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The Mechanical Activation of mTOR Signaling: An Emerging Role for Late Endosome/Lysosomal Targeting

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Abstract

It is well recognized that mechanical signals play a critical role in the regulation of skeletal muscle mass, and the maintenance of muscle mass is essential for mobility, disease prevention and quality of life. Furthermore, over the last 15 years it has become established that signaling through a protein kinase called the mammalian [or mechanistic] Target of Rapamycin (mTOR) is essential for mechanically-induced changes in protein synthesis and muscle mass, however, the mechanism(s) via which mechanical stimuli regulate mTOR signaling have not been defined. Nonetheless, advancements are being made, and an emerging body of evidence suggests that the late endosome/lysosomal (LEL) system might play a key role in this process. Therefore, the purpose of this review is to summarize this body of evidence. Specifically, we will first explain why the Ras homologue enriched in brain (Rheb) and phosphatidic acid (PA) are considered to be direct activators of mTOR signaling. We will then describe the process of endocytosis and its involvement in the formation of LEL structures, as well as the evidence which indicates that mTOR and its direct activators (Rheb and PA) are all enriched at the LEL. Finally, we will summarize the evidence that has implicated the LEL in the regulation of mTOR by various growth regulatory inputs such as amino acids, growth factors and mechanical stimuli.

Keywords

mTOR; lysosome; skeletal muscle; mechanotransduction; protein synthesis; hypertrophy

Introduction

Comprising ~45% of the body's mass, skeletal muscles are not only the motors that drive locomotion, but they also play a critical role in breathing, whole body metabolism and maintaining quality of life (Seguin and Nelson 2003; Izumiya et al. 2008). Indeed, both sedentary and active adults will lose 35–40% of their skeletal muscle mass by the age of 80, and this loss in muscle mass is associated with disability, loss of independence, an increased risk of morbidity and mortality, as well as an estimated \$18.5 billion in annual healthcare costs in the United States alone (Janssen et al. 2004; Seguin and Nelson 2003; Proctor et al. 1998; Pahor and Kritchevsky 1998). Moreover, the loss of skeletal muscle mass is associated with a wide array of diseases including myopathies, cancer and HIV (Fearon et al. 2013; Dudgeon et al. 2006; Moylan and Reid 2007). Thus, the development of pharmacological therapies that can maintain or restore muscle mass would be highly beneficial to multiple clinically relevant populations (Hurley et al. 2011). However, in order

to develop such therapies, we will first need to have a comprehensive understanding of the molecular mechanisms that regulate skeletal muscle mass.

One of the most potent environmental regulators of skeletal muscle mass is mechanical loading, with an increase in mechanical loading resulting in an increase in muscle mass (i.e. muscle hypertrophy) and a decrease in mechanical loading resulting in the loss of muscle mass (i.e. muscle atrophy) (Goldberg et al. 1975). It has also been demonstrated that mechanical load-induced changes in muscle mass are highly associated with changes in the rate of protein synthesis. For example, muscle hypertrophy induced by increased mechanical loading is accompanied by an increase in the rate of protein synthesis, while muscle atrophy induced by a decrease in mechanical loading is accompanied by a decrease in the rate of protein synthesis (Vandeburgh 1987; Vandeburgh et al. 1999; Goldberg 1968; Fitts et al. 2000; Goodman et al. 2012). Thus, changes in the rate of protein synthesis appear to play a fundamental role in the mechanical regulation of muscle mass. However, the molecular mechanisms through which mechanical stimuli regulate changes in the rate of protein synthesis, and ultimately muscle mass, have only been vaguely defined.

