STATE-OF-THE-ART REVIEW

Cellular and molecular pathways controlling muscle size in response to exercise

Michael Attwaters and Simon M. Hughes

Randall Centre for Cell and Molecular Biophysics, School of Basic and Medical Biosciences, King’s College London, UK

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Correspondence
S. M. Hughes, Randall Centre for Cell and Molecular Biophysics, School of Basic and Medical Biosciences, King’s College London SE1 1UL, UK
Email: s.hughes@kcl.ac.uk

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From the discovery of ATP and motor proteins to synaptic neurotransmitters and growth factor control of cell differentiation, skeletal muscle has provided an extreme model system in which to understand aspects of tissue function. Muscle is one of the few tissues that can undergo both increase and decrease in size during everyday life. Muscle size depends on its contractile activity, but the precise cellular and molecular pathway(s) by which the activity stimulus influences muscle size and strength remain unclear. Four correlates of muscle contraction could, in theory, regulate muscle growth: nerve-derived signals, cytoplasmic calcium dynamics, the rate of ATP consumption and physical force. Here, we summarise the evidence for and against each stimulus and what is known or remains unclear concerning their molecular signal transduction pathways and cellular effects. Skeletal muscle can grow in three ways, by generation of new syncytial fibres, addition of nuclei from muscle stem cells to existing fibres or increase in cytoplasmic volume/nucleus. Evidence suggests the latter two processes contribute to exercise-induced growth. Fibre growth requires increase in sarcolemmal surface area and cytoplasmic volume at different rates. It has long been known that high-force exercise is a particularly effective growth stimulus, but how this stimulus is sensed and drives coordinated growth that is appropriately scaled across organelles remains a mystery.

Introduction
The regulation of cell size is a fundamental aspect of biology but remains poorly understood. The importance of cell size control is underscored by the evolutionarily conserved mechanisms that regulate cell growth, perturbations in which result in disease [1]. Growth pathways are activated during development

Abbreviations
4EBP, eukaryotic initiation factor 4E-binding proteins; ActRIIB, activin receptor IIB; Ankrd2, ankyrin repeat domain 2; Arpp, ankyrin repeat protein with PEST and proline-rich region; BFR, blood flow restriction; Ca 2+, calcium; CaM, calmodulin; CaMK, calmodulin-dependent protein kinase; CARP, cardiac ankyrin repeat protein; CNTF, ciliary neurotrophic factor; CsA, cyclosporin A; DARP, diabetes-associated ankyrin repeat protein; DGKζ, diacylglycerol kinase ζ; DHRP, dihydropyridine receptor; DTA, diphtheria toxin A; ERK, extracellular signal-regulated kinase; eEF2, eukaryotic elongation factor 2 FAK, focal adhesion kinase; HIF-1α, hypoxia-inducible factor α; IGF-1, insulin-like growth factor 1; IL, interleukin; IRS1, insulin receptor substrate 1; JNK, Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MARP, muscle ankyrin repeat protein; Meqf2, myocyte enhancer factor 2; MLP, muscle LIM protein; MS, mechanosensitive; MuSC, muscle stem cells; MuSK, muscle-specific kinase; NDS, nuclear domain size; NFAT, nuclear factor of activated T cells; NMJ, neuromuscular junction; p70S6k, ribosomal protein S6 kinase; PA, phosphatidic acid; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator 1 alpha; PKC, protein kinase C; PLD, phospholipase D; ROS, reactive oxygen species; SERCA, sarcoplasmic reticulum Ca2+ATPase; SR, sarcoplasmic reticulum; SRF, serum response factor; TGF-β, transforming growth factor-β; TORC1, target of rapamycin complex 1; TRP, transient receptor potential.
and in adulthood by instructive signals from hormones and additional factors that can impinge on these mechanisms, such as nutrients and mechanical loading [2]. In adults, many cells maintain their size for years but retain the ability to proliferate and form new tissue. Some cells, however, can alter their size after permanent withdrawal from the cell cycle. The mature skeletal muscle fibre is one such example of a nondividing cell that can alter its size dramatically. Following exercise, particularly high-force exercise, adult muscle will grow due to enlargement of existing muscle fibres [3]. By contrast, during periods of inactivity, such as bed rest or exposure to a weightless environment, individual muscle fibres atrophy [3]. Despite impressive historical progress, however, it remains unclear at molecular and cellular levels exactly how muscle use and, in particular, exercise regulate muscle size and strength (Fig. 1).

Growth of whole muscle might arise from an increase in the size of existing muscle fibres (i.e. hypertrophy) and/or new fibre formation (i.e. hyperplasia) (Fig. 2). Whereas the ability of existing fibres to hypertrophy with exercise is unquestioned [4], whether hyperplasia also contributes to growth is unclear. Early animal studies reported an increase in fibre number based on indirect counts of fibres from histological cross-sections [5,6], but these estimates can be influenced by changes in muscle architecture [7] and longitudinal growth of intrafascicularly terminating fibres [5]. Direct counts of muscle fibres using nitric acid digestion techniques, where each fibre is teased apart and individually counted, revealed no evidence of hyperplasia after overload [8–10], or only a small (9%) increase in fibre number [11]. An exception appears to be when avian wing muscles are chronically stretched, resulting in a ~50% increase in total fibre number [12], but this effect may be species- or stimulus-specific and/or explained by longitudinal splitting of existing fibres [13,14]. Moreover, support for hyperplasia in humans is limited [15]. What is unchallenged is that enlargement of existing fibres contributes substantially to muscle growth in both animal and human studies of resistance exercise [4].

Each muscle fibre is a multinucleate syncytial cell, generated by the fusion of precursor myocytes. Hypertrophic growth of muscle fibres in response to overload is accompanied by an increase in myonuclear content [16], believed to be donated by satellite cells, the resident tissue-restricted muscle stem cells (MuSC) [17]. This observation lends credence to the myonuclear domain hypothesis, which states that each nucleus can support a finite maximal volume of cytoplasm and that growth beyond a certain limit requires nuclear donation from myonuclear fusion into the growing fibre (Fig. 2). Genetic cell lineage tracing experiments have confirmed that MuSC contribute nuclei to muscle fibres following exercise [18,19]. Evidence for a role in subsequent growth comes from the findings that overload-induced muscle hypertrophy is blunted upon diphtheria toxin (DTA)-mediated ablation of the MuSC pool [20,21] or when MuSC fusion is blocked through Myomaker deletion [22]. However, at least in
mature adult mice, hypertrophy in the short term (2 weeks) can proceed in the absence of MuSC [21,23]. This is in line with a model in which initial growth occurs without myonuclei addition, causing domain size increase, which triggers MuSC recruitment to return domain size to a set point. Non-fusion-mediated communication between MuSC and muscles fibres may also contribute to growth, although this area of research is in its infancy [24,25]. How MuSC are activated and contribute to growth, and how myonuclear domain size is controlled [26,27] remain unclear.

