

# Chapter 5

## Targeting the Type I TGF- $\beta$ Receptor for Treating Caveolin-3-Deficient Autosomal Dominant Limb-Girdle Muscular Dystrophy Type 1C and Muscle Wasting Disorders

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**Abstract** Caveolin-3, the principal scaffold protein in sarcolemmal caveolae, regulates signal transduction and vesicular trafficking. Dominant-negative mutations in the caveolin-3 gene (CAV3) cause autosomal dominant limb-girdle muscular dystrophy 1C (LGMD1C) and autosomal dominant rippling muscle disease (AD-RMD). Myostatin, a member of the muscle-specific transforming growth factor (TGF)- $\beta$  family, negatively regulates muscle growth and volume. We recently showed that wild-type caveolin-3 binds to and inhibits TGF- $\beta$  type I receptor (T $\beta$ RI), thereby suppressing intracellular TGF- $\beta$  signaling. In contrast, LGMD1C-causing mutant caveolin-3 activates T $\beta$ RI, resulting in muscle atrophy. Recently, small-molecule compounds suppressing activation of T $\beta$ RI, also known as activin receptor-like kinase 5 (ALK5), have been developed as anticancer agents. Oral administration of a T $\beta$ RI inhibitor, Ki26894, ameliorates muscle atrophy and weakness in a caveolin-3-deficient LGMD1C mouse model. The therapeutic effect of Ki26894 is associated with a reduction in TGF- $\beta$  signaling and an increase in the number of muscle precursor satellite cells. This suggests that the caveolin-3/T $\beta$ RI signaling pathway plays an important role in the pathogenesis of LGMD1C and that it regulates skeletal muscle size by controlling the number of muscle precursor cells. Consequently, drugs that target the TGF- $\beta$  pathway may have therapeutic potential for diseases characterized by muscle atrophy.

**Keywords** Caveolin-3 • LGMD1C • AD-RMD • ALK5 • T $\beta$ RI inhibitors

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## 5.1 Mutations in the Caveolin-3 Gene Cause Autosomal Dominant Limb-Girdle Muscular Dystrophy 1C and Autosomal Dominant Rippling Muscle Disease