Over the last 20 years, a protein kinase called the mammalian [or mechanistic] Target of Rapamycin (mTOR) has become well regarded as a major regulator of protein synthesis and cell size (Sengupta et al. 2010). Furthermore, numerous studies have shown that mechanical stimulation leads to the activation of mTOR signaling, and signaling through mTOR is necessary for a mechanically-induced increase in protein synthesis and the concomitant hypertrophic response (Bodine et al. 2001; Goodman et al. 2011; Hornberger et al. 2004; Drummond et al. 2009; Kubica et al. 2005). In addition, it has also been shown that the activation of mTOR signaling is sufficient to induce an increase in muscle protein synthesis and muscle fiber hypertrophy (Goodman et al. 2010). For these reasons, it has become widely accepted that mTOR plays a fundamental role in the mechanical regulation of protein synthesis and muscle mass; however, the mechanism(s) through which mechanical stimuli regulate mTOR signaling remain unclear (Hornberger 2011). Nevertheless, advancements are being made, and an emerging body of evidence suggests the late endosome/lysosomal (LEL) system might play a key role in this process. Therefore, the purpose of this review is to summarize this body of evidence. Specifically, we will first explain why the Ras homologue enriched in brain (Rheb) and phosphatidic acid (PA) are considered to be direct activators of mTOR signaling. We will then describe the process of endocytosis and its involvement in the formation of LEL structures, as well as the evidence which indicates that mTOR and its direct activators (Rheb and PA) are all enriched at the LEL. Finally, we will summarize the evidence that has implicated the LEL in the regulation of mTOR by various growth regulatory inputs such as amino acids, growth factors and mechanical stimuli.

Rheb and PA: The Direct Activators of mTOR

It is well recognized that mTOR can sense and respond to a very wide range of different stimuli, but surprisingly, only two molecules have been shown to function as direct activators of mTOR signaling (Sengupta et al. 2010; Hornberger 2011). These molecules include the lipid second messenger PA and the Ras-related GTPase Rheb. In the following sections we will summarize the evidence which indicates that these molecules can function as direct activators of mTOR signaling.

Rheb, like other members of the Ras superfamily, is a GTP-binding protein that possesses GTPase activity (Aspuria and Tamanoi 2004). The GTPase activity of Rheb, and its subsequent GTP/GDP-bound state, is regulated by the GTPase activating protein (GAP) tuberlin (TSC2). Based on current evidence, it appears that the GAP domain of TSC2 stimulates Rheb's intrinsic GTPase activity, and in turn, converts active GTP-Rheb into

inactive GDP-Rheb (Zhang et al. 2003; Inoki et al. 2003; Tee et al. 2003). For example, in cells lacking TSC2, Rheb is highly confined to its GTP-bound state and this is associated with a robust activation of mTOR signaling (Garami et al. 2003). It has also been shown that the overexpression of Rheb is sufficient to activate mTOR signaling and that the knockdown of Rheb inhibits mTOR signaling (Long et al. 2005a; Tabancay et al. 2003; Sengupta et al. 2010). Furthermore, Rheb can bind to the catalytic domain of mTOR, and most importantly, GTP-, but not GDP-, bound Rheb can directly activate mTOR kinase activity *in-vitro* (Long et al. 2005b; Sancak et al. 2007; Sato et al. 2009). In other words, several lines of evidence indicate that when Rheb is in its GTP-bound state it can directly activate mTOR signaling.

PA is a glycerophospholipid whose intracellular concentration can be regulated by 5 distinct classes of enzymes. These enzymes include phospholipase D (PLD) which synthesizes PA from phosphatidylcholine (PC), lysophosphatidic acid acyltransferases (LPAAT) which synthesize PA from lysophosphatidic acid (LPA), and the diacylglycerol kinases (DAGK) which synthesize PA from diacylglycerol (DAG) (Foster 2007; Wang et al. 2006). Furthermore, the concentration of PA can also be controlled by enzymes that degrade PA which includes the conversion of PA to LPA by A type phospholipases (PLA), and the conversion of PA to DAG by phosphatidic acid phosphatases (PAP) (Wang et al. 2006; Aoki et al. 2007; Carman and Han 2006). To date, numerous studies have shown that the stimulation of cells with exogenous PA, or the overexpression of PA-generating enzymes, can increase mTOR signaling (Avila-Flores et al. 2005; Tang et al. 2006; O'Neil et al. 2009; You et al. 2012; Foster 2007). Conversely, blocking the generation of PA has been reported to inhibit the activation of mTOR that occurs in response to various types of stimuli (Fang et al. 2001; Ballou et al. 2003; Hornberger et al. 2006; Takahara et al. 2006; Ha et al. 2006). Mechanistically, PA has been shown to bind to the FKBP12-Rapamycin binding (FRB) domain of mTOR, and like GTP-Rheb, it can directly activate mTOR kinase activity *in-vitro* (You et al. 2012; Yoon et al. 2011b; Fang et al. 2001; Veverka et al. 2008). To the best of our knowledge, GTP-Rheb and PA are the only molecules that can directly activate mTOR, and as we will describe below, both of these molecules appear to be enriched at the LEL.

