Signaling pathways controlling skeletal muscle mass

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Abstract

The molecular mechanisms underlying skeletal muscle maintenance involve interplay between multiple signaling pathways. Under normal physiological conditions, a network of interconnected signals serves to control and coordinate hypertrophic and atrophic messages, culminating in a delicate balance between muscle protein synthesis and proteolysis. Loss of skeletal muscle mass, termed “atrophy”, is a diagnostic feature of cachexia seen in settings of cancer, heart disease, chronic obstructive pulmonary disease, kidney disease, and burns. Cachexia increases the likelihood of death from these already serious diseases. Recent studies have further defined the pathways leading to gain and loss of skeletal muscle as well as the signaling events that induce differentiation and post-injury regeneration, which are also essential for the maintenance of skeletal muscle mass. In this review, we summarize and discuss the relevant recent literature demonstrating these previously undiscovered mediators governing anabolism and catabolism of skeletal muscle.

The IGF1/P13K/Akt hypertrophy signaling pathways

Skeletal muscle hypertrophy, characterized in the adult mammal by an increase in the size of pre-existing myofibers (rather than hyperplasia, or an increase in the number of myofibers), involves a shift towards protein synthesis and away from protein degradation. Hypertrophy can be induced by multiple anabolic stimuli – among the most studied of which is insulin-like growth factor 1 (IGF1) (Bodine et al., 2001b; Rommel et al., 2001). Signaling via IGF1 is mediated first by IGF1 ligand binding to its receptor, the tyrosine kinase IGF1 receptor (IGF1R). This binding induces trans-phosphorylation of the dimeric receptor, and the resultant phosphorylated tyrosines create a docking site for the recruitment of the Insulin Receptor Substrate 1 (IRS1) (Bodine et al., 2001). IRS1 phosphorylation is required for most of the downstream signalings, and its phosphorylation is thus a key, highly regulated step in the modulation of IGF1 signaling. The IGF1 pathway can be inactivated by targeting IRS1 for ubiquitin-mediated degradation – ubiquitination of IRS1 has been reported after prolonged insulin stimulation, in which IRS1 was degraded in a phosphatidylinositol-3 kinase (P13K)-sensitive fashion, in various cell lines (Haruta et al., 2000; Lee et al., 2000; Tzatsos and Kandror, 2006; Xu et al., 2008; Zhande et al., 2002). However, these multiple reports differed as to the pathways downstream of PI3K that mediate IRS1 turnover (Haruta et al., 2000; Tzatsos & Kandror, 2006; Xu et al., 2008; Zhande et al., 2002). Xu et al. (2008) and Tzatsos & Kandror (2006) suggest that the PI3K/Akt/mamalian target of rapamycin (mTOR) pathway activates IRS1 degradation, while others reported that the activation of PI3K independent of mTOR signaling is required for IRS1 degradation (Zhande et al., 2002).

A variety of E3 ubiquitin ligases have been demonstrated to serve as IRS1 ligases, although under distinct conditions. The first pair was SOCS1 and SOCS3, which were shown to promote degradation of IRS1 and IRS2, and which may mediate inflammation-induced insulin resistance (Rui et al., 2002). The cullin 7 complex, containing the E3 ligase Fbxw8, was shown to be activated by an mTOR-dependent negative feedback loop after which it could degrade IRS1 (Xu et al., 2008). Cbl-b was then reported to degrade IRS1 in settings of muscle atrophy (Nakao et al., 2009).

As for IGF1 induced phosphorylation of IRS1, the E3 ligase that modulates IRS1 levels after IGF1R phosphorylation of IRS1 is the Fbxo40–SCF complex (Shi et al., 2011) (Figure 1). IRS1 is rapidly degraded after IGF1 stimulation, and this limits downstream activation of the IRS1/PI3K/Akt pathway. A screen for the causative mechanism demonstrated that knockdown of the Fbox-containing protein Fbxo40 resulted in a sparing of IRS1 even after IGF1 stimulation of the pathway (Shi et al., 2011). Fbxo40 null mice experience a more rapid increase in mass during their growth phase where IGF1 levels are high, and these animals have enhanced muscle size. Fbxo40, thus, is a physiologic regulator of IGF1 signaling; it is noteworthy that the Fbxo40–SCF complex can induce rapid turnoff of the IGF1 pathway only if IRS1 cannot be replenished by new protein synthesis. Thus, the mechanism seems to be a checkpoint to stop IGF1 signaling under those conditions where the muscle is incapable of responding to an
anabolic signal due to a lack of protein. In those settings, where the protein synthesis is turned off, IRS1 cannot be regenerated after Fbxo40-mediated degradation, and the IGF1 pathway is thus short-circuited (Figure 1). A distinct protein, MG53, has recently been shown to mediate the degradation of IRS1 when it is bound to the Insulin Receptor (IR), but not when bound to the IGF1 Receptor (IGF1R) (Song et al., 2013). As such, the primary substrate of MG53 appears to be the IR itself (Song et al., 2013). By mediating the turnover of the IR and the associated IRS1, MG53 can, therefore, inhibit myogenesis (Yi et al., 2013).