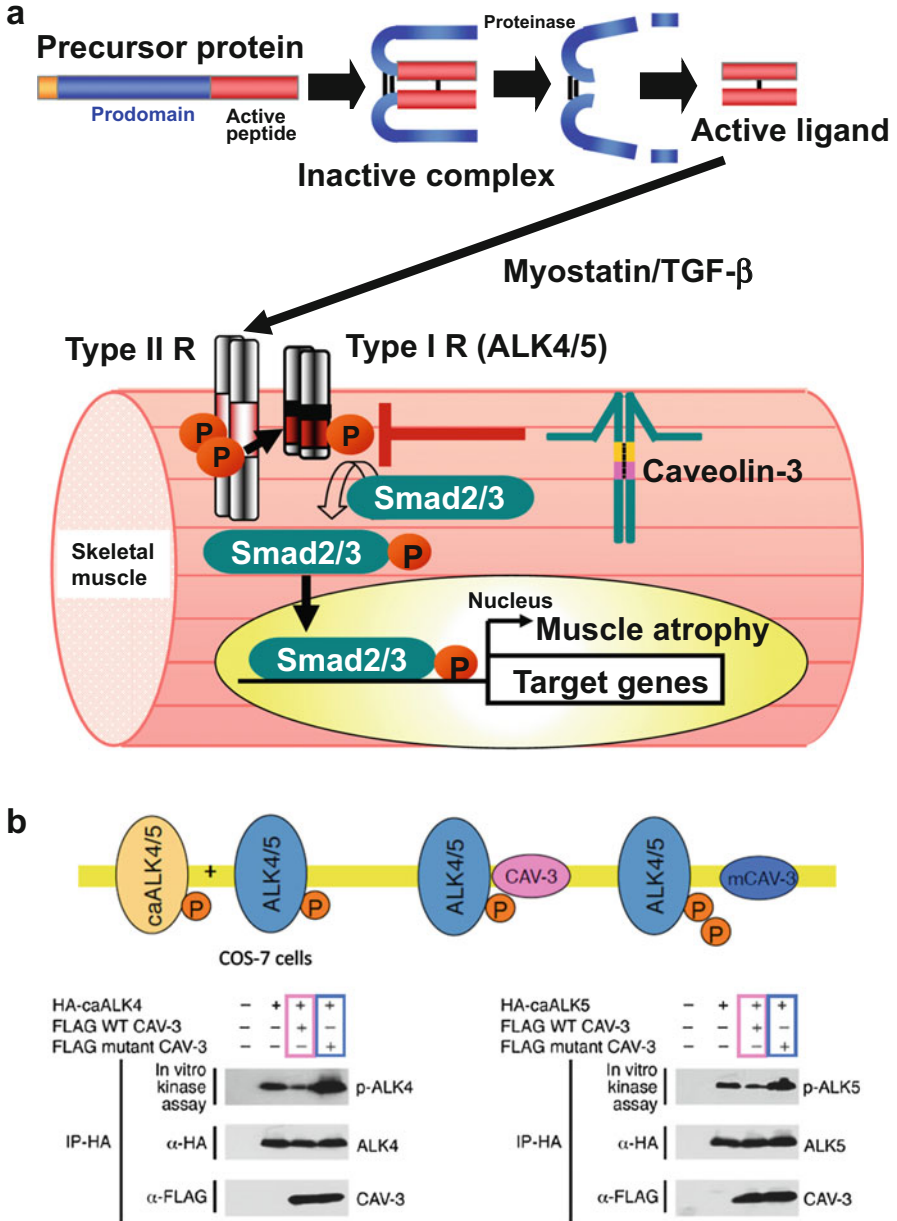
Caveolins, 18–24-kDa integral membrane proteins, play a crucial role in the formation of caveolae, flask-shaped pits in the plasma membrane, in terminally differentiated cells, such as adipocytes, endothelial cells, and skeletal muscle cells [1, 2]. Caveolin-1 and caveolin-2 are co-expressed and form hetero-oligomers in non-muscle cells, whereas caveolin-3 forms homo-oligomers in muscle cells [3, 4]. De novo synthesized caveolins assemble to form ~350-kDa oligomers in the endoplasmic reticulum (ER) and are subsequently targeted to the plasma membrane via the trans-Golgi network. Caveolins participate in diverse cellular processes, including vesicular trafficking, lipid metabolism, and signal transduction [1–4]. They bind to and regulate specific lipid and lipid-modified signaling proteins, including cholesterol, G-proteins, G-protein-coupled receptors, Src family kinases, Ha-Ras, and nitric oxide synthases [3, 4]. The interaction between caveolins and these molecules is mediated by a caveolin-binding motif on the target protein and a scaffolding domain in caveolins [5]. Numerous recent *in vitro* studies have demonstrated an important role of caveolins in regulating signaling by these various effectors. However, very few studies have examined the role of caveolins in signal transduction pathways *in vivo* [2, 3].

Mutations in the caveolin-3 gene (*CAV3*) have been identified in autosomal dominant limb-girdle muscular dystrophy 1C (LGMD1C) and autosomal dominant rippling muscle disease (AD-RMD) [6]. These mutations cause a significant reduction in sarcolemmal caveolin-3 protein levels and, to a lesser extent, mistargeting of the mutant protein to the Golgi complex from the sarcolemma [6–8]. The loss of caveolin-3 by mutations in *CAV3* in LGMD1C/AD-RMD patients may also impact the function of caveolin-3-binding molecules. Indeed, we and another group have shown that the enzymatic activity of neuronal nitric oxide synthase, which is suppressed by caveolin-3 *in vitro*, is elevated in skeletal muscles in a transgenic mouse model of LGMD1C and in LGMD1C/AD-RMD patients [7, 8]. Cytokine-induced nitric oxide production is increased in C2C12 myoblast cells transfected with LGMD1C/AD-RMD-causing mutant caveolin-3, compared with cells transfected with wild-type caveolin-3 [8]. Src, a membrane tyrosine kinase that regulates the balance between cell survival and cell death, is highly activated and accumulates in the perinuclear region, instead of the plasma membrane, in cells transfected with mutant LGMD1C/AD-RMD-causing caveolin-3 [9]. Furthermore, muscle-specific phosphofructokinase, a glycolytic enzyme, is significantly reduced in cells transfected with LGMD1C/AD-RMD-causing mutant caveolin-3, likely because of enhanced ubiquitin-mediated proteasomal degradation [10]. Of note, dysferlin, a protein involved in skeletal muscle repair, is deficient in LGMD2B/Miyoshi myopathy and is mistargeted to the cytoplasm in skeletal muscle of LGMD1C/AD-RMD patients, probably due to misdelivery of mutant caveolin-3 to the trans-Golgi network [11]. We previously showed that the ER stress molecule