The Late Endosome/Lysosomal System (LEL)

The LEL, as defined in this review, comprises the late endosome, the lysosome, and the hybrid organelle that results from the fusion of the late endosome and the lysosome. The formation and function of these subcellular organelles is best understood by describing the dynamic process of endocytosis. As shown in Figure 1, the endocytic pathway involves the uptake of plasma membrane, including integral proteins and their associated ligands, into primary endocytic vesicles which, in turn, are delivered to larger vesicular structures known as early endosomes (Huotari and Helenius 2011). The early endosomes are marked by the presence of the cytosolic protein Rab5 and act as the sorting center for the endocytic pathway. Specifically, the early endosomes recycle the majority of internalized material back to the plasma membrane with the help of recycling endosomes, and they also deliver a small fraction of this material to late endosomes (Huotari and Helenius 2011; van Ijzendoorn 2006). Late endosomes, also known as multivesicular bodies, are derived from the early endosomes, maintain a relatively acidic pH (6.0 – 4.9) and can be characterized by the presence of Rab7 (Maxfield and Yamashiro 1987; Rink et al. 2005; Luzio et al. 2007). Late endosomes also contain a membrane bound glycoprotein called lysosome associated membrane protein-2 (LAMP2). After further maturation, the late endosomes fuse with lysosomes to form a hybrid organelle. Lysosomes are characterized by the presence of integral membrane proteins such as LAMP2, are more acidic than late endosomes (pH 5.0 – 4.6) and contain acid hydrolase enzymes (Luzio et al. 2007). The resulting late endosome/lysosome hybrid organelle is responsible for the degradation of the original endocytosed material. After the degradation of endocytosed material is complete, the lysosome reforms

from the hybrid organelle (Bright et al. 1997; Luzio et al. 2000; Luzio et al. 2007). Thus, in the most simple of terms, the LEL can be described as membranous vesicular structures that play a critical role in the complex process of protein trafficking and degradation. For more in depth reviews on the formation and functions of the LEL, the reader is referred to the following excellent reviews (Huotari and Helenius 2011; Luzio et al. 2007).

Markers of the LEL

Evidence for a possible role of the LEL in the regulation of mTOR signaling has largely been determined through colocalization studies that utilized protein markers for the LEL such as Rab7, and the more commonly used marker LAMP2. Rab7 is a small GTPase which is recruited specifically to late endosomes during their maturation process and is necessary for the fusion of the late endosome with the lysosome (Bucci et al. 2000). LAMP2 is an integral lysosomal membrane protein that functions to protect the lysosomal membrane and it has also been implicated in the regulation of lysosomal enzyme targeting, autophagy, lysosomal biogenesis and cholesterol transport (Eskelinen et al. 2003; Schneede et al. 2011; Cuervo and Dice 1996; Fujiwara et al. 2013). Importantly, while the majority of Rab7 and LAMP2 are found in LEL structures, due to the constant processes of maturation, fusion, fission and recycling, a small fraction of these proteins may also be found in other non-LEL structures (Lippincott-Schwartz and Fambrough 1987; Fukuda 1991). Nonetheless, both Rab7 and LAMP2 are currently the most commonly used markers for identifying the LEL.

