-3 als een potentieel farmacologisch doelwit hierin. Hoofdzakelijk is bediscussieerd of de bijdragen van de regulerende rol van GSK-3 en myonucleaire aanwas aan spiermassaherstel worden onderschat besluitend dat beide punten verder onderzoek vereisen. Rede hiervoor zijn dat enerzijds gebaseerd op de huidige, tevens in de literature beschreven, indicatieve uitkomsten dat spier-specifieke GSK-3β-inactivatie, behalve atrofie door niet belasten van spieren, diverse voor spieren atrofische effecten van katabole factoren kan doen reduceren en anderzijds dat door afwezigheid van GSK-3β er een verbetering is in het begin stadium van spierherstel. Daarmee aanleiding geeft om GSK-3 verder te onderzoeken als een potentieel farmacologisch doelwit ter beïnvloeding skeletspiermassa plasticiteit mogelijk in combinatie met andere componenten in een therapeutische multimodale behandelingsaanpak.

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# List of abbreviations

| | |
|---|---|
| 4E-BP1 | eIF4E-binding protein 1 |
| AIDS | acquired immunodeficiency syndrome |
| ALP | autophagy-lysosomal pathway |
| AMPK | adenosine monophosphate-activated protein kinase |
| ANOVA | analysis of variance |
| APC | adenomatous polyposis coli |
| AR | androgen receptor |
| ARBP | attachment region binding protein |
| ATCC | American Type Culture Collection |
| ATF-4 | activating transcription factor 4 |
| Atg | autophagy gene |
| ATP | adenosine-5'-triphosphate |
| AU | arbitrary units |
| Bax | Bcl-2-associated X protein |
| BBP | bruto binnenlands product |
| BCA | bicinchoninic acid assay |
| BCAA | branched-chain amino acid |
| BCAT2 | branched-chain amino acid transaminase 2 |
| Bcl-2 | Beclin-2 |
| Bim | Bcl-2-binding protein |
| BL | baseline |
| BMI | body mass index |
| Bnip3 | Bcl-2 and 19 kDa-interacting protein 3 |
| BSA | bovine serum albumin |
| BW | body weight |
| C/EBP-β | CCAAT/enhancer-binding protein β |
| C2C12 | C2C12 skeletal muscle cell line derived from *Mus musculus* |
| ca | constitutively active (e.g. caGSK-3β) |
| cDNA | copy DNA |
| CHF | congestive heart failure |
| CHIR | CHIR99021, GSK-3β inhibitor |
| CK1α | casein kinase 1α |
| CLP | cecal ligation and puncture |
| CM | conditioned medium |
| COPD | chronic obstructive pulmonary disease |
| COX | cyclooxygenase |
| CRF | chronic renal failure |
| CRP | c-reactive protein |
| CSA | cross-sectional area |
| Cys | cysteine |
| DAPI | 4',6-diamidino-2-phenylindole |
| DC | detergent compatible |
| Dex | dexamethasone |
| DM | differentiation medium |
| DMEM | Dulbecco's modified eagle medium |
| DMSO | dimethyl sulfoxide |

| | |
|---|---|
| dn | dominant-negative (e.g. dnGSK-3β) |
| DNA | deoxyribonucleic acid |
| DR4/5 | death receptor 4/5 |
| dsDNA | double-stranded DNA |
| DSHB | Developmental Studies Hybridoma Bank |
| DTT | dithiothreitol |
| Dvl | dishevelled |
| E3α-II | ubiquitin ligase (a.k.a. UBR2) |
| EDL | extensor digitorum longus |
| EDTA | ethylenediaminetetraacetic acid |
| EGF | epidermal growth factor |
| eIF | eukaryotic initiation factor |
| ER | endoplasmatic reticulum |
| ERK | extracellular signal regulated kinase |
| ESC | embryonic stem cells |
| Fasl | Fas ligand |
| FBS | fetal bovine serum |
| FDA | Food and Drug Administration |
| FEV1 | forced expiratory volume in 1 s |
| FFM | fat-free mass |
| FoXO | Forkhead Box O |
| FRAT | frequently rearranged in advanced T-cell lymphomas (a.k.a GBP) |
| fw | forward |
| Fz | frizzled |
| Gabarapl1 | GABA receptor associated protein 1 |
| Gadd45a | growth arrest and DNA damage-inducible 45a |
| GAPDH | glyceraldehyde 3-phosphate dehydrogenase |
| GBP | GSK-3-binding protein |
| GC | glucocorticoid |
| GDP | guanosine diphosphate / gross domestic product |
| Glul | glutamate-ammonia ligase |
| GM | growth medium |
| GOI | gene of interest |
| GR | glucocorticoid receptor |
| GRE | glucocorticoid responsive element |
| GS | glycogen synthase |
| GSK-3 | glycogen synthase kinase-3 |
| GSK-3β$^{-/-}$ | GSK-3β-null or GSK-3β knockout |
| GSK-3β$^{+/-}$ | retaining 1 GSK-3β allele |
| GSK-3β$^{+/+}$ | GSK-3β WT |
| GSK-3β$^{fl/fl}$ | double floxed GSK-3β |
| GTP | guanosine triphosphate |
| h | hour |
| HMK | halomethylketone |
| HS | hindlimb suspension |
| HSF-1 | heat shock factor 1 |