Considerable progress has been made in identifying signalling networks involved in exercise-induced muscle hypertrophy, many of which converge on TORC1 to regulate protein synthesis and ubiquitination or autophagy to control protein degradation (see below for detailed description; these have been described in some excellent reviews [28–32]). However, a fundamental question that remains to be answered is precisely how exercise initially triggers the activation of these pathways to control fibre growth. Theoretically, the initial growth trigger might arise at any of the following major steps in the excitation–contraction cascade: (i) muscle action potential pattern, initiated by neurotransmitter action due to action potential in motor nerve, (ii) rise in cytoplasmic free calcium (Ca^{2+}) concentration after release from the sarcoplasmic reticulum (SR), (iii) conformational change on the thin filament permitting actomyosin ATPase activation and facilitating rounds of cross-bridge cycling, (iv) generation of the mechanical force that produces muscular work or (v) Ca^{2+} uptake into SR driven by SERCA, catalysing further ATP turnover (Fig. 3A). Resolving the trigger(s) of muscle growth requires the careful elimination of specific elements of neuromuscular activity to control and isolate the influence of each aspect of the excitation–contraction process. This is experimentally difficult to achieve and commonly overlooked. In the literature, various modes of overload, including synergist ablation, electrical stimulation or resistance exercise, are often referred to as a ‘mechanical stimulus’ (e.g. [33–36]). Yet, mechanical factors are almost never controlled independently from other correlates of neuromuscular activation. The complex relationship between action potentials, cytoplasmic Ca^{2+} status, mechanical force and energy utilisation makes it difficult to separate mechanotransduction mechanisms from the wider context of the muscle fibre electrophysiology and biochemistry. As a case in point, high-force muscle contractions are a more potent growth stimulus than low-force contractions, but this does not necessarily speak to the importance of force per se because the magnitude of force output is tightly controlled by changes in motor unit recruitment [37] and firing frequency [38]. Hence, resistance exercise and synergist ablation are not clean models of mechanical activation, as changes in the nervous input, Ca^{2+} signalling and energy status might each also influence muscle signalling and growth.

Fig. 2. Distinct cellular processes in three modes of muscle growth. Skeletal muscle can grow in three ways, or modes. Growth resulting from formation of new muscle fibres (Mode 1) predominantly occurs during early life, in utero in mammals, and contributes little to postnatal size control in mammalian species. Subsequently, muscle size is regulated by addition of new nuclei to pre-existing muscle fibres through activation and fusion of muscle stem cells (MuSC) (Mode 2). Growth of pre-existing fibres also occurs by increase in nuclear domain size (NDS, red boxes), the volume of cytoplasm per nucleus (Mode 3). Atrophy can readily reverse Mode 3, but whether and how nuclei might be lost from fibres is unclear. During sarcopenia of ageing muscle, some ‘reversal’ of all three modes appears to occur, although the mechanism(s) and the role of reducing exercise remain to be determined.
In view of these considerations, the aim of this review was to address formally the evidence for and against each aspect of ‘muscle activity’ as a growth trigger. But first, we outline the local and systemic control of muscle growth, which provides the context for the effects of exercise, before considering each potential activity sensor in turn.

**Systemic versus local control of skeletal muscle size**

Circulating hormones and nutrients influence muscle growth and development throughout life. Such systemic control is exemplified by starvation, where adaptive hormonal and metabolic responses drive the release of gluconeogenic amino acids from the muscle for glucose production in the liver and kidneys, ultimately providing fuel for vital organs [39]. An obvious feature of starvation is muscle wasting, but it is not only in extreme scenarios that muscle mass is controlled by systemic influence. During development, the appropriate balance of circulating growth factors, mitogens and nutrients all impact on muscle mass and function [40,41]. Hormones and nutrient availability continue to control growth during postnatal development and in adulthood. Moreover, proper nutrition supports muscle maintenance in the elderly, mitigating sarcopenia [42]. Thus, although the focus of the present review is on local control of muscle growth, that is, hypertrophy of individual exercised muscle fibres or whole muscles, local adaptation occurs in the context of ongoing systemic control over whole-body muscle mass, which may alter local responses to exercise stimuli.

In general, when considering the specific effect of exercise, one assumes a stable baseline steady state, in which protein synthesis is balanced by degradation; this steady-state turnover is thought to facilitate repair and adaptation. At least in adults, however, inhibition of steady-state protein degradation alone is insufficient to account for the rapid hypertrophy of muscle in exercise-triggered growth scenarios [43]. Thus, while alterations in catabolic pathways may contribute to exercise-induced growth, we focus on anabolic pathways as the major exercise drivers of growth. Catabolic pathways, which can be highly activated in atrophic conditions such as denervation, bed rest, cancer cachexia or ICU-acquired muscle weakness, have been extensively reviewed elsewhere [44,45].

**How systemic signals control muscle mass**

At a cellular level, how growth is controlled is not clear. It is often assumed that growth simply requires more synthesis of cellular components, and the primary focus has been on proteins. However, to grow...
cells must increase their plasma membrane area, lipid content and balance between cell surface and internal membranes. How such changes are coordinated with protein synthesis change during growth of muscle is essentially unexplored. It is conceivable that the growth-initiating event is increased in cell volume and that protein metabolism then adapts to fill the expanded cytoplasm with contents, much as one’s shopping is limited by the size of one’s basket. How physiological stimulators of muscle growth expand basket and shopping in parallel is an important future area of study.

Despite the above consideration, net loss or gain of muscle protein depends on the balance between rates of protein synthesis and protein degradation. A net gain of muscle protein (e.g. during growth) results when synthesis exceeds degradation, whereas net loss of muscle proteins (e.g. during starvation) occurs when breakdown exceeds synthesis. Muscle protein metabolism is affected by various factors, including time of day, feeding, disease and ageing. The action of circulating hormones is a key controller of protein turnover in each situation. Known hormonal factors implicated in muscle size control include testosterone [46], insulin and insulin-like growth factor 1 (IGF-1) [47–50], myostatin [51], β-adrenergic signalling [52,53] and glucagon [54,55]. Among the most studied of these stimuli are IGF-1 and myostatin and, as we shall see, their downstream signalling pathways, or even they themselves acting as autocrine regulators, may be involved in the local growth mechanism. The following sections give an overview of the mechanisms of growth control by IGF-1 and myostatin.

**IGF-1/PI3K/Akt/TOR**

The IGF-1 pathway is initiated by IGF-1-like ligands binding to their receptors, which in turn induce receptor transphosphorylation and recruitment of the insulin receptor substrate 1 (IRS1) [56]. Downstream of this mechanism is the PI3K/Akt pathway, one of the branches of which involves the activation of TOR signalling [48,57]. Overexpression of IGF-1 [49,50,58] or Akt [57,59,60] results in skeletal muscle hypertrophy and increased force output. These effects are thought to be largely mediated through TOR, an evolutionarily conserved serine/threonine kinase found in two complexes, TORC1 and TORC2. Whereas the consequences of TORC2 signalling are only beginning to be unravelled [61], TORC1 is known as a key regulator of cell growth [62], primarily by stimulating protein synthesis. Major downstream TORC1 targets in this respect are the 70 kD ribosomal protein S6 kinase 1 (p70S6k1) and eukaryotic initiation factor 4E-binding proteins (4EBPs) [63]. TOR is required during development, as global knockout mice die during early embryogenesis [64,65]. Mice lacking TOR specifically in muscle from birth are viable but exhibit severe myopathy and premature death [66]. By contrast, recent work has challenged the notion that TORC1 signalling is needed for the maintenance of muscle mass in adulthood, as muscle atrophy does not result after inducible deletion of raptor, a major TORC1 component needed for TOR activity, in muscle [67–69]. As described below, however, there is strong evidence that TOR plays a major role in overload-induced muscle growth (see ‘Muscle activity as the key local regulator of muscle size’). These findings taken together lead to the interesting hypothesis that there is a fundamental difference in how muscle mass is maintained (TOR-independent) from the mechanism of exercise-driven muscle growth (TOR-dependent).

**Myostatin is a negative regulator of muscle size**

Myostatin, a member of the transforming growth factor-β (TGF-β), is a negative regulator of muscle mass. Natural genetic mutations in myostatin result in a profound hypertrophic phenotype that is conserved across multiple species [70–74]. In the mature animal, transient overexpression of myostatin results in skeletal muscle atrophy [75], whereas its inhibition produces hypertrophy [76]. Mechanistically, myostatin binds to the activin receptor IIB (ActRIIB), leading to the initiation of intracellular signalling via phosphorylation and activation of the transcription factors Smad2 and Smad3 [51]. Once activated, Smad2 and Smad3 translocate to the nucleus to inhibit the transcription factor JunB, a positive regulator of muscle growth [77,78]. While the precise mechanism of muscle mass regulation is not well understood, some evidence indicates that myostatin signalling impinges on the Akt/TOR pathway [77,79]. Hence, there may be multiple signalling pathways, which interact with each other to control growth.