The LEL as a Potential Site for the Regulation of mTOR Signaling

Recent immunohistochemical studies have demonstrated that mTOR colocalizes with Rab7 and LAMP2 in both muscle and non-muscle cells (Sancak et al. 2008; Sancak et al. 2010; Jacobs et al. 2013; Flinn et al. 2010; Narita et al. 2011; Zoncu et al. 2011; Yoon et al. 2011a). Furthermore, Rheb has been shown to colocalize with Rab7, and biochemical studies have recently shown that the LEL is highly enriched with PA (Sancak et al. 2008; Zhao et al. 2012; Saito et al. 2005). The fact that mTOR, and its direct activators, are located at the LEL suggests that the LEL might function as a key integration site for various upstream pathways that regulate mTOR signaling. Indeed, functional evidence to support this point was presented in a recent study which demonstrated that forced targeting of mTOR to the LEL is sufficient to activate mTOR signaling in amino-acid starved HEK293T cells (Sancak et al. 2010). Specifically, in this study, the regulatory-associated protein of mTOR (Raptor) was fused with the last 15 amino acids of Rheb (Raptor-Rheb15) that contains a farnesylation site (termed the CAAX box) which confers its targeting to membranous structures such as the LEL. Since Raptor can directly bind to mTOR, the targeting of Raptor to the LEL also results in an enhanced association of mTOR with the LEL. Indeed, the overexpression of Raptor-Rheb15 resulted in an enhanced colocalization of mTOR to LAMP2-positive structures and this was associated with the robust activation of mTOR signaling. Furthermore, overexpression of a second Raptor fusion protein which lacked the Rheb CAAX box (Raptor-Rheb15 Δ CAXX) did not increase mTOR colocalization with LAMP2, and it was unable to promote the activation of mTOR signaling (Sancak et al. 2010). Combined, these results indicate that, at least in amino acid starved cells, the enhanced targeting of mTOR to the LEL is sufficient to activate mTOR signaling.

Recently, we also used the Raptor-Rheb15 and Raptor-Rheb15 Δ CAXX fusion proteins to determine if forced targeting of mTOR to the LEL would be sufficient to activate mTOR signaling in skeletal muscle *in vivo*. To accomplish this, we used electroporation to transfect mouse Tibialis Anterior (TA) muscles with plasmid DNA encoding the Raptor-Rheb15 or Raptor-Rheb15 Δ CAXX fusion proteins along with a Myc-tagged variant of the ribosomal S6 kinase (p70^{S6k}) as a reporter for mTOR signaling. Changes in p70^{S6k} Threonine 389

(p70^{S6k} T389) phosphorylation were then used as a marker of mTOR activity (Hornberger et al. 2007; Goodman et al. 2010). As shown in Figure 2, muscles overexpressing Raptor-Rheb15 revealed a large (~5 fold) increase in p70^{S6k} T389 phosphorylation when compared with muscles overexpressing wild-type Raptor. Furthermore, the increase in mTOR signaling was not observed in muscles overexpressing Raptor-Rheb15ΔCAXX. These results are highly consistent with the previous observations reported in HEK293T cells and, therefore, further support the conclusion that an enhanced association of mTOR with the LEL is sufficient to activate mTOR signaling. With this point in mind, we will now examine the evidence that has implicated the LEL in the regulation of mTOR signaling by growth factors, amino acids, and finally, mechanical stimuli.

Growth Factors

Current evidence indicates that growth factors, such as insulin or IGF-1, regulate mTOR signaling via a phosphoinositide 3-kinase (PI3K)/protein kinase B (PKB)-dependent mechanism that primarily involves changes in the GTP-loading state of Rheb. Specifically, it is thought that growth factors promote a PKB-dependent increase in the phosphorylation of TSC2 and that this, in turn, inhibits the ability of TSC2 to function as a GAP for Rheb. As a result, Rheb accumulates in its active GTP-bound state and subsequently promotes the activation of mTOR signaling (Inoki et al. 2002; Manning et al. 2002; Potter et al. 2002; Inoki et al. 2003). Support for this model has come from several studies which indicate that growth factor-induced changes in the phosphorylation of TSC2 regulate its GAP activity towards Rheb *in-vivo* (Manning et al. 2002; Zhang et al. 2009; Cai et al. 2006; Inoki et al. 2002). However, the molecular mechanisms that are responsible for this inhibition remain a subject of intense debate. For example, growth factor induced changes in the phosphorylation of TSC2 do not alter TSC2's GAP activity when measured *in-vitro* (Cai et al. 2006; Huang and Manning 2008). Therefore, it appears that changes in the phosphorylation of TSC2 do not inhibit TSC2's intrinsic GAP activity, but instead, somehow impair its ability to effectively function as a GAP for Rheb.