In skeletal muscle, downstream of IGF1 induced IRS1 phosphorylation, there is a subsequent stimulation of the PI3K/Akt pathway, resulting in the parallel phosphorylation and activation of TORC1 signaling, in one distinct pathway, and inhibition of glycogen synthase kinase 3β (GSK3β) in the other pathway (Rommel et al., 2001) (Figure 1). Genetic activation of IGF1 (Coleman et al., 1995; Musaro et al., 2001) or Akt (Lai et al., 2004) has been shown to be sufficient in inducing muscle hypertrophy, as demonstrated by tissue-specific transgenic mouse models. Lai et al. produced an inducible constitutively active Akt model, which allowed for the demonstration that even in an adult animal, relatively short-term activation of Akt resulted in doubling of skeletal muscle mass (Lai et al., 2004). In a distinct transgenic mouse model, Akt was inducibly activated only in fast glycolytic muscle, and this more restricted activation was nevertheless shown to be sufficient to reduce fat mass (Izumiya et al., 2008). This effect was either due to a simple tradeoff in calories, given the enhanced requirements of the hypertrophic pathways.
TORC1 signaling and its regulation by MNK2 in skeletal muscle

As mentioned earlier, one of the branches of the Akt pathway that mediates skeletal muscle hypertrophy is the activation of mTOR signaling (Figure 1). Once activated, mTOR exists as two distinct complexes, TORC1 and TORC2. TORC1 is characterized by the presence of regulatory-associated protein of mTOR (RAPTOR) (Kim et al., 2001), whereas TORC2 binds rapamycin-insensitive companion of mTOR (RICTOR) instead (Guertin et al., 2006; Sarbassov et al., 2005). TORC2, mostly insensitive to the pharmacologic agent rapamycin with effects observed only after long-term treatment, phosphorylates Akt on serine 473 as a part of a required feedback loop (Lamming et al., 2012; Schalm et al., 2003). TORC1, sensitive to inhibition by rapamycin treatment, propagates downstream signaling through the phosphorylation and activation of p70S6K, and inhibition of 4E-BP1 (also called PHAS-1), with downstream targets including the ribosomal protein S6 (RPS6) and the eukaryotic translation initiator eIF4E (Sarbassov & Sabatini, 2005; Sarbassov et al., 2005) (Figure 1). The effect of rapamycin treatment on TORC1 targets does, however, vary significantly with some substrates, such as 4E-BP1, being largely resistant. Of note, in contrast, ATP-competitive mTORC1 inhibitors block the phosphorylation of all mTORC1 phosphorylation sites, regardless of their rapamycin sensitivity (Kang et al., 2013).

Phosphorylation and inhibition of 4E-BP1 is tightly controlled by TORC1 (Gingras et al., 1998), and results in the release of 4E-BP1-dependent inhibition of the translation initiator eIF4E (Brunn et al., 1997; Hara et al., 1997; Lawrence et al., 1997). Following dissociation from 4E-BP1, eIF4E binds with eIF4G and eIF4A forming the eIF4F complex, a key first step required for translation. Both the formation and the activity of the eIF4F complex are dependent on free eIF4E and the phosphorylation state of eIF4F. The amount of free eIF4E is correlated to the relative degree of 4E-BP1 phosphorylation; when it is complexed with the non-phosphorylated form of 4E-BP1, then eIF4E cannot bind eIF4G (Berset et al., 1998; Gingras et al., 2001). eIF4G, acting as a scaffold, links eIF4E with other members of the eIF4F complex, including mitogen-activated protein kinase-interacting kinases (or MNKs), which are responsible for directly phosphorylating and activating eIF4E at serine 209; however, it is not clear whether this phosphorylation event is necessary for the assembly of the translational initiation complex, or indeed what the effect of MNK phosphorylation is on eIF4E activity (Prevot et al., 2003a,b; Pyronnet et al., 1999; Schepfer et al., 2001). Previous research indicated that both MNK1 and MNK2 bind eIF4G near serine 1108, a key residue whose phosphorylation is increased in an mTOR-dependent manner following IGF1 expression (Schepfer et al., 2001).