**Muscle activity as the key local regulator of muscle size**

The extent of hypertrophy in response to manipulation of systemic signals differs between different muscles. For instance, myostatin deficiency preferentially results in fast type muscle hypertrophy. Yet, the myostatin signal has been removed from all muscles of the body, begging the question: Why are different muscles affected differently? One possibility is that local mechanisms are critically important. This view is supported...
by the simple observation that exercise-induced growth is restricted to the exercised muscle(s), despite large elevations in systemic hormones [80]. A role for circulating signals in local muscle growth might be permissive, rather than instructive, which is consistent with evidence that a minimal basal level of testosterone is needed to support local muscle hypertrophy in response to overload [81,82]. The general bodily hormonal status may modulate the gain on how sensitive a muscle is to changes in local signals that are intrinsically driven by excitation–contraction coupling of the exercised muscle(s).

Systemic and local inputs appear to share similar targets, particularly the TOR pathway, suggesting a critical control point in size regulation. This may be because certain growth factors known to regulate TOR, such as IGF-1, are also produced locally [83], suggesting autocrine/paracrine control of muscle size (Fig. 3B). Support for this notion comes from the finding that muscle-restricted IGF-1 overexpression leads to fibre hypertrophy [49,58]. However, this model has been challenged by Spangenberg et al. [84] who showed that transgenic mice expressing a muscle-specific dominant negative IGF-1 receptor have no hindrance in their ability to undergo exercise-induced muscle growth. IGF-1 signalling was also not necessary for exercise-induced TOR activation, suggesting an alternative upstream mechanism [84]. Nonetheless, skeletal muscle-specific knockout of PDK1, a major component in the PI3K signalling pathway, results in muscle atrophy under basal conditions and impairs overload-induced hypertrophy [85]. Activation of the TORC1 pathway occurs minutes after the onset of muscle overload [86,87] and correlates with the induction of hypertrophy [88]. Bodine and colleagues first observed that inhibition of TORC1 activity by rapamycin could prevent muscle hypertrophy in response to synergist ablation overload [57]. The inhibitory action of rapamycin on activity-stimulated growth and muscle protein synthesis was later confirmed by independent laboratories [89–91]. Low doses of rapamycin similarly prevented resistance exercise-induced muscle protein synthesis in humans [90]. Moreover, genetic inhibition of TORC1 by muscle-specific conditional deletion of raptor reduced synergist ablation-induced muscle hypertrophy in mice [67]. Thus, TORC1 is considered essential for exercise-induced muscle hypertrophy, although whether TOR activation is the key causal route to growth remains undetermined.

Several lines of evidence suggest the importance of TOR-independent pathways. Unlike the synergist ablation overload model, fibre growth in response to electrical stimulation training is only partially (50%) blocked by rapamycin treatment [92]. Moreover, despite blunting the hypertrophic response, TORC1 inhibition by raptor deletion in mice does not prevent stimulated increase in protein synthesis [67]. Thus, the case is strong for TORC1 as a major regulator of exercise-induced muscle growth, but additional TOR-independent mechanisms may also be important. This raises interesting questions about the identity of potential TOR-independent events that might be involved in the growth process. If future work can define such pathways, the mechanism by which exercise triggers muscle growth may become clearer. One possibility is activity of the mitogen-activated protein kinase (MAPK) pathway(s) in response to exercise. MAPKs are a family of evolutionarily conserved serine/threonine kinases subdivided into three major classes: the extracellular signal-regulated kinases 1 and 2 (ERK1/2), c-Jun N-terminal kinases (JNK) and p38-MAPKs (p38). One of the earliest reported responses downstream of muscle activity in the hypertrophic response was upregulation of the c-Fos and c-Jun proto-oncogenes [93,94]. Such ‘immediate-early’ responses are known to arise from signalling through the AP1 protein, itself a dimer of c-Fos and c-Jun proteins [95,96]. Thus, these early data suggested activation of the MAPK pathway(s). Exercise is a potent stimulus for ERK1/2 and JNK1/2 phosphorylation (activation) in animals and humans [97,98]. In support of a role in muscle hypertrophy, loss of JNK1 and JNK2 from skeletal muscle entirely prevents muscle fibre growth in response to synergist ablation overload, showing that some level of JNK activity is required [99]. Once activated, there are several potential mechanisms by which JNK might trigger skeletal muscle hypertrophy, including activation of transcription factors with roles in muscle growth [100] and crosstalk with TOR signalling [101]. Thus, coordinated JNK/TOR signalling may positively regulate muscle hypertrophy in response to exercise. The requirement for ERK1/2 or p38 in exercise-induced muscle growth awaits muscle-specific loss-of-function studies.

Another pathway of possible importance is Hippo-related mechanosensing [102]. Hippo pathway signalling through Yap and Wwtr1/Taz proteins can modulate various muscle-active transcription factors, such as Tead proteins that were originally described as regulators of a common motif in the regulatory elements of muscle-specific genes [103]. Yap activity is required for normal MuSC and C2C12 myoblast behaviour [104–106] and can be a positive regulator of muscle fibre size [107,108], possibly acting through maintenance of the neuromuscular junction [109]. Wwtr1 function in muscle size control remains to be
investigated. In Drosophila, loss of function of the sole Yap/Wwtr1 orthologue yorkie reduces flight muscle fibre size [110]. In summary, the role of Hippo signalling in exercise-driven muscle growth remains to be determined.

Whereas the pathways mentioned are likely significant influences on muscle size homeostasis, the primary trigger/s of growth in response to exercise must be somewhere in the excitation–contraction coupling cascade (Fig. 3A). We begin at the level of the motor nerve, the first step in the road to muscle contraction.

Electrical signals from the motor nerve

Over half a century ago, the cross-innervation experiments of Buller, Eccles and Eccles provided compelling evidence for the control of muscle phenotype by the nervous system [111]. Their findings showed that the contractile characteristics of the slow-twitch soleus muscle became ‘faster’ when reinnervated by nerve fibres normally supplying the fast-twitch flexor digitorum longus muscle; the converse change occurred for fast muscle innervated by slow nerve fibres. Transient atrophy occurring prior to successful cross-innervation was recovered in both cases. Two hypotheses were put forward to explain these findings. The first hypothesis was that the determining factor is the activity pattern imposed by the nerve on the muscle. The second hypothesis, termed the neurotrophic hypothesis, stated that muscle phenotype is controlled by nonelectrical signals from the motor nerve (Fig. 3B). An example of this type of neurotrophic control is the interaction between nerve-derived agrin, a glycoprotein, and its muscle membrane receptor, a process required for the formation of the neuromuscular junction (NMJ) [112,113]. Local signalling may also explain the synthesis of distinct myosin isoforms in muscle fibres at regions of ectopic innervation [114]. Putting these local effects aside, however, the neurotrophic hypothesis for the regulation of muscle size has been discarded in part because, despite exhaustive searches, no nerve-derived molecule has been demonstrated to be essential for inducing hypertrophy.

Compelling evidence for the importance of impulse activity came from Lomo and colleagues in 1974 [115], who showed that direct electrical stimulation of denervated muscle restores muscle mass. Thus, muscle size can be controlled by the pattern of electrical activity alone in the absence of the motor nerve and independent of putative neurotrophic factors. Subsequent work in denervated muscles confirmed that nerve-like activity could restore normal force output and fibre size [116,117]. Later sections of this review focus on how muscle activation could be sensed through calcium (Ca$^{2+}$), ATP turnover or mechanical stress, in order to trigger hypertrophy. It is worth considering, however, the possibility of an activity-dependent trigger for growth that is upstream of depolarisation-induced Ca$^{2+}$ release. For example, the sodium–potassium pump, in addition to maintaining the electrochemical gradient, is an important signal transducer capable of affecting gene expression and cardiomyocyte size [118] and could theoretically be involved in signalling for muscle growth upon activation by exercise (Fig. 3B).