Intriguingly, like mTOR, Rheb and PA, TSC2 was recently shown to colocalize with the LEL (Dibble et al. 2012; Jacobs et al. 2013). It has also been demonstrated that growth factor stimulation causes TSC2 to translocate from a crude membrane to cytosolic fraction. Furthermore, this translocation event requires the phosphorylation of TSC2 on residues that lie within the RxRxxS*/T* consensus motifs that are commonly utilized by members of the AGC family of kinases such as PKB (Cai et al. 2006; Miyazaki et al. 2010). Based on these points, we reasoned that growth factor-induced changes in the phosphorylation of TSC2 might cause it to translocate away from the LEL, and therefore, spatially inhibit the ability of TSC2 to effectively function as a GAP for Rheb at the LEL (Jacobs et al. 2013). Therefore, to begin testing this hypothesis, we examined the localization of TSC2 in skeletal muscles. Consistent with our previous report, we found that TSC2 is enriched at the LEL under basal conditions (Jacobs et al. 2013). Furthermore, we also discovered that the association of TSC2 with the LEL is almost completely abolished after insulin stimulation (Fig. 3). Hence, it can be argued that an emerging body of evidence suggests that growth factors induce a phosphorylation-dependent translocation of TSC2 away from the LEL and this, in turn, enables the LEL-associated Rheb to accumulate in its active GTP-bound state and subsequently promote the activation of the LEL-associated mTOR.

Changes in the GTP loading state of Rheb are considered to be the primary mechanism through which growth factors regulate mTOR signaling, however, PA might also play a role in this process. For example, insulin stimulation has been shown to induce a rapid increase in PA and a variety of growth factors can induce an increase in PLD activity (Song et al. 1994; Plevin et al. 1991; Sa and Das 1999; Karnam et al. 1997; Banno et al. 2003; Seymour

et al. 1996; Farese et al. 1984). Furthermore, a pool of PLD was recently identified at the LEL (Yoon et al. 2011a). This is important because it raises the possibility that growth factor-induced increases in PLD activity might lead to an accumulation of PA specifically at the LEL. In addition, it has been shown that Rheb can physically interact with, and activate, PLD in a GTP-dependent manner (Sun et al. 2008). Thus, it's tempting to suggest that a growth factor-induced increase in GTP-Rheb at the LEL would not only help to directly activate the LEL-associated mTOR, but it would also promote an increase in the activity of the LEL-associated PLD. As a result, PA levels at the LEL would become further elevated, and therefore, help to amplify the effects of GTP-Rheb on mTOR signaling. In summary, our knowledge of how growth factors regulate mTOR signaling is already quite advanced, and the vast majority of studies have suggested that TSC2-dependent changes in the GTP-loading state of Rheb are the primary mechanism through which growth factors regulate mTOR. Nonetheless, there are still many gaps in our knowledge, and the LEL will likely become the focal point of future studies that are aimed at filling these gaps.

Amino Acids

It has long been known that mTOR signaling is regulated by amino acids, with amino acid depletion reducing mTOR signaling and increased amino acid availability leading to increased mTOR signaling [for review see (Jewell et al. 2013)]. For many years, the mechanism(s) via which amino acids regulate mTOR signaling remained quite elusive. For example, unlike growth factors, multiple studies demonstrated that amino acids do not alter the GTP-bound state of Rheb (Roccio et al. 2006; Nobukuni et al. 2005). However, it had also been concluded that Rheb is necessary for amino acids to activate mTOR signaling (Saucedo et al. 2003; Zou et al. 2011; Sancak et al. 2010). Combined, these studies suggested that amino acids regulate mTOR signaling through a pathway that is distinct from growth factors, yet it still involves Rheb.