A novel function for MNK2 on this phosphorylation site was only recently shown (Hu et al., 2012). Hu et al. reported an inverse relationship between the activity of MNK2 and eIF4G Ser1108 phosphorylation both in vitro and in vivo (Hu et al., 2012). In the presence of IGF1, overexpression of MNK2, but not MNK1, blocked eIF4G Ser1108 phosphorylation independent of Akt activation while siRNA knockdown of MNK2 overcame rapamycin-mediated inhibition of the phosphorylation event, suggesting that MNK2 negatively influences IGF1-Akt signaling downstream of mTOR (Hu et al., 2012). Similarly, in MNK2 knockout mice, but not in those lacking MNK1, phosphorylation of eIF4G Ser1108 was elevated. Since MNK2 is a kinase, and since its activation resulted in a decrease as opposed to an increase in eIF4G phosphorylation, the implication was that there was a kinase substrate that was inhibited by MNK2, which in turn was responsible for phosphorylating eIF4G. The serine–arginine-rich protein kinases (SRPKs) were identified through an siRNA screen as a possible link between MNK2 and eIF4G phosphorylation (Hu et al., 2012). MNK2 expression was shown to specifically block SPRK-mediated phosphorylation of eIF4G on Ser1108 via an allosteric effect.

MNK2 was further shown to bind RAPTOR, giving a mechanism by which it inhibits TORC1 mediated phosphorylation p70S6K (Hu et al., 2012). As such, MNK2 appears to exert distinct, antagonistic effects, not shared by its closest paralog, MNK1, on both eIF4G Ser1108 phosphorylation and p70S6K activation, thereby acting as a downstream inhibitor of IGF1/Akt/mTOR hypertrophy signaling. It is worth noting that while both MNK1 and MNK2 are expressed in skeletal muscle, only MNK2 was induced in two animal models of atrophy, denervation-induced atrophy and dexamethasone-induced atrophy (Figure 1).

In addition and independent of its activation of mTOR, Akt phosphorylates and inactivates GSK3β (Alessi et al., 1996; Cross et al., 1995; Morisco et al., 2000; Rommel et al., 2001), which in turn promotes the activity of the translation initiator eIF2B (Hardt et al., 2004). Together, the pathways converge to enhance translation initiation and elongation and, thus, protein synthesis. GSK3β has other inhibitory effects on skeletal muscle, which are thus also reversed by phosphorylation of Akt. These include GSK3β-mediated phosphorylation of the transcription factor NFAT (Crabtree & Olson, 2002; Rommel et al., 2001). When GSK3β is inhibited by Akt signaling, the dephosphorylated NFATc1 and c3 proteins can translocate to the nucleus, where their activity enhances myoblast differentiation and fiber-type switching to the slow/oxidative phenotype (Frady et al., 2000; Horsley & Pavlath, 2002; Horsley et al., 2001). Akt-mediated inhibition of GSK3β does not, however, necessitate fiber-type switching, given that adult activation of Akt is not accompanied by a change in fiber-type composition (Blaauw et al., 2009).

MuRF1 and MAFbx mediated induction of muscle atrophy

In contrast to hypertrophy, skeletal muscle atrophy is characterized by a shift toward protein degradation, resulting from cachectic stimuli such as inflammatory cytokines and glucocorticoids. Proteolysis, as observed in atrophy, has been shown to occur in part due to the activation of ubiquitin-mediated proteasomal degradation (Mitch & Goldberg, 1996). Multiple models of muscle atrophy, including denervation, high-dose dexamethasone treatment, treatment with
inflammatory cytokines, and simple immobilization, all induce transcriptional upregulation of MuRF1 and MAFbx (also called atrogin-1), genes that encode for E3 ubiquitin ligases (Bodine et al., 2001a; Gomes et al., 2001). Supporting their prominent roles, under atrophic conditions mice null for either gene (MuRF1 /– or MAFbx /–) exhibit a resistance to muscle mass loss as compared to wild-type control littermates (Bodine et al., 2001a).