Other voltage-, ion- or force-dependent channels have barely been investigated.

An additional important aspect of activity-induced growth is the probability that muscle fibre activity also elicits cell-autonomous change in secretion of signalling molecules derived from the fibre [119]. Examples include IGF-1 [49,83], myostatin [120] and muscle-derived agrin [121], which may autochinely affect the fibre itself and, through local paracrine signalling, its associated MuSC and even adjacent fibres (Fig. 3B).

Calcium signalling as a hypertrophy trigger

Ca$^{2+}$ is a major cytoplasmic second messenger in all eukaryotic cells and has distinct effects depending on cell type, developmental stage and physiological condition. In skeletal muscle, myoblast proliferation, apoptosis, differentiated fibre character and contraction are all Ca$^{2+}$-regulated. Under resting conditions, the cytosolic Ca$^{2+}$ concentration measured in single muscle fibres is 30–50 nm [122]. Action potential-triggered T-tubule membrane depolarisation causes structural changes to voltage-sensitive dihydropyridine receptors (DHPR) that lead to opening of physically linked ryanodine receptors in the sarcoplasmic reticulum, which release stored Ca$^{2+}$ (Fig. 3A). Each action potential also activates a small influx of Ca$^{2+}$ across the cell surface from the extracellular space [123]. These mechanisms permit a massive (~100 fold) rise in cytoplasmic free Ca$^{2+}$ concentration resulting in rapid binding of Ca$^{2+}$ to troponin C on the thin filament and activation of actomyosin contraction.

Beyond its direct involvement in the excitation–contraction command, Ca$^{2+}$ is a critical component of numerous signalling pathways, controlling the activity of diverse downstream effectors that regulate gene expression. These signalling cascades are likely to be central to the maintenance of phenotypes in both fast
and slow muscle fibres [124]. However, isolating the role of Ca$^{2+}$ per se is difficult due to the complex interaction among Ca$^{2+}$, tension development and energy metabolism. For example, treatment of mice with the Ca$^{2+}$ chelator BAPTA-AM prevents muscle hypertrophy in response to overload [86], yet this effect might be explained by a secondary consequence of reduced force output or less ATP turnover. One attempt to isolate the effects of Ca$^{2+}$ has involved incubating muscles with caffeine, which releases Ca$^{2+}$ from the sarcoplasmic reticulum, at concentrations that raise cytosolic Ca$^{2+}$ to levels too low to cause muscle contraction and without a decrease in high-energy phosphates [125–127]. These experiments have demonstrated a role for basal Ca$^{2+}$ levels in glucose transport and activation of calmodulin-dependent protein kinase (CaMK)-II and p38 MAPK signalling. However, determining whether Ca$^{2+}$ alone is sufficient to induce muscle growth requires the normal, contraction-driven Ca$^{2+}$ release without the confounding effects of actomyosin contraction. A valuable approach in this respect is the use of certain small molecule inhibitors of myosin, which block actomyosin contraction, and thus tension development, without affecting Ca$^{2+}$ transients [128–130]. A recent study using this approach provided some evidence that both Ca$^{2+}$ release and actomyosin contraction are prerequisites for an increase in protein synthesis in rat muscle subjected to electrical stimulation ex vivo [131]. Whether the growth of muscle in vivo is similarly dependent on the concomitant signals from Ca$^{2+}$ and tension remains to be determined.

**Ca$^{2+}$-sensitive regulation of muscle transcription could promote growth**

Distinct firing patterns cause different dynamic patterns of Ca$^{2+}$ rise in fibres [132–134]. How distinct patterns of Ca$^{2+}$ rise affect downstream signalling in adult muscle fibres is, in general, poorly known. Moreover, studies on people and animal models suggest that repeated high-force exercise bouts, ‘reps’, are key to driving muscle growth [135–137]. The intense firing required to drive the high-force contractions close to tetany that typically stimulate muscle hypertrophy causes a prolonged high level of Ca$^{2+}$ during the actual contraction, but nevertheless last for short absolute periods of seconds to minutes. Ca$^{2+}$ rapidly returns to baseline when contraction stops; so how the triggered signalling persists over hours to days to drive growth is a key aspect of the growth problem.

Various Ca$^{2+}$-sensitive targets have been identified in skeletal muscle that could record prior Ca$^{2+}$ signals, some of which have been implicated in muscle hypertrophy. One example is Mef2 (myocyte enhancer factor 2), a family of transcription factors that can work in concert with MyoD and Myogenin to promote muscle differentiation and gene expression [138]. Mef2 is indirectly responsive to multiple Ca$^{2+}$-regulated signals, including synergistic activation by calcineurin and other Ca$^{2+}$-regulated signalling molecules [139, 140]. A series of mouse overexpression and knockdown experiments revealed that Mef2 is a positive regulator of skeletal muscle hypertrophy in adult muscle fibres [122]. In addition, Mef2 has been shown to promote skeletal muscle growth in zebrafish larvae, an effect dependent upon muscle activity [141]. Mef2 and other Ca$^{2+}$-regulated transcription factors, such as NFATs, act cooperatively to activate the transcription of peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC-1α), a major transcriptional coactivator involved in adaptation to endurance exercise [140]. A splice isoform of PGC-1α (PGC-1α4) has been implicated in muscle fibre size control, as there is a large increase in fibre cross-sectional area and strength after PGC-1α4 overexpression [142]. PGC-1α4 does not regulate most known PGC-1α targets involved in oxidative metabolism, but rather induces IGF-1 via GPR56 and represses myostatin signalling [142, 143]. Among different modes of exercise, activation of PGC-1α4 was confined to resistance-type training protocols associated with muscle fibre hypertrophy [142]. PGC-1α4 is itself regulated by MEK/ERK signalling that is activated during resistance exercise [144, 145]. Moreover, the role of PGC-1α4 appears more complex than originally described and is suppressed by interleukin-6 (IL-6) [146, 147]. Thus, Mef2-PGC-1α may link Ca$^{2+}$ signalling to overload-induced muscle growth (Fig. 4), although proof of the role of PGC-1α4 awaits muscle- and isoform-specific knock-out studies.

Calcium can also activate serum response factor (Srf)-dependent transcription through a mechanism involving CaMKII [148] (Fig. 4). Srf cooperates with other transcription factors in myogenesis [149] and is essential for skeletal muscle development, as evidenced by muscle-specific loss of Srf function in mice [149, 150]. In mature muscle, Srf is crucial for hypertrophic growth. Tamoxifen-inducible deletion of Srf in muscle fibres blunted hypertrophy induced by synergist ablation [151]. In that model, the effect of Srf was suggested to involve interleukin-4 and IL-6, which signal in a paracrine manner to control MuSC fusion and differentiation [151]. In support of these data is the finding that MuSC-specific loss of Srf prevents overload-induced hypertrophy by blocking MuSC fusion, possibly by affecting the expression of actin genes and...
Calcineurin/NFAT activity as a hypertrophy candidate

One of the most studied Ca$^{2+}$ effector molecules in muscle is calcineurin, a Ca$^{2+}$/calmodulin(CaM)-dependent serine/threonine phosphatase [153]. Calcineurin exists in the cytoplasm as a heterodimer of two subunits, termed A and B. Calcineurin A has a catalytic domain and three carboxy-terminal regulatory domains, with, respectively, calcineurin B binding, CaM binding and autoinhibitory capacity [154]. The calcineurin B subunit contains four Ca$^{2+}$-binding EF-hand motifs. Upon elevation of Ca$^{2+}$ levels, CaM/Ca$^{2+}$ binding causes a conformational shift that displaces the autoinhibitory domain and permits activation of the phosphatase [155]. Calcineurin activity can be blocked by two chemically distinct immunosuppressant drugs, cyclosporin A (CsA) and FK506, which bind to cyclophilin and FKBP, respectively, forming inhibitory complexes that bind to calcineurin and block its phosphatase activity [156]. Calcineurin is involved in cytokine gene expression in B and T cells through its action upon nuclear factor of activated T-cell (NFAT) transcription factors [157]. Under basal conditions, the NFATs are localised to the cytoplasm in a phosphorylated state. An increase in intracellular Ca$^{2+}$ activates calcineurin’s phosphatase activity, which dephosphorylates NFAT family members, among other proteins, and facilitates their rapid translocation to the nucleus (Fig. 4). Once in the nucleus, NFATs bind cooperatively with other transcription factors to control prolonged gene expression outlasting the triggering Ca$^{2+}$ stimulus in multiple cell types [155,157]. Calcineurin and various NFAT isoforms are abundantly expressed in muscle [158], which provided the first indication that a similar mechanism may operate upon activation of muscle to control muscle-specific gene expression and growth.