GRP78 is upregulated in parallel with the gene dosage of mutant caveolin-3 (CAV3<sup>P104L</sup>) in skeletal muscle in a rodent model of LGMD1C [12]. Despite the recent insight into the function of wild-type and mutant caveolin-3, the pathogenic mechanisms underlying LGMD1C/AD-RMD remain fully understood.

## 5.2 Myostatin is a Promising Therapeutic Target for Muscle Wasting Disorders

Myostatin, a muscle-specific TGF- $\beta$  family member, was discovered as a negative regulator of muscle growth and muscle volume. Overexpression of myostatin causes severe muscle atrophy, whereas targeted disruption of the myostatin gene increases skeletal muscle mass in mice [13, 14]. Like most other TGF- $\beta$  family members, myostatin is synthesized as a precursor that undergoes proteolytic processing to generate a peptide with an N-terminal prodomain and a biologically active C-terminal disulfide-linked dimer [15, 16] (Fig. 5.1a). In the circulating inactive state, the N-terminal prodomain suppresses the biological activity of the C-terminal dimer [17, 18]. Once the prodomain is cleaved by BMP-1/tolloid-like proteases in the extracellular matrix, the C-terminal active dimer binds to and phosphorylates the activin receptor IIB/IIA (ActRIIB/IIA), a receptor type II serine/threonine kinase [18, 19]. This, in turn, recruits and phosphorylates activin receptor-like kinase 4/5 (ALK4/5), a receptor type I serine/threonine kinase, at the plasma membrane [16, 18–20]. The activation of a heteromeric complex of receptor type II and type I serine/threonine kinases results in the phosphorylation of intracellular effectors, including the receptor-regulated Smads (R-Smads), such as Smad2/3 [16, 21]. Phosphorylated R-Smads translocate to the nucleus from the cytoplasm, where they regulate the transcription of specific target genes leading to skeletal muscle atrophy [16]. In 2002, Khurana's group reported that administration of a neutralizing antibody against myostatin ameliorated symptoms in dystrophin-deficient *mdx* mice, including muscle atrophy, muscle weakness, and dystrophic changes [22]. However, a clinical trial using a humanized neutralizing antibody against myostatin showed no significant improvement in muscle strength in patients with Becker-type muscular dystrophy (BMD), facioscapulohumeral muscular dystrophy (FSHD), or LGMD [23]. Recently, highly potent myostatin signal blockers have been developed for clinical application in patients with muscle wasting disorders. A clinical trial of an antibody against the receptor type II serine/threonine kinase is currently ongoing in patients with sporadic inclusion body myositis (sIBM). This antibody, named bimagrumab, was designated as a breakthrough therapy by the US Food and Drug Administration (FDA) in 2013 [24, 25].



**Fig. 5.1** Caveolins regulate myostatin and related TGF- $\beta$  signaling at the type I receptor level. (a) Schematic representation of myostatin and related TGF- $\beta$  signaling and caveolin-3. Type I serine/threonine receptor (R) controls intramuscular signaling by activating R-Smad (Smad2/3). Caveolin-3 suppresses the type I receptor. (b) In vitro autophosphorylation of constitutively active type I TGF- $\beta$  receptors, ALK4 and ALK5. Cell lysates from COS-7 cells cotransfected with FLAG-tagged wild-type or mutant caveolin-3 (CAV3<sup>P104L</sup>) and HA-tagged constitutively active ALK4 (HA-caALK4) or ALK5 (HA-caALK5) were immunoprecipitated with anti-HA agarose. The in vitro kinase reaction was initiated by the addition of kinase reaction buffer and [ $\gamma$ -<sup>32</sup>P] ATP.