Muscle RING finger-containing protein 1 (MuRF1) encodes a protein that contains four domains. The most NH3-terminal domain is a RING-finger (Borden & Freemont, 1996; Saurin et al., 1996), which is required for MuRF1’s ubiquitin ligase activity, since this is the domain which binds to an E2 protein, which in turn mediates transfer of ubiquitin to the substrate (Joazeiro et al., 1999). The next domain, downstream of the RING, is a ‘‘B-box’’, which can mediate self-association – the B-box in MuRF1 self-associates into dimers with high affinity (Mrosek et al., 2008). Next in MuRF1, there is a ‘‘coiled-coil domain’’, which may be required for the formation of heterodimers between MuRF1 and itself, in addition to the related MuRF2 protein (Witt et al., 2008). Additional evidence that MuRF1 and MuRF2 work together was provided by a double-knockout, which showed actual hypertrophy of cardiac muscle (Witt et al., 2008), and not just blockade of atrophy. Further evidence for MuRF1/MuRF2 interactions came from a double knockout (DKO) study, which demonstrated that in these animals there was a profound loss of type II fibers (Moriscot et al., 2010). Proteins that have a RING domain, a B-box, and a coiled-coil domain are now known as TRIM proteins (for tripartite motif; Meroni & Diez-Roux, 2005). The fourth, less recognized domain of MuRF1, is a ‘‘MuRF domain’’, which is shared by all three MuRF proteins, MuRF1, MuRF2, and the less-studied MuRF3 (Gregorio et al., 2005).

MuRF1 is localized to the sarcomere. This was originally suggested by virtue of its binding to the very large myofibrillar protein titin, at the M line (Centner et al., 2001; McElhinny et al., 2002; Pizon et al., 2002). However, there is no evidence that titin is actually a substrate for MuRF1. MuRF1 also physically binds myosin heavy chain (MyHC), as demonstrated by immunoprecipitation of epitope-tagged MuRF1 protein, which brings down the MyHC protein (Clarke et al., 2007). Unlike the case of titin, which also binds MuRF1, MyHC is also a substrate of MuRF1, demonstrated by the finding that MuRF1 can directly ubiquitinate MyHC (Clarke et al., 2007). Furthermore, MuRF1 null mice demonstrate sparing of MyHC during atrophy, and knockdown of MuRF1 results in an increase of MyHC (Clarke et al., 2007).

Later, it was shown that additional myosin domain-containing proteins in the thick filament of muscle were also degraded by MuRF1, including myosin light chain and myosin binding protein C (Cohen et al., 2009). Therefore, MuRF1 induces muscle atrophy, at least in part, by directly attacking the thick filament of the sarcomere and causing the proteolysis of myosin proteins.

Muscle atrophy Fbox protein (MAFbx) contains an Fbox domain, a motif seen in a family of E3 ubiquitin ligases called SCFs (for Skp1, Cullin, and Fbox). The Fbox containing proteins are not enzymes themselves, but instead bring substrates to the E2 by virtue of the Fbox binding to the Skp1–Cullin complex. A RING-containing protein, Rbx1, is also a part of this complex, which is responsible for activating the E2 (Kamura et al., 1999). Fbox containing proteins usually bind a substrate only after that substrate has first been posttranslationally modified – for example, by serine or tyrosine phosphorylation (Winston et al., 1999).

Substrates have been suggested for MAFbx, including MyoD (Lagirand-Cantaloube et al., 2009; Tintignac et al., 2005) and calcineurin (Li et al., 2004). However, it has not yet been shown whether protein is ubiquitinated either by MAFbx in skeletal muscle or under atrophy conditions. MAFbx has been convincingly shown to be an E3 ligase for ElF3-f, a protein initiation factor (Li, 2007). This finding suggests that MAFbx activity results in muscle atrophy through the downregulation of protein synthesis.

Proinflammatory cytokines, such as TNF-z, TWEAK, or IL-1, signal into two established pathways: the NF-κB pathway and the p38 MAP kinase. These two signaling mediators are required to upregulate the expression of the key E3 ligases, MuRF1, which mediate sarcomeric breakdown and inhibition of protein synthesis (Clarke et al., 2007; Cohen et al., 2009), and MAFbx, which control protein synthesis by ubiquitination of ElF3c (Csibi et al., 2009; Lagirand-Cantaloube et al., 2008; Sanchez et al., 2013). MuRF1 is upregulated in multiple settings of muscle atrophy (Bodine et al., 2001a) and is responsible for mediating the ubiquitination of the thick filament of the sarcomere – MyHC (Clarke et al., 2007) – and other thick filament components (Cohen et al., 2009). The cytokine TWEAK, in particular, induces MuRF1 upregulation via NF-κB, resulting in MyHC loss (Mittal et al., 2010). Inhibition of classical NF-κB is sufficient to significantly decrease tumor-induced muscle loss, at least in mice, in part, by inhibiting the upregulation of MuRF1 (Cai et al., 2004; Moore-Carrasco et al., 2007). This inflammatory pathway is activated in the setting of inflammatory cachexia, with examples including pulmonary cachexia, where the inflammation is present (Langen et al., 2012), and joint inflammation (Ramirez et al., 2011).