Calcineurin is now understood to be involved in several aspects of muscle development (for a review, see [159]). The first described role of calcineurin in skeletal muscle fibres was its control over fibre-type-specific contractile proteins [160,161]. Later work showed that functional overload of muscle by synergist ablation results in activation of NFATc1 and its nuclear localisation [162], indicating that calcineurin/NFAT signalling is responsive to growth signals in vivo. However, while several studies have implicated calcineurin-NFAT signalling in cardiac muscle hypertrophy [163–167], attempts to identify a role in skeletal muscle hypertrophy have yielded opposing conclusions. In vitro studies first showed that IGF-1-induced myotube growth was prevented by calcineurin inhibition [47,168], but this effect was later explained by a block on muscle differentiation and/or fusion of myocytes into myotubes, rather than a direct inhibition of myotube hypertrophy [48,169]. In vivo, loss of calcineurin function by CsA treatment had no effect on muscle mass or fibre cross-sectional area of the mouse plantaris or soleus under normal weight-bearing conditions [170–172]. In addition, mice lacking both isoforms of the calcineurin catalytic subunit (Aα and Aβ) exhibited no alterations in the weight of several muscle groups [173], suggesting that calcineurin activity is not necessary to maintain muscle size under normal contractile conditions. In the context of muscle overload, some studies have reported that calcineurin inhibitors reduce the magnitude of muscle fibre hypertrophy in both fast and slow fibres [170,171], whereas others have reported no such effect [57,172,174].

The lack of consensus may be explained by differing degrees of calcineurin inhibition with different drug treatments (range: 20%-65% inhibition). Nonetheless, an 80% reduction in calcineurin activity in mice with
Additional Ca\(^{2+}\)-dependent pathways are underexplored

Most work on Ca\(^{2+}\)-regulated muscle growth has focused on calcineurin signalling. However, other Ca\(^{2+}\)-dependent molecules may also be important, including CaMKs and protein kinase C (PKC). CaMKs are a family of serine/threonine protein kinases that are activated in a Ca\(^{2+}\)- and CaM-dependent manner. Several CaMK isoforms have been identified, although CaMKII appears to be the major isoform expressed in skeletal muscle [178]. CaMKII has 12 subunits arranged as two six-subunit rings in a hub-and-spoke conformation. Upon binding of the Ca\(^{2+}\)/CaM complex to the CaM-binding domain of CaMKII, it is activated by autophosphorylation and is then active independent of elevated Ca\(^{2+}\) concentration, at least for a period of 2–3 min [179,180]. CaMKII activity has been shown to increase in hypertrophied muscle during stretch overload [181] and during exercise in humans or rodents [182,183]. Once activated, CaMKII leads to Mef2-mediated transcription, either directly by deactivating HDAC4 [184] or by inhibiting GSK3\(\beta\) [185]. CaMKII also activates SRF [148]. Transcriptional activity through p38 MAPK can also be initiated by CaMKII in response to increases in cytosolic Ca\(^{2+}\) [126]. Despite these correlative data, however, we are unaware of any study that has used genetic loss- or gain-of-function experiments to address the role of CaMKs in skeletal muscle hypertrophy.

The PKCs are a family of enzymes involved in metabolism, differentiation and cellular growth. In rat skeletal muscle, PKC is activated in response to electrical stimulation in vivo [186]. Moreover, compensatory hypertrophy by synergist ablation overload is accompanied by increased PKC activity [187]. Despite these correlations, however, the role of PKC in triggering muscle hypertrophy has received little attention. One recent study implicated PKC in muscle hypertrophy through a mechanism involving G\(\alpha\)i2 [188]. It was shown that rapamycin and PKC inhibitors prevented muscle hypertrophy induced by constitutively active G\(\alpha\)i2. These findings are consistent with a study of cardiac muscle hypertrophy in transgenic mice with cardiac-specific overexpression of a constitutively active mutant of PKC, in which heart mass was increased by 28% with normal cardiac function [189]. Thus, PKC may have a role in stimulating skeletal muscle hypertrophy in response to exercise (Fig. 4). Again, this hypothesis must be tested by muscle-specific removal of PKC activity during overload.

The jury is out on calcium-triggered skeletal muscle growth

Changes in intracellular Ca\(^{2+}\) concentration are a prime candidate for mediating the effects of neuromuscular activity on muscle phenotype (Fig. 4). Evidence for the involvement of Ca\(^{2+}\) in regulating slow/oxidative fibre-type programme is compelling, yet the importance of Ca\(^{2+}\) signalling in skeletal muscle hypertrophy is less clear. Several Ca\(^{2+}\)-regulated transcription factors, such as Srf, have been implicated in muscle hypertrophy, providing a theoretical mechanism by which exercise-induced Ca\(^{2+}\) flux might be linked to growth. The underlying Ca\(^{2+}\) signalling pathway/s remain to be fully elucidated.

Energy stress as a hypertrophic stimulus

Muscle activation is tremendously energetically taxing, with ATP turnover rate increasing 100-fold during
maximal exercise [190]. The two main energy demands during maximal exercise are the myosin ATPase, which provides the immediate source of energy for cross-bridge cycling and is thought to use about 50–70% of the ATP, and SERCA, which is necessary for pumping Ca^{2+} back into the sarcoplasmic reticulum and uses 30–50% of ATP [191] (Fig. 5A,B). As initial ATP is depleted within seconds, metabolism of creatine phosphate, glycogen and lipid is utilised successively to regenerate ATP and maintain energy supply. Accompanying metabolic changes, including increase in AMP and metabolite accumulation, could serve as activity-dependent triggers for persistent changes in muscle, including hypertrophy (Fig. 5C). Importantly, in experiments where myosin function is blocked by small molecule inhibitors to separate the effects of calcium and force [129,131,192], any effects of myosin inhibitors on muscle growth could be secondary to inhibition of ATP utilisation by the myosin ATPase.

Control by AMP concentration [or AMP/ATP ratio]

Whereas ADP is the direct product of ATP hydrolysis, to maximise ATP availability, ADP is rapidly converted to AMP by the adenylate kinase reaction (2ADP ↔ ATP + AMP). In exercising muscle, elevations in AMP activate the enzyme called AMP-activated protein kinase (AMPK), considered to be the ‘energy sensor’ of the cell. AMPK is a heterotrimeric protein comprised of a catalytic subunit (α) and two regulatory subunits (β and γ). Its activation involves binding of AMP or ADP to the regulatory γ-subunit, resulting in conformational changes that cause allosteric activation, as well as facilitating phosphorylation (and activation) of the catalytic subunit by various upstream kinases [193]. As the first-in-line sensor of increased ATP turnover, AMPK has the potential to be a rapid regulator of muscle adaptations.