### 5.3 Caveolin-3 Suppresses the Type I TGF- $\beta$ Receptor: Relationship to the Pathogenesis of Caveolin-3-Deficient LGMD1C

Recently, caveolin-1 has drawn considerable attention as a critical regulator of TGF- $\beta$  signaling. Caveolin-1 binds to and suppresses activation of the TGF- $\beta$ 1 receptor type I serine/threonine kinase (T $\beta$ RI), which induces growth arrest in non-muscle cells [26]. Caveolin-1 facilitates ligand-bound T $\beta$ RI internalization and ubiquitin-mediated degradation [27]. In addition, caveolin-1 interacts with type II and type I receptors for bone morphogenic proteins (BMPs) in vivo [28]. These findings indicate that caveolin-1 regulates TGF- $\beta$  signaling at the receptor level.

Based on the role of caveolin-1 in non-muscle cells, we postulated that caveolin-3 likely inhibits myostatin and TGF- $\beta$  signaling in muscle cells. Indeed, we found several caveolin-3 binding motifs in the cytoplasmic kinase domain of the T $\beta$ RI [29]. We cotransfected caveolin-3 and a constitutively active T $\beta$ RI in COS-7 monkey kidney cells (Fig. 5.1b). We found that caveolin-3 colocalized and co-immunoprecipitated with the T $\beta$ RI. Furthermore, phosphorylation of the T $\beta$ RI was decreased by wild-type caveolin-3 and, conversely, increased by the P104L disease-causing mutant caveolin-3 in vitro [3]. These results indicate that wild-type caveolin-3 inhibits activation of the T $\beta$ RI, whereas mutant caveolin-3 enhances activation. Genetic introduction of the myostatin prodomain or administration of type II decoy receptor (ActRIIB-Fc) drastically rescues muscle atrophy in a LGMD1C mouse model (CAV3<sup>P104L</sup>). This therapeutic effect is associated with a decrease in TGF- $\beta$  signaling, which is elevated in mutant mouse muscle [29]. Thus, caveolin-3 normally suppresses myostatin and TGF- $\beta$  signaling at the type I receptor level, thereby preventing muscular atrophy. These findings suggest that excessive TGF- $\beta$  signaling, triggered by caveolin-3 deficiency, could participate in the pathogenesis of muscular atrophy in LGMD1C.

### 5.4 T $\beta$ RI Inhibitors Potentially Suppress Multiple TGF- $\beta$ Signaling Pathways

Small-molecule compounds antagonizing TGF- $\beta$  signaling at type I receptors (T $\beta$ RI) have recently been developed as targeted drugs for advanced cancer [30]. Tumor cells in advanced stages become refractory to TGF- $\beta$ -induced growth