Activation of Akt can in turn inhibit the transcriptional upregulation of MAFbx and MuRF1 normally seen during atrophy (Rüegg & Glass, 2011). Their normal upregulation was demonstrated to require the FOXO (also known as Forkhead) family of transcription factors (Sandri et al., 2004; Stitt et al., 2004). FOXO transcription factors are excluded from the nucleus when phosphorylated by Akt and translocate to the nucleus upon dephosphorylation (Brunet et al., 1999). The translocation and activity of FOXO transcription factors are required for upregulation of MuRF1 and MAFbx – in the case of FOXO3, the activation was demonstrated to be sufficient to induce atrophy (Mammucari et al., 2007; Zhao et al., 2007), a finding that was subsequently supported by the transgenic expression of FOXO1, which also resulted in an atrophic phenotype (McLoughlin et al., 2009; Southgate et al., 2007).

MST1, a kinase that is highly expressed in the skeletal muscle, is up-regulated in fast but not slow skeletal muscle upon denervation (Wei et al., 2013). Deletion of the MST1 kinase significantly blocked loss of skeletal muscle normally caused by denervation and decreased the expression of
MAFbx, along with LC3. These effects of MST1 are apparently due to its ability to phosphorylate FOXO3a at Ser207, promoting its nuclear translocation in atrophic fast-dominant muscles (Wei et al., 2013).

**Glucocorticoid-mediated activation of MuRF1 transcription**

High concentrations of glucocorticoids can induce muscle atrophy, in part by upregulation of MuRF1 and MAFbx (Wray et al., 2003; Stitt et al., 2004). It was shown that glucocorticoids synergize with FOXO1 in inducing transcription of the MuRF1 gene (David et al., 2008; Zhao et al., 2009). Indeed, sepsis induces MuRF1 activation in part by glucocorticoid activation of its ligand-dependent transcription factor, the glucocorticoid receptor (GR) (Smith et al., 2010). Target genes of the GR were identified in the skeletal muscle. One such gene, KLF15, was found to upregulate the expression of both MAFbx and MuRF1, resulting in myotube atrophy (Shimizu et al., 2011).

In addition to the regulation by Akt/FOXO signaling, MuRF1 and MAFbx transcription can be at least partially inhibited by the activation of TORC1 (Herningtyas et al., 2008), although this evidence is not sufficient to block cachexia, since supplementation by amino acids, which induce TORC1 activation, cannot block muscle atrophy seen in cachexia. However, it has been reported that mTOR activation inhibits GR (Shimizu et al., 2011), which gives one mechanism by which mTOR signaling blocks the upregulation of these E3s. While activation of TORC1 might not be sufficient to block MuRF1 and MAFbx upregulation, the inhibition of mTOR independently can induce activation of these E3 ligases. This was shown, for example, by the use of AMPK, which can block mTOR signaling, and which is sufficient to upregulate MuRF1 and MAFbx (Tong et al., 2009).

TORC2, in a positive feedback loop, phosphorylates Akt at serine 473, thereby permitting maximum Akt activation (Sarbassov & Sabatini, 2005; Sarbassov et al., 2005). Recently Bentzinger et al. (2013) demonstrated that deletion or knockdown of RAPTOR, resulting in an inhibition of TORC1 signaling, was sufficient to increase muscle atrophy (Bentzinger et al., 2013). Surprisingly, a sustained activation of TORC1 actually caused muscle atrophy, due to the suppressed phosphorylation of Akt via feedback inhibition by mTORC1 (Bentzinger et al., 2013). Indeed, this negative feedback signaling, resulting in a blockade of Akt, is due to a mechanism involving feedback phosphorylation and inhibition of the upstream mediator IRS by p70S6K downstream of mTOR (Tremblay & Marete, 2001), causing inhibition of PI3K and, therefore, Akt activation. In the recent study by Bentzinger et al., this feedback loop resulted in a paradoxical activation of MuRF1 and MAFbx, since in this case FOXO signaling was derepressed. This surprising finding seems to indicate that long-term stimulation of TORC1, caused for example by amino acid-mediated stimulation without coincident activation of Akt, which can be induced by exercise-mediated activation of IGF1, might paradoxically result in muscle atrophy – giving a mechanism whereby it may be counterproductive to eat protein without exercising. Indeed, this same group also showed that sustained activation of TORC1 could eventually result in actual myopathy (Castets et al., 2013). Another mechanism for this effect is a disregulation of autophagy, which is usually induced when mTOR signaling is blocked (Mordier et al., 2000), and which is required for the normal maintenance of skeletal muscle (Sandri, 2013).