Active AMPK appears to suppress muscle growth under certain circumstances. This response reflects the role of AMPK as an energy-conserving enzyme, inhibiting energy-requiring processes such as protein synthesis. Indeed, AMPK can inhibit TOR by directly phosphorylating upstream proteins in the TORC1 activation pathway, including TSC2 and the TORC1 subunit raptor [194], as well as TOR itself on Thr2446 [195]. AMPK also inhibits eukaryotic elongation factor 2 (eEF2) at Thr56, reducing its binding to the ribosome and therefore slowing the rate of translation elongation [196]. These mechanisms are thought to underpin the marked reduction in protein synthesis when muscle cells are treated with AICAR, an AMPK activator [197]. Further support for a role of AMPK in suppressing protein synthesis and growth is the finding that genetic disruption of either the α or β AMPK catalytic subunits stimulates muscle hypertrophy [198,199]. Moreover, AMPK activation by AICAR attenuates TOR signalling and muscle hypertrophy in response to overload [200,201].

Despite these findings, uncertainties regarding the precise role of AMPK remain. For instance, AMPKα1-null mice have smaller muscles, indicating a requirement for AMPK in muscle development [202]. The exact role of AMPK in blunting overload-induced muscle protein synthesis in vivo has also been called into question. A recent study demonstrated that protein synthesis was blunted in AMPKα2-null mice subjected to repeated muscle contractions [203]. Moreover, TOR-p70S6K signalling was coactivated with AMPKα2 immediately postcontraction, reinforcing previous work showing that TOR activity and AMPK

![Fig. 5. Energy stress and muscle size regulation.](image-url)
activity are not mutually exclusive [204,205], as originally thought [206]. Future work involving conditional muscle- and isoform-specific genetic knockout of AMPK in the context of overload-induced hypertrophy is required.

The metabolite hypothesis

It has been hypothesised that the build-up of metabolites, such as lactate and reactive oxygen species (ROS), might themselves serve as triggers of muscle growth [207,208]. Indirect evidence for this hypothesis comes from training methods that exaggerate metabolic stress, such as by blood flow restriction (BFR). BFR training involves occlusion to restrict venous return and reduce blood inflow during exercise (e.g. by application of a tourniquet around the proximal end of a limb). Occlusion during overload amplifies metabolic ‘stress’ through two mechanisms, firstly by increasing reliance on glycolytic metabolism due to localised hypoxia, and secondly by impeding the removal of metabolic by-products through venous outflow, resulting in heightened accumulation and trapping of metabolites [209,210]. It has been repeatedly observed that BFR augments muscle hypertrophy in response to low-load exercise [211,212], an effect that has been suggested to result from an anabolic effect of metabolite build-up [213,214]. An additional possibility is that local hypoxia is a stimulus for growth, as hypoxia-inducible factor 1α (HIF-1α) overexpression in rat muscle stimulates hypertrophy [215]; the role of HIF-1α signalling for exercise-induced hypertrophy remains largely unexplored.

In support of a role of metabolites in stimulating muscle growth is the finding that some metabolites have anabolic properties. Of these, lactate appears to have the most potential as a molecule capable of triggering muscle hypertrophy. Supplementing C2C12 cells with lactate increases signalling through TORC1 [216] and stimulates myotube differentiation and hypertrophy [217,218]. In vivo, lactate supplementation improved regeneration after muscle damage [217] and stimulated muscle hypertrophy in the absence of exercise [219], perhaps by activating TOR signalling [220]. One problem is that there are no known means for cells to sense lactate; instead, as lactate can be used by oxygenated cells to generate ATP, it is possible that anabolic effects observed in vitro could simply reflect greater nutrient supply upon exposure to lactate [221]. Reactive oxygen species (ROS), which can be promoted by lactate activity [222], have also been implicated in muscle growth. However, the evidence is mixed, with studies showing that antioxidant supplementation slightly attenuates overload-induced muscle growth in rats [223] but not in humans subjected to strength training [224,225]. Other metabolites that have been shown to have anabolic effects upon supplementation in vivo include phosphatidic acid [226,227] and nitric oxide [86,228].

Metabolites as indirect, rather than direct, controllers of growth

An alternative hypothesis is that metabolites indirectly promote muscle growth by potentiating motor unit recruitment [229]. Metabolite accumulation produces an acidic intramuscular environment, which serves as a stimulus for metaboreceptors and group III and IV afferent fibres [230]. With sufficient sensory stimulation, a net inhibitory effect on the active alpha motor neurons can ensue [231], leading to increased motor neuron recruitment to minimise functional weakening [232]. This hypothesis is supported by the fact that BFR combined with low-load exercise increases electromyography amplitude of the target muscle [233,234]. Hence, by augmenting neuromuscular activation, metabolic stressors might stimulate growth not through a direct effect but instead by indirectly activating a larger proportion of muscle fibres, thereby increasing the extent of potential remodelling in the whole muscle [229]. Nonetheless, some uncertainty remains. For example, it was recently shown that BFR training selectively hypertrophied type I muscle fibres [235], a finding that appears to contradict the hypothesis that metabolic molecules simply serve to increase high-threshold fast motor unit activation.

Mechanosignalling as the hypertrophic stimulus

A highly coordinated interplay between mechanical and biochemical inputs controls the shape and size of tissues throughout development and in adulthood [236]. Mechanotransduction is particularly relevant in the musculoskeletal system, where mechanical stimuli, whether from externally applied or internally generated forces, elicit adaptive responses to maintain normal tissue homeostasis. Such adaptation is exemplified by the larger bone size in the dominant arm of tennis players caused by the mechanical strains of repetitive tennis strokes [237]. A prevailing hypothesis in the skeletal muscle literature is that mechanical factors play an important or even essential role in hypertrophic growth. The contention is that mechanical signals, such as actomyosin-driven force, are sensed by the muscle fibre and are subsequently converted into
biochemical signals, many of which converge on TORC1 to regulate translation initiation [207]. A mechanism of growth that is controlled by force makes evolutionary sense, given that force production is the main aim of muscle activation and that muscle mass is strongly associated with maximal force output; coupling force to growth allows a feedback control to satisfy the requirements of functional demand. The following section examines the evidence for mechanical regulation of muscle hypertrophy.

**Muscle stretch is a specific mechanical stimulus and a poor model of exercise**

Early evidence for the mechanical control of muscle size came from the observation that stretching myotubes in culture stimulates hypertrophy [238,239]. In vivo, immobilisation atrophy can be attenuated when the atrophying muscle is stretched [240–242], which preserves muscle mass, fibre cross-sectional area, fibre length and total protein content [242,243]. Stretch of immobilised muscle has even been reported to produce hypertrophy after ~1 week [244,245], although this effect is transient, and muscles ultimately show overall atrophy [246]. Nonetheless, hypertrophy can follow stretch in nonatrophying muscle; cumulative evidence from several laboratories has shown that in vivo stretch of avian wing muscle results in pronounced muscle hypertrophy [12,247].

The above findings have been put forward as evidence for a mechanical stimulus controlling growth. A criticism of this interpretation is a lack of control over changes in neuromuscular activity, since stretched muscles are more neurologically active than shortened ones [246,248]. In the few studies that have silenced nerve input, stretch still protects against muscle atrophy, but the rescue is far from complete [243,249,250]. Hence, some growth may be due to electrical activity in fibres, rather than a mechanical mechanism. An additional criticism is the questionable ecological relevance of chronic muscle stretch. Stretching a muscle for several days is profoundly different from intermittent bouts of exercise performed in humans or animal models of resistance training. Importantly, a distinction should be made between mechanical stress, that is force generated when a muscle contracts, and stretch, which is a change in strain and may or may not be accompanied by a change in stress. Further, as bones elongate during development, stretch clearly increases muscle mass by adding nuclei and sarcomeres at the ends of fibres [251–253], a quite distinct cellular mode of growth from the increase in fibre width observed after training. In a passive stretch situation, to generate comparable amounts of stress as that occurring in an active contraction, the muscle must be stretched beyond a physiologically relevant distance and there is potential for the response to structural damage to hamper the interpretation of signalling mechanisms. Moreover, muscle growth can occur in response to isometric contractions, where no changes in muscle length occur [254]. Studies employing stretch as a growth stimulus should be interpreted with these considerations in mind.