In 2008, a major component of this distinct mechanism was uncovered when Dr. Sabatini's lab demonstrated that amino acids regulate the association of mTOR with the LEL via a mechanism that is dependent on another family of Ras-related GTPases called the Rag GTPases (Sancak et al. 2008). Specifically, it was shown that mTOR translocates away from the LEL when cells are deprived of amino acids. On the other hand, stimulating cells with amino acids promotes an increase in the association of mTOR with LEL. Furthermore, it was determined that amino acids regulate the activity/GTP-loading state of the Rags, and that constitutive activation of the Rags prevents the dissociation of mTOR from the LEL, and the inhibition of mTOR signaling, that normally occurs in response to amino acid deprivation (Sancak et al. 2010; Sancak et al. 2008; Kim et al. 2008). Based on these observations, it was proposed that amino acid/Rag-dependent changes in mTOR signaling are primarily regulated by spatially controlling the ability of mTOR to connect with its direct activators (PA and Rheb) at the LEL. Indeed, this concept gained further support when it was demonstrated that the forced targeting of mTOR to the LEL with Raptor-Rheb15 also prevented the inhibition of mTOR signaling that occurs in response to amino acid deprivation (Sancak et al. 2010).

In addition to controlling the association of mTOR with the LEL, it also appears that amino acids can regulate the concentration of PA at the LEL. For example, a recent study demonstrated that amino acid stimulation promotes an increase in PLD1 activity and the association of PLD1 with the LEL. It was also shown that PLD1 contributes to the amino acid-induced increase in mTOR signaling, and this effect is driven through a pathway that lies in parallel to the Rag pathway (Yoon et al. 2011a). In other words, current evidence suggests that amino acids control mTOR signaling by regulating both the association of mTOR with the LEL and the concentration of PA at the LEL.

Mechanical Stimulation

As highlighted in the introduction, the molecular mechanisms that are responsible for the mechanical activation of mTOR signaling remain vaguely defined. For example, it has been demonstrated that, unlike growth factors and nutrients, mechanical stimuli activate mTOR signaling through a PI3K-independent mechanism (Hornberger et al. 2004; O'Neil et al. 2009; Hornberger and Chien 2006). The components of this PI3K-independent mechanism have not been identified, but several studies suggest that PA might be involved. For instance, various forms of mechanical stimuli can promote an increase in the intracellular concentration of PA (Hornberger et al. 2006; O'Neil et al. 2009; Cleland et al. 1989). Furthermore, just like mechanical stimulation, PA can promote an increase in mTOR signaling via a PI3K-independent mechanism (O'Neil et al. 2009). Finally, it has been shown that 1-butanol, which inhibits the synthesis of PA by PLD, can prevent the mechanically-induced increase in PA and mTOR signaling (Hornberger et al. 2006; O'Neil et al. 2009). Based on these results, it would appear that the synthesis of PA by PLD is necessary for the mechanical activation of mTOR signaling. However, more recent studies have shown that some of 1-butanol's biological effects cannot be explained by the inhibition of PLD activity (Su et al. 2009). Moreover, the time course of PLD activation does not adequately correspond with the increase in PA concentration, and hence, it has been argued that a mechanically-induced increase in PLD activity may not fully explain how mechanical stimuli promote an increase in PA (Hornberger 2011; Hornberger et al. 2006). Therefore, at this time, it can be concluded that there are several lines of evidence which suggest that PA plays a role in the mechanical activation of mTOR signaling, but further studies are needed to fully establish the validity of this mechanism.