**Recently discovered E3 ligases that regulate muscle mass and differentiation**

While acute atrophy results in upregulation of MuRF1 and MAFbx, which are sufficient to cause breakdown of the myosin-containing thick filament of the sarcomere and protein translation factors like eIF3f (Sanchez et al., 2013), respectively; other E3 ligases come into play during muscle atrophy. Such E3 ligases include the already-mentioned Fbx-containing protein Fbxo40, which degrades IRS1 upon IGF1 signaling (Shi et al., 2011), TRIM32, an E3 ligase that degrades actin (Kudryashova et al., 2005) and desmin (Cohen et al., 2012). Loss of desmin is responsible for a particular form of limb girdle muscular dystrophy (Frosk et al., 2002). In contrast to MuRF1, whose deletion seems to spare muscle and block atrophy, loss of TRIM32 results in pathologic, or dystrophic, skeletal muscle. The E3 ligase Trip12, a HECT domain E3 ubiquitin ligase, has been recently shown to bind and induce the polyubiquitination of a protein called Sox6, a transcription factor which plays a role in fiber-type switching (An et al., 2013). Knockdown of Trip12 in myotubes resulted in an increase in Sox6 protein levels and a concurrent decrease in slow fiber-specific Myh7 expression, along with a coincident increase in the fast fiber-specific marker, Myh4 (An et al., 2013).

An interesting recent finding is that an E3 ligase called Mul1 controls “mitophagy” – the turnover of mitochondria (Lokireddy et al., 2012). Loss of mitochondria was noted early on in settings of muscle atrophy (Pellegrino & Franzini, 1963), and this loss has been thought to contribute to the phenotype, due to the decreased ability to generate ATP, among other sequelae. Overexpression of Mul1 was sufficient for the induction of mitophagy in skeletal muscle myotubes, and suppression both protected against mitophagy and partially rescued the muscle subjected to atrophy-inducing stimuli (Lokireddy et al., 2012).

**Myostatin, activin, and other TGFβ family members**

Myostatin, or growth and differentiation factor 8 (GDF-8), is a member of the transforming growth factor-β (TGF-β) superfamily that acts as a negative regulator of muscle growth (Lee & McPherron, 1999, 2001; McPherron & Lee, 1997, 2002; McPherron et al., 1997, 1999). Genetic mutations in MSTM and therapeutic inhibition against myostatin result in an increase in the overall skeletal muscle mass, a phenotype conserved across multiple species, including mice, cattle, and humans (Grobet et al., 1997; Lee, 2007; McPherron & Lee, 1997; McPherron et al., 1997). Once bound to its type I and type II receptors, Activin Receptor II A or B (ActRIIA or B) and Activin-Like Kinase-4 or 5 (ALK-4 or 5), respectively, intracellular signaling is initiated via phosphorylation and activation of the transcription factors Smad2 and 3, which translocate to the nucleus and...
activate target genes (McCroskery et al., 2003; Rebbapragada et al., 2003). The existence of non-Smad-mediated pathways has been only recently reported and the identities of downstream targets of myostatin intracellular signals are unclear (Huang et al., 2007; Philip et al., 2005).

In skeletal muscle, myostatin negatively regulates Akt signaling (Sartori et al., 2009; Trendelenburg et al., 2009). Interestingly, IGFl can rescue this effect on Akt; when myostatin and IGFl are given together, Akt phosphorylation is indistinguishable versus myotubes stimulated with IGFl alone (Trendelenburg et al., 2009), despite the fact that there is no obvious direct effect of IGFl on myostatin-mediated Smad signaling (Sartori et al., 2009; Trendelenburg et al., 2009). One mechanism by which IGFl may inhibit or at least restrict Smad2/3 activation is by TORC1 signaling. The roles of TORC1 and TORC2 in myostatin’s inhibition of muscle growth were investigated, and an increase in myostatin-induced Smad2 phosphorylation was observed following inhibition of TORC1 (by siRNA knockdown of RAPTOR), potentiating myostatin’s inhibitory effects on muscle (Trendelenburg et al., 2009). An additional component of a myostatin-Akt cross-talk model may, therefore, include a feedback loop, with TORC1 capable of negatively influencing myostatin signaling. Although myostatin and IGFl have antagonistic effects on mTOR phosphorylation, it is not clear whether the regulation of mTOR represents a necessary central nexus of myostatin-induced effects or, rather, plays a more supportive role, downstream of Akt (Figure 1). Recent studies have shown that blocking mTOR activity does not fully prevent the increases in protein synthesis and hypertrophy phenotype associated with myostatin inhibition (Sartori et al., 2009; Welle et al., 2009).