**Force is a good candidate for activity-driven muscle hypertrophy**

Teasing out the role of force *per se* requires an approach in which electrophysiological and mechanical factors are manipulated independently. To our knowledge, Eftestøl et al. [254] are the only authors to have attempted to achieve this *in vivo*. In their elegant study, the muscles of two groups of rats were stimulated with identical nerve stimulation patterns, but one group performed isometric contractions (high load), while the other group performed high-velocity concentric contractions (low load) [254]. The low-load condition produced 50–60% of the peak force produced in the high-load group. In this sense, the rat muscles were exposed to standardised electrical activity but with different mechanical conditions. It was found that a twofold greater muscle hypertrophy and more myonuclear accretion resulted in the high-load group, supporting the argument that high mechanical force is a trigger for muscle growth [254]. While this study strongly suggests that force is a key aspect of muscle contraction driving hypertrophy of fibres and myonuclear recruitment, possible criticisms are that a) during the training regime the rats that experienced high and low force may have differed in muscle activity or other behaviour between training bouts and b) that the high- and low-force muscles were held in a distinct state of stretch during the training bouts, although as the low-force group were more stretched this would not be predicted to explain the observed difference in hypertrophy [254]. Nevertheless, this important study failed to find clear evidence of the signalling mechanism inside muscle driving growth.

Some data point to a role for TOR downstream of the mechanical stimulus. However, it must be emphasised that there is no *a priori* reason to assume that TOR is not activated by any of the myriad signals activated by exercising muscle that are known to stimulate TOR activity in other cell types, such as nutrient uptake [62]. Nonetheless, support for a mechanically sensitive TOR comes from the observations that TOR
is activated in myotubes stretched in culture [255] and in muscle removed from the animal and stretched ex vivo [192]. Stronger evidence comes from the finding that rat muscle subjected to excitation-induced contractions activates TOR signalling only when actomyosin is permitted to generate force [192]. Electrical activity combined with myosin inhibitors blocked contraction and failed to activate TORC1 signalling at 1 hour poststimulation [192]. However, whether the actomyosin-dependent activation of TORC1 in these studies would be sufficient to stimulate muscle growth remains unknown. Pertinently, recent studies have questioned the extent to which protein synthesis and translation initiation signalling are predictive of muscle growth triggered by repeated bouts of activity [256,257]. Indeed, the study of Eftestøl and colleagues showed that mechanical load per se is not predictive of activation of Akt or TORC1 [254]. Precisely how exercise triggers TORC1 signalling is an exciting avenue for future investigations.

It has also been postulated that MAPK is activated by mechanical force [258], a hypothesis supported by the positive relationship between muscle tension and MAPK phosphorylation [259]. Further support for the mechanical activation of MAPK comes from findings that ERK1/2, p38 and JNK are activated upon muscle stretch [99]. Still, whether MAPK is activated by the physical forces produced during exercise is unknown. Interestingly, it was recently shown that mice with loss of p38α exhibit resistance to denervation-induced muscle atrophy through a mechanism that involves CaMK2B, a member of the CaMKII family [260]. Hence, both force and Ca2+-dependent mechanisms are implicated in MAPK activity and muscle growth. Resolution of this complex situation will require analysis of muscle growth in response to precisely defined mechanosignals in animals lacking specific MAPK genes, and after elimination of confounding correlated signals.

**Potential mechanisms of mechanosensing in skeletal muscle**

Muscle fibres transmit force from sarcomere to sarcomere and then both laterally through Z-lines and linearly at fibre ends to extracellular matrix, ultimately pulling on the tendon. These extensive and complex mechanochemical connections between the force-producing sarcomere and the rest of the muscle cell present a challenge when attempting to identify individual components in the force transduction pathway that are responsible for sensing mechanical signals and stimulating growth. Nonetheless, mostly indirect evidence has highlighted several candidate mechanosensory components within the muscle, as described below.

**Integrins and other force sensors at the sarcolemma**

Integrins, composed of α and β subunit heterodimers that span the cell membrane, have a key role in transmitting physical forces bidirectionally between the actin cytoskeleton and the extracellular matrix to regulate various cell functions [261]. The predominate integrin in mature skeletal muscle is α7β1 integrin, which is enriched at the myotendinous and neuromuscular junctions and at costameres, protein assemblies that connect the Z-disc to the extracellular matrix. A role for α7β1 integrin in load-mediated muscle hypertrophy is possible, because transgenic overexpression of α7 in fibres augments TOR signalling and muscle growth in response to downhill running in mice [262,263]. However, the molecular mechanism of this effect is unclear. Overexpression of integrin protects against postexercise sarcolemma damage [262,264], which might establish a healthy microenvironment that is conducive to hypertrophy, irrespective of any mechanotransduction mechanism [263]. Focal adhesion kinase (FAK), a tyrosine kinase that binds to the cytoplasmic portion of β1 integrin, is perhaps the best candidate for a mediator of mechanosignalling through integrin in skeletal muscle. Knockdown of FAK in C2C12 myotubes reduced IGF-1-stimulated growth, which correlated with a reduction in TSC2/TOR/p70S6K1 pathway [265]. In vivo analysis of the role of FAK is limited because FAK null mice are embryonic lethal [266]. Nonetheless, in vivo evidence for an anabolic role of FAK signalling comes from the finding that FAK overexpression after pCMV:FAK transfection leads to exaggerated load-induced TORC1 signalling [267]. These findings provide some evidence that an integrin-FAK-mediated mechanotransduction mechanism links to TORC1 signalling, although the precise mechanobiology remains to be determined.

Force application to integrins can activate mechanosensitive (MS) ion channels [268]. MS channels are expressed abundantly in skeletal muscle across all stages of development and may have a role in sensing physical activity. Indeed, muscle hypertrophy and TORC1 activation are prevented when overloaded mice are treated with streptomycin, an antibiotic capable of inhibiting the activation of several types of MS ion channels [269,270]. Genetic loss-of-function studies are needed to extend these findings and identify the
channel(s) required for growth. Candidate MS channels with sensitivity to streptomycin are the Piezo proteins, gigantic Ca\(^{2+}\) channels that have clear mechanosensory functions in a range of cell types [271,272]. Piezo1 is expressed in skeletal muscle, where its activation and subsequent Ca\(^{2+}\) influx promote RhoA/ROCK-mediated actomyosin assemblies during myotube formation [273]. But how such Ca\(^{2+}\)-driven signalling could function in the context of the dramatic Ca\(^{2+}\) changes that trigger muscle contraction is unclear. Transient receptor potential (TRP) channels also communicate with integrin [274] and have been implicated in overload-induced muscle growth [86]. Thus, in this model, integrins may play a role in mechanotransduction through MS channels beyond any role as a direct transducer.

Emerging evidence suggests that phosphatidic acid (PA), a mechanically generated derivative of membrane lipids [275], has an important mechanotransduction role in muscle, although its link to integrin is uncertain. PA can be produced in muscle as a result of activation of either phospholipase D, an enzyme found in the Z-disc, or diacylglycerol kinase-ζ, an enzyme that can bind to syntrophins on the sarcolemma [276], in response to mitogenic actions of various growth factors and hormones [277,278]. Several forms of stimuli, including passive stretch of muscle \textit{ex vivo} [279] and electrical stimulation [226], increase PA concentration in muscle fibres by an unknown mechanism. It has been shown that PA can directly bind to TOR in the FKBP12/rapamycin binding (FRB) domain, an interaction that activates TOR kinase activity [144,226]. Indeed, in cultured muscle fibres, increasing the concentration of PA is sufficient to phosphorylate p70S6K (Thr389), whereas PA-sufficient to phosphorylate p70S6K (Thr389), whereas PA increase PKC, future work needs to identify upstream inputs to DGKζ, including its potential to interact with integrins.