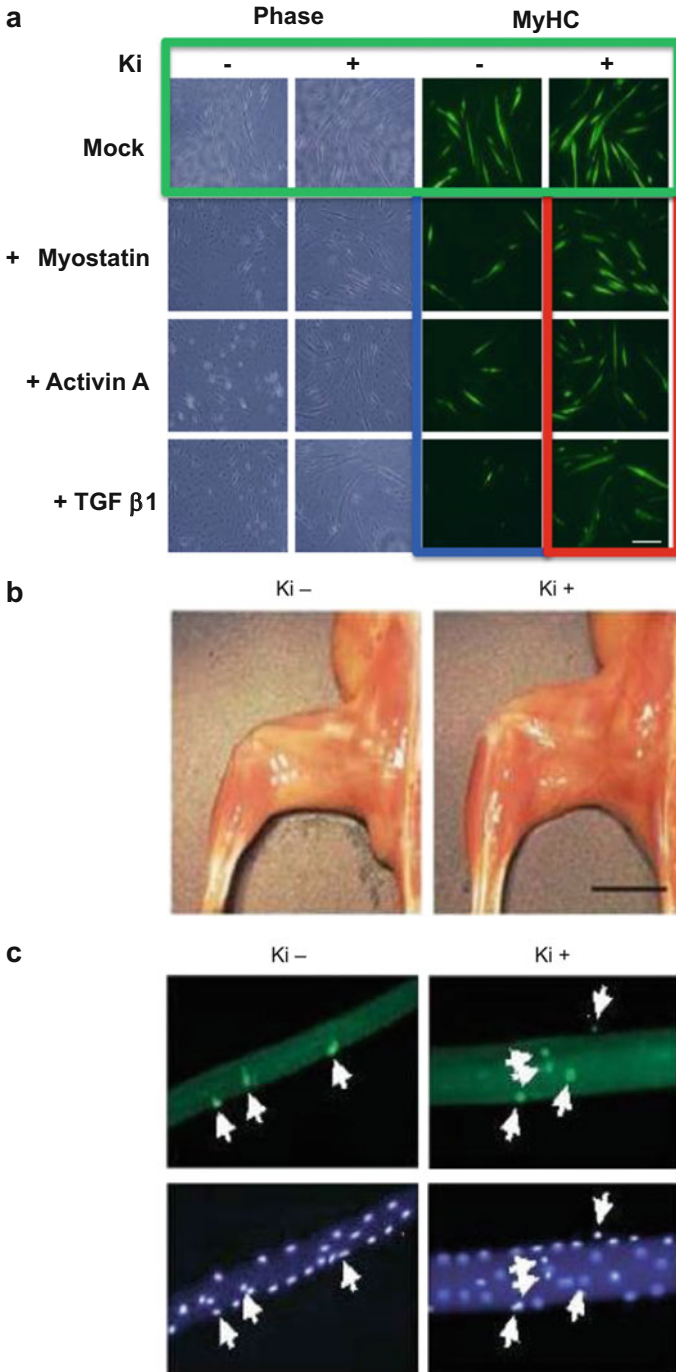
**Fig. 5.1** (continued) Phosphorylated caALK4 and caALK5 were detected by autoradiography. Immunoprecipitated caALK4, caALK5, and caveolin-3 were analyzed by immunoblotting with anti-HA ( $\alpha$ -HA) or anti-FLAG ( $\alpha$ -FLAG) mAb. Bands corresponding to phosphorylated type I receptor (in vitro kinase assay), total type I receptor ( $\alpha$ -HA), and caveolin-3 ( $\alpha$ -FLAG) are shown

arrest, but often overexpress TGF- $\beta$ 1, TGF- $\beta$ 2, and TGF- $\beta$ 3 [31]. TGF- $\beta$  family members promote the epithelial–mesenchymal transition, immunosuppression, and angiogenesis, resulting in tumor growth and metastasis [31]. Each member of the TGF- $\beta$  family binds to the type II receptor, which, in turn, recruits a T $\beta$ RI. Seven different T $\beta$ RI—activin receptor-like kinases 1–7 (ALK1–7)—determine the intracellular signaling specificity of the 33 members of the TGF- $\beta$  family. Small-molecule T $\beta$ RI inhibitors were originally developed to compete with the binding of adenosine triphosphate (ATP) to the kinase domain of ALK5, the T $\beta$ RI for TGF- $\beta$ 1–3 [15, 16]. T $\beta$ RI inhibitors have been found to suppress tumor enlargement and metastasis in the advanced stages of cancer in animals [32, 33]. Notably, these inhibitors also suppress a similar kinase domain in ALK4, the type I receptor for activin, and potentially block ALK4/5, the type I receptor for myostatin [30]. However, the effects of T $\beta$ RI inhibitors on activin, myostatin, and TGF- $\beta$ 1–3 signaling in muscle remain unclear.