| | |
|---|---|
| Hsp90 | heat shock protein 90 |
| $IC_{50}$ | half maximal inhibitory concentration |
| IGF | insulin-like growth factor |
| IGF-IR | IGF-I receptor |
| IL-1β | interleukin-1β |
| IMPA | inositol monophosphatase |
| IP | immunoprecipitation |
| IR | insulin receptor |
| IRS-1 | insulin receptor substrate 1 |
| IVC | inspiratory vital capacity |
| kDa | kilodalton |
| KI | knock-in |
| KLF-15 | Krüppel-like factor 15 |
| KO | knock-out |
| L6 | L6 skeletal muscle cell line derived from Rattus norvegicus |
| LC3(b) | microtubule-associated proteins 1A/1B light chain 3(B) |
| LPS | lipopolysaccharide |
| LRP5/6 | lipoprotein receptor-related protein 5/6 |
| LSD | least statistical difference |
| LY | LY294002, PI-3K inhibitor |
| Lys | lysine |
| MAFbx | muscle atrophy F-box (a.k.a. atrogin-1) |
| MAPK | mitogen-activated protein kinase |
| MCK | muscle creatine kinase |
| MGF | mechano growth factor |
| MGSK-3β KO | muscle-specific GSK-3β knockout |
| MHC | myosin heavy chain |
| min | minute |
| MIRKO | muscle insulin receptor expression |
| MLC | myosin light chain |
| mM | millimolar |
| MMP | matrix metalloproteinase |
| MRF | muscle regulatory factor |
| mRNA | messenger RNA |
| mTOR | mammalian target of rapamycin |
| mTORC1 | raptor-mTOR complex 1 |
| mTORC2 | rictor-mTOR complex 2 |
| MuRF1 | muscle-specific RING finger protein 1 |
| MYH | myosin heavy chain |
| MyHC-f | myosin heavy chain fast |
| MyLC-1 | myosin light chain 1 |
| MyLC-3 | myosin light chain 3 |
| $Na_3VO_4$ | sodium orthovanadate |
| ND | not detectable / nandrolone |
| NFAT | nuclear factor of activated T cells |
| NFDM | non-fat dried milk |

| | |
|---|---|
| NF-κB | nuclear factor-κB |
| nM | nanomolar |
| NMDA | N-methyl-D-aspartate |
| NS | non-significant |
| OCT | Optimal Cutting Temperature compound |
| OXPHEN | oxidative phenotype |
| p- | phospho |
| $p70^{S6K}$ | p70S6 kinase |
| $p90^{RSK}$ | AGC kinase of the RSK family |
| PAGE | polyacrylamide gel electrophoresis |
| PAX7 | Paired Box 7 |
| PBL | problem-based learning |
| PBS | phosphate-buffered saline |
| PCNA | proliferating cell nuclear antigen |
| PCR | polymerase chain reaction |
| PDGF | platelet-derived growth factor |
| PDK | phosphoinositide-dependent kinase |
| PFA | paraformaldehyde |
| PGC-1(α) | peroxisome proliferator-activated receptor γ coactivator 1(α) |
| PI-3K | phosphatidylinositol 3-kinase |
| PKA | protein kinase A |
| PKB | protein kinase B |
| PKC | protein kinase C |
| PMSF | phenylmethylsulfonyl fluoride |
| PP1 | protein phosphatase 1 |
| PP2A | protein phosphatase 2A |
| PRED | prednisolone |
| qPCR | quantitative reverse transcription PCR |
| RA | rheumatoid arthritis |
| RCT | randomized controlled trial |
| REDD1 | regulated in development and DNA damage responses 1 |
| RG | reference gene |
| RL | reloading |
| RLU | relative light unit |
| RNA | ribonucleic acid |
| ROS | reactive oxygen species |
| RPL13A | ribosomal protein L13A |
| RT-qPCR | reverse transcription quantitative polymerase chain reaction |
| rv | reverse |
| SD | standard deviation |
| SDS | sodium dodecyl sulfate |
| SE(M) | standard error of the mean |
| Ser | serine |
| Sgg | shaggy |
| siRNA | small interfering RNA |
| STAT | signal transducer and activator of transcription |

| | |
|---|---|
| TBS | Tris-buffered saline |
| Tcf/Lef-1 | T-cell factor/lymphocyte-enhancer factor 1 |
| TDZD | thiadiazolidinone |
| Thr | threonine |
| TIP60 | histone acetyl transferase of the MYST family |
| T-loop | activation loop (in GSK-3 protein) |
| TNF | tumor necrosis factor |
| TnI | troponin I |
| tPK1 | human tau protein kinase |
| TRAIL | TNF-related apoptosis-inducing ligand |
| $tRNA_i^{Met}$ | initiator methionine transfer RNA |
| TSC1/2 | tuberous sclerosis protein 2 |
| Tyr | tyrosine |
| ULK1 | serine/threonine-protein kinase involved in autophagy |
| UPS | ubiquitin 26S-proteasome system |
| v/v | volume concentration |
| vc | vehicle |
| w/v | weight/volume percentage |
| WCL | whole cell lysate |
| WHO | World Health Organization |
| Wnt | Wingless/int |
| WT | wild-type |
| zw3 | zeste-white 3 |
| β2M | β2 microglobulin |
| βArr2 | β-arrestin 2 |
| β-gal | β-galactosidase |
| µM | micromolar |
| µm | micrometer |