Titin – force sensing in the sarcomere

Not only the muscle membrane experiences forces. When muscle contracts it can either shorten, generating acceleration but little force, or produce high force in the loaded isometric state when muscle contracts. High force is thought to trigger hypertrophy. As sarcomeres within a myofibril all generate force in the same direction and are arrayed throughout the length of the fibre, they are a prime candidate to contain a force sensor capable of triggering hypertrophy. Titin, a giant protein integral to myofibrils and stretching across an entire half sarcomere, is a candidate for such mechanosensitivity [280]. Mounting evidence indicates that titin participates in various signalling pathways by forming several ‘signalling hubs’ along its length. Titin has been shown to interact with over 20 other proteins, linking titin to various stress signalling pathways [281]. One titin region that is particularly engaged in protein–protein interactions is the M-band region of titin, which contains a unique pseudokinase domain that is suggested to stretch during active muscle contraction [282]. Upon stretch \textit{in vitro}, this titin kinase domain recruits a Nrb1-p62-MURF1/Srf signalling complex [280]. As MURF1 is a known driver of protein degradation [283], and Srf is a muscle transcription factor that correlates with hypertrophy [151,284], it is possible that titin regulates muscle size through effects on one or both molecules.

A second domain of titin, the N2A region, is another putative transducer of mechanosignals. The muscle ankyrin repeat proteins (MARPs) are a family of titin-associated molecules, consisting of three members: cardiac ankyrin repeat protein (CARP), ankyrin repeat domain 2/ankyrin repeat protein with PEST and proline-rich region (Ankrd2/Arpp), and diabetes-associated ankyrin repeat protein (DARP). Ultrastructural techniques in heart muscle tissue have shown that all three MARP proteins bind to the I-band region of titin N2A epitopes and that passive stretch results in the accumulation of CARP and DARP in the nucleus [285]. MARP family proteins are upregulated in mice after a single bout of eccentric contractions [286]. Causative data are, however, lacking; triple MARP knockout mutant mice show no defect in fibre size of the soleus muscle [287]. The responsiveness of these mice to an overload stimulus has not been reported.

In addition to titin, there are likely additional molecular force sensors within the sarcomere. Increasing evidence in cardiac muscle points to protein structures within the Z-disc and the I-band in mechanosensation controlling downstream effects, including those that regulate gene expression and
molecular tools not previously explored. Bioengineering tools, such as microfabrication technology, may serve as useful models of the biophysical properties of muscle for the study of mechanosignalling to MuSC [291–294]. One difficulty is that putative mechanosensory elements in muscle fibres also play important structural roles; genetic disruption of these structural elements results in disease, such as muscular dystrophies, limiting the applicability of loss-of-function approaches to study the signalling roles of structural features. Novel molecular tools not previously used in muscle biology may help address these issues, such as the application of local stresses on the sarcolemma using magnetic beads [295] or precise genome-edited mutagenesis of signalling regions of structural proteins.

**Clarification of the definition and complexity of skeletal muscle hypertrophy**

A central area for future work is the study of how a growing muscle is assembled. Cell growth is much more complicated than the balance between rates of synthesis and degradation of cellular constituents. Multiple aspects of growth must be controlled simultaneously including not only the synthesis and accumulation of various macromolecules and proteins but also their precise localisation and organisation within the cell. Fluid content of the cell must be concomitantly regulated, necessitating tight control of osmotic pressure. In skeletal muscle, the situation is even more complex because fibre size can increase by both radial and elongation growth, with differential effects on muscle function. Sarcomeric material can be added in parallel or in series within existent myofibrils (the latter may be termed sarcomerogenesis) or entirely new myofibrils can be synthesised (i.e. myofibrillogenesis). An increase in cell mass additionally involves an expansion of noncontractile cell constituents, such as sarcoplasmic reticulum, t-tubule, plasma membrane and sarcoplasm. Some progress has been made in this respect, for example the potential role of muscle agrin in activity-dependent regulation of the costameric cytoskeleton of the growing muscle fibre [121], but we are far from understanding which signals drive each aspect of hypertrophy. It is important to note that, during radial growth, the various cellular components do not scale in direct proportion to one another; surface area increases in direct proportion to radius, whereas fibre cross-sectional area, and hence volume, increases as the square of the radius [27]. Hence, cell growth involves a complex and coordinated assembly process; the challenge is to determine which signals regulate which aspect and how these signals interact during growth.

The choice of measurement for quantifying growth is an important aspect when studying muscle hypertrophy. Many studies rely on acute measures of muscle protein synthesis as a proxy for growth, but this approach can be problematic. Acute measures of protein synthesis do not correlate with chronic muscle hypertrophy [256,296], possibly because some changes in protein synthesis are driven by repair of muscle damage for the purpose of muscle remodelling, rather than growth [257,297]. Thus, studies measuring acute responses should be confirmed with long-term hypertrophic outcomes. Another measurement issue is the common use of changes in muscle mass as an indication of hypertrophy, particularly in stretch studies [241,244,245,249]. It has been observed, both in developing and in exercised situations, that sarcomeres are added in series to stretched fibres and lost from shortened ones [298–300]. Such sarcomere addition, if unaccounted for, could appear to increase muscle mass while the fibres simultaneously declined in cross-sectional area through disuse atrophy. We argue that measurements of both fibre cross-sectional area and length, accompanied by functional assessments, should be employed for the assessment of muscle hypertrophy.

**Conclusions and future directions**

How skeletal muscle senses activity to trigger exercise-induced responses, including growth, is a fundamental question that remains poorly answered. There are perhaps four reasons for this. Firstly, there is a lack of compelling proof for which ‘cause(s) of contraction’
How does exercise control muscle size?

Calcium flux, ATP turnover, mechanical stress provide the signal for growth and/or other adaptations. This is primarily due to the difficulty of finding systems in which each component of activity can be independently controlled. Secondly, while muscle fibres have multiple sensors for each ‘cause of contraction’, it remains unclear which are essential to mediate the muscle size-regulating system. Thirdly, although there is evidence that multiple intracellular signalling pathways (e.g. TORC1, MAPK, Smad2/3) can affect muscle growth, it is unclear how these pathways cooperate in the overall size control scheme. Lastly, although in our view it is likely that the sensing is done within the muscle fibres themselves, hypertrophic responses include recruitment of nuclei from MuSC (and, debatably, loss of nuclei from fibres during atrophy) and how this is coordinated with fibre size regulation is unresolved.

Mechanical stimuli are perhaps the best candidates for triggers of muscle hypertrophy, although the mechanisms of mechanosensation in muscle are only beginning to be elucidated. It is likely that mechanical signals do not operate in isolation and are part of an intricate multifaceted signalling system with both redundancy and crosstalk. Calcium signalling may act as an upstream regulator of muscle growth, working in concert with mechanical signals to drive anabolism in response to muscle overload [131]. The increased energy consumption of active muscle, involving ATP use and metabolite accumulation, might indirectly facilitate growth by augmenting neuromuscular activity, although there is little evidence for energy demand as a direct trigger of hypertrophy. It is highly likely that whole-body hormonal balance has a fundamental permissive role in muscle size control, but appears unlikely to target specific muscles. Finally, the possibility of nonelectrical nerve-derived regulation of growth cannot be entirely ruled out, although what is certain is that electrical activation of muscle plays a fundamental role in muscle hypertrophy. The challenge for scientists of the future is to elucidate how each of these growth triggers interacts to control the assembly of the fascinating and powerful molecular machine that is the functionally hypertrophied muscle fibre. Understanding the mechanisms of muscle growth is critical for developing therapeutic strategies to prevent weakening in ageing-related muscle loss, cardiovascular disease, cachexia and other diseases affecting muscle.

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Conflicts of interest

The authors declare they have no conflict of interest.

Author contributions

MA wrote the initial draft after discussion with SMH. Both authors edited the manuscript.

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M. Attwaters and S. M. Hughes

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