Myostatin’s negative regulation of skeletal muscle growth may be due, in part, to its interference with myoblast differentiation (Lin et al., 2002; McPherron et al., 1999; Rios et al., 2001). Trendelenburg et al. (2009) reported that treatment of primary human skeletal myoblasts and myotubes with physiologic concentrations of myostatin resulted in an inhibition of differentiation. siRNA-mediated knockdown of Smad2 and Smad3 was shown to be sufficient to inhibit myostatin signaling and rescue differentiation (Trendelenburg et al., 2009; Sartori et al., 2009). Previous research has suggested that other members of the TGF-β superfamily may cooperate with myostatin in regulating differentiation. Specifically, expression of the TGF-β inhibitor follistatin, coupled with myostatin inhibition, exhibits a synergistic effect on increasing muscle mass. TGF-β1, GDF-11, and Activin A, all members of the TGF-β superfamily, have been shown to block muscle differentiation with similar or even greater potencies than that of myostatin (Trendelenburg et al., 2009; Figure 1). It is currently unclear whether endogenous levels of these molecules are capable of modulating skeletal muscle and, as such, further studies are required to determine the specific roles and physiological importance of TGF-β molecules in skeletal muscle.

Whether or not myostatin directly induces atrophy signaling is somewhat less clear. One study showed that rather than inducing MuRF1 and MAFbx, myostatin signaling actually decreased transcription of these genes, along with other genes normally induced upon muscle differentiation (Trendelenburg et al., 2009). The conclusion from that study, therefore, was that myostatin induces muscle atrophy both by blocking Akt-mediated protein synthesis and by downregulating genes required for muscle homeostasis normally induced upon differentiation, even in post-differentiated muscle fibers. Other studies, however, have shown that myostatin, albeit at quite high concentrations, can in fact induce upregulation of the E3 ligases (Lokireddy et al., 2012; Sartori et al., 2009).

Myostatin itself can be regulated by multiple mechanisms, including via the CCAAT/enhancer (David et al., 2010), hypoxia (Hayot et al., 2011), and microRNA27-a (David & Amanda, 2010). Furthermore, it has been shown that inflammatory signaling, downstream of cytokine activation, induces endogenous expression of the TGFβ family member Activin, demonstrating an important instance of cytokine/TGFβ signaling (Trendelenburg et al., 2012; Figure 1).

**G-Protein induced activation of hypertrophy signaling**

Independent of IGFl-mediated mTOR activation, signaling through heterotrimeric guanine nucleotide-binding proteins (G protein)-coupled receptors (GPCRs) (Pierce et al., 2002) has emerged as a novel mechanism in the regulation of skeletal muscle hypertrophy (Jean-Baptiste et al., 2005). Upon ligand binding, GPCRs undergo a conformational shift, permitting their activation and signaling via recruitment of intracellular heterotrimeric G proteins. Activation of four G protein-coupled receptors, CRFR2, β2-AR, the LPA receptor, and Fzd7, have shown to induce skeletal muscle hypertrophy (Lynch & Ryall, 2008; Rebecca & Randi, 2012). More recently, Minetti et al. (2011) demonstrated that a G protein, specifically Gzι2, was essential for the induction of muscle hypertrophy mediated by LPA receptor signaling.

G proteins, expressed in multiple tissue types including skeletal muscle, consist of a GTP-binding alpha subunit (Gz) and a heterodimer of beta and gamma subunits (Gβγ) – once activated, Gz subunits bind GTP, thereby releasing bound Gβγ subunits and allowing Gz to mediate downstream signaling. Among the four classes of Gz subunits, Gzα proteins (Gzα1, Gzα2, and Gzα3) are widely distributed and highly homologous, capable of regulating key signaling mediators such as phospholipase C and protein kinase C (PKC) (Wettschureck & Offermanns, 2005). Previous research has suggested a possible link between G protein and Akt signaling events, with GPCR β2-AR-mediated skeletal muscle hypertrophy, at least, accompanied by the activation of Akt in a manner dependent of mTOR (Kline et al., 2007; Koopman et al., 2010).