Unlike PA, the potential role of Rheb in the mechanical activation of mTOR has been almost completely unexplored. Nonetheless, recent work from our lab suggests that mechanical stimulation might regulate mTOR signaling, at least in part, by controlling the amount of GTP-Rheb at the LEL. For example, we determined that, like growth factors, mechanical stimulation induces an increase in the phosphorylation of TSC2 on residues that lie within RxRxxS*/T* motifs (Jacobs et al. 2013). Furthermore, we found that mechanical stimulation causes TSC2 to translocate from a crude membrane to cytosolic fraction, and just like insulin, mechanical stimulation almost completely abolished the association of TSC2 with the LEL (Jacobs et al. 2013). Thus, it appears that changes in the GTP-loading state of the LEL-associated Rheb could play an important role in the pathway through which mechanical stimuli regulate mTOR signaling.

Another piece of evidence which implicates the LEL in the mechanical activation of mTOR comes from our finding that mechanical stimulation promotes an increase in the association of mTOR with the LEL (Jacobs et al. 2013). Currently, the mechanism(s) responsible for this effect is not known, but indirect evidence suggests that the Rags might be involved. For example, previous studies have shown that the intracellular concentration of amino acids are elevated after a bout of mechanical stimulation, and presumably, this would lead to a Rag-dependent increase in the association of mTOR with the LEL (MacKenzie et al. 2009). Alternatively, the enhanced association of mTOR with the LEL might involve a currently undefined mechanism. For example, we have determined that mechanical stimulation not only promotes an increase in the association of mTOR with the LEL, but it also leads to an increase in the number of LAMP2 positive, and mTOR positive, punctate structures (Fig. 4). Currently, the potential significance of this observation is not readily apparent, but we have highlighted this data because it indicates that mechanical stimulation alters the properties of the LEL, and such changes might ultimately be linked to the pathway through which mechanical stimuli regulate mTOR signaling.

A Conceptual Model of How Mechanical Stimuli Could Regulate mTOR Signaling at the LEL

Based on the aforementioned body of knowledge, we developed a conceptual model in which the LEL could serve as a major regulatory center for controlling the mechanical activation of mTOR signaling (Jacobs et al. 2013). Specifically, as shown in Figure 5, our model predicts that, in a basal state, the LEL is already enriched with PA, mTOR, Rheb and TSC2. Under these conditions, the presence of TSC2 keeps the LEL-associated Rheb in its inactive GDP-bound state, and thus, signaling by mTOR is relatively inactive. In response to mechanical stimulation, the association of mTOR with the LEL becomes further enhanced. In addition, TSC2 becomes phosphorylated and we have proposed that this change in phosphorylation causes TSC2 to dissociate from the LEL. As a result, the LEL-associated Rheb is now able to obtain its active GTP-bound state and, in-turn, promote the activation of the LEL-associated mTOR. Furthermore, we have previously demonstrated that various types of mechanical stimuli promote an increase in PA and, presumably, at least some of this increase will occur at the LEL (O'Neil et al. 2009; Hornberger et al. 2006; You et al. 2012). If this is correct, then an increase in PA at the LEL would be expected to further amplify the activation of mTOR signaling.

Clearly, our conceptual model is based on several assumptions that will need to be addressed in future studies. However, we cannot ignore the rapidly growing body of evidence that has implicated the LEL in the regulation of mTOR signaling. As described above, a role for the LEL in regulation of mTOR by amino acids has already become firmly established, and we suspect that future studies will also establish that the LEL plays a key role in the regulation of mTOR by growth factors and mechanical stimuli.

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Figure 1. Endocytosis and the Late Endosome/Lysosomal System

Endocytosis begins with the uptake of plasma membrane into primary endocytic vesicles. These vesicles are then delivered to the early endosome which is characterized by the presence of Rab5 on its cytosolic membrane. The early endosome sorts the endocytosed material and returns the majority of endocytosed material to the plasma membrane either directly, or with the assistance of the recycling endosome which is characterized by the presence of Rab11 (van Ijzendoorn 2006). The remaining material that has been targeted for degradation is delivered by the early endosome to the late endosome. The late endosome is characterized by the presence of Rab7 and multivesicular bodies. It also contains the lysosomal membrane protein LAMP2 which is delivered, along with endocytosed material, to the lysosome through the formation of a hybrid organelle. The hybrid organelle degrades the endocytosed material and the lysosome then reforms (Huotari and