## 5.5 A T $\beta$ RI Inhibitor Ameliorates Muscle Atrophy and Weakness in a Rodent Model of Caveolin-3-Deficient LGMD1C by Impacting Muscle Precursor Cells

Myostatin suppresses the differentiation of C2C12 myoblasts exposed to low-serum conditions [34]. Using an efficient retrovirus-mediated gene transfer system [35], we assessed the effect of a T $\beta$ RI inhibitor, Ki26894 [33], on the differentiation of C2C12 myoblasts expressing TGF- $\beta$ 1 or activin A, as well as myostatin (Fig. 5.2a) [36]. Transferring C2C12 myoblasts expressing an empty vector from high-serum (growth) to low-serum (differentiation) media caused them to fuse and form multinucleated myotubes. We then stained the cells with antibodies against myosin heavy chain (MyHC). Adding Ki26894 to the culture media enhanced myoblast fusion and myotube formation (Fig. 5.2a, green). In comparison, myotube formation was impaired in C2C12 myoblasts expressing myostatin, activin A, or TGF- $\beta$ 1 (Fig. 5.2a, blue), compared with controls harboring an empty vector. Notably, Ki26894 alleviated the impairment in myotube formation induced by the TGF- $\beta$  family members (Fig. 5.2a, red). T $\beta$ RI inhibitors enhance myoblast differentiation *in vitro* by suppressing the activity of several members of the anti-myogenic TGF- $\beta$  family, including activin, TGF- $\beta$ 1, and myostatin. Ki26894 also prevented the impairment in myotube formation induced by LGMD-causing mutant caveolin-3 [36].

We orally administered Ki26894 to a rodent model of caveolin-3-deficient LGMD (CAV3<sup>P104L</sup>) from 6 to 16 weeks of age. Muscle atrophy was reduced in Ki26894-treated animals compared with untreated mice (Fig. 5.2b). Ki26894 increased muscle weight, muscle strength, and myofiber size, as well as centrally nucleated regenerative myofiber number in the mouse [36]. Furthermore, Ki26894



**Fig. 5.2** T $\beta$ RI inhibitor Ki26894 (Ki) prevents muscular atrophy in a caveolin-3-deficient LGMD1C mouse model. (a) Ki26894 suppresses the impairment in myogenesis produced by myostatin, activin A, and TGF- $\beta$ 1. C2C12 myoblasts expressing an empty vector were grown in



ameliorated the reduction in the number of satellite cells attached to isolated single myofibers in the mice (Fig. 5.2c). Ki26894 also increased muscle mass and strength and elevated the number of satellite cells, in wild-type littermate mice. These findings indicate that T $\beta$ RI inhibitors strongly antagonize anti-myogenic TGF- $\beta$  signaling and alleviate muscle atrophy.

## 5.6 Future Directions

As mentioned above, caveolin-3 regulates skeletal muscle volume by suppressing multiple anti-myogenic TGF- $\beta$  signaling pathways. Pharmacological intervention strategies based on T $\beta$ RI inhibitors are expected to prevent the progression of muscle weakness better than those based on inhibitors that suppress myostatin signaling alone. An increase in the number of satellite cells and the enhancement of myoblast differentiation induced by this type of inhibitor suggests that they may hold substantial therapeutic potential for the treatment of muscle atrophy in various diseases, including muscular dystrophies, sarcopenia, and cancer cachexia. To evaluate their effect on muscle performance, T $\beta$ RI inhibitors should be tested in large-animal models of muscular atrophy. However, much additional study is required before clinical application of these compounds. For example, it is necessary to define the optimal, but nontoxic, dosage of these drugs for the treatment of individual patients with muscular atrophy. To facilitate this, we have developed an *ex vivo* myostatin activity assay as a convenient real-time biomonitoring system to determine how myostatin signaling is affected by the administration of these drugs in individual patients [36]. Furthermore, novel delivery systems targeting skeletal muscles also require development before the full potential of these drugs can be realized.

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**Fig. 5.2** (continued) DMEM containing 10 % FBS (growth medium) and then differentiated in DMEM containing 2 % horse serum (differentiation medium), without (–) or with (+) 10 nM Ki26894 for 6 days. The cells were then stained with the muscle differentiation marker myosin heavy chain (MyHC). (b) Oral administration of Ki26894 ameliorates muscular atrophy. Appearance of de-skinned hind limb from 16-week-old mice treated without (–) or with (+) Ki26894. Scale bar, 5 mm. (c) Fluorescence images of satellite cells attached to single myofibers isolated from the EDL muscles of a rodent model of caveolin-3-deficient LGMD (CAV3<sup>P104L</sup>) at 16 weeks of age. The white arrow indicates caveolin-1-positive satellite cells



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