Constitutively active Gzι2 by itself was sufficient to promote hypertrophy in cultured myotubes as well as in mouse models (Minetti et al., 2011). However, while rapamycin and PKC inhibitors blocked the resulting hypertrophic phenotype, PI3K inhibitors did not have an effect. Consistent with these data, Gzι2 activity drove phosphorylation of targets downstream of mTOR, specifically p70S6K and GSK3β, but not that of Akt, suggesting a linear pathway between the G protein and the mTOR via PKC. Surprisingly, the results offer a novel mechanism for Gzι-mediated hypertrophy signaling in skeletal muscle that is dispensable of PI3K and Akt.
In addition to its role in hypertrophy, GPCR signaling may also directly influence the atrophy program in skeletal muscle. In multiple rodent models of atrophy, including unloading and aging, ligands for the GPCRs promoted atrophy resistance (Carter & Lynch, 1994; Kline et al., 2007). Similarly, β2-AR agonists exhibit an inhibitory effect on muscle atrophy in cancer cachexia models (Carbó et al., 1997; Costelli et al., 1995). Minetti et al. (2011) demonstrated that activation of Gzii2 can block the up-regulation in the expression MuRF1 and MAFbx associated with proinflammatory cytokine TNFα-induced atrophy. In contrast to the Gzii2/PKC signaling for hypertrophy, this appears to be PKC-independent, mediated instead by HDAC4 localization/activity – Gzii2 drove HDAC4 cytoplasmic localization, thereby preventing its nuclear functions (Figure 1).

PGC-1α, mitochondria, and sarcopenia
Mitochondrial oxidative metabolism and energy transduction pathways are critical for skeletal muscle function, and it has been recognized for quite some time that another major effect of long-term muscle atrophy is a decrease in mitochondria (Pellegrino & Franzenzi, 1963). The transcriptional coactivator peroxisome proliferator-activated receptor-γ coactivator-1z (PGC1z) is sufficient to induce mitochondriogenesis (Wu et al., 1999), along with other effects on muscle, including fiber-type switching (Puigserver & Spiegelman, 2003). There are two reports that over-expression of PGC1z can also result in the sparing of skeletal muscle under atrophy conditions, perhaps by negative regulation of FOXO signaling (Brault et al., 2010; Sandri et al., 2006). Paradoxically, a signaling pathway that increases mitochondriogenesis via PGC1z is the activation of AMPK, which at the same time actually decreases protein synthesis, by blocking mTOR (Bolster et al., 2002; Mounier et al., 2009). Therefore, it would be important to determine whether it is possible to positively modulate the PGC1z pathway without interfering with protein synthesis, since there is a net loss of protein in atrophy, and therefore, an additional inhibition in synthesis pathways may not be desirable.

In addition to the transcriptional co-activator PGC1z, the expression of genes important for mitochondrial biogenesis is also in part controlled by the estrogen-related receptor (ERR) subfamily of nuclear receptors (Soriano et al., 2006). PGC-1z and ERRs, working together, induce the expression of a muscle-specific protein, Perm1, which regulates the expression of genes with roles in glucose and lipid metabolism, energy transfer, and contractile function (Cho et al., 2013).

The age-related loss of skeletal muscle is called “sarcopenia” (Glass & Roubenoff, 2010; Hughes & Roubenoff, 2000). This loss of muscle mass and function results in frailty of the elderly, a considerable degree of morbidity, such as an enhanced risk of falls, and the loss of the ability to maintain an independent lifestyle. In an unbiased survey of gene changes which occur upon sarcopenia in rats, the most downregulated pathway was that associated with PGC1z and mitochondriogenesis (Ibebujo et al., 2013). While this simply establishes an association, when transgenic mice overexpressing PGC1z in skeletal muscle were followed in an aging colony, they were found to be resistant to the onset of sarcopenia (Wenz et al., 2009); even more provocative was the supplemental figure in this study, demonstrating that the transgensics had a significant increase in life-span. This last piece of data is especially surprising, since PGC1z was only overexpressed in the muscle; the implication is that the muscle may be a source of secreted “myokines” which have a protective effect on the rest of the organism.

The anabolic sex hormone testosterone is also capable of inducing PGC1z, in addition to IGF-1 (Ibebujo et al., 2011); testosterone and the consequent activation of the Androgen Receptor provide one of the few known mechanisms that can simultaneously induce mitochondriogenesis and anabolism via the IGF1/Akt pathway (Ibebujo et al., 2011).

Conclusion
It has only been in the last 15 years or so that the signaling pathways controlling skeletal muscle mass and function have begun to be elucidated. Therefore, it should not be surprising that new mechanisms and refinements to these pathways continue to be discovered, and indeed very recently quite considerable progress has been made in this area. Still, the fact remains that, in contrast to other well-studied diseases, there is an almost entire lack of approved medications for skeletal muscle disease – despite the great need accentuated by an aging population, where muscle frailty and weakness are an almost universal sign of “normal aging”. Further, the finding that in settings such as cancer, the simple treatment of cachexia – distinct from the tumor itself – can increase survival which in turn greatly increases the interest in preserving skeletal mass and function in settings of disease. The enhanced understanding of the mechanisms controlling skeletal muscle maintenance gives increased hope that such treatments will be developed in the near future.

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Declaration of interest
Both authors are employees of Novartis Institutes for Biomedical Research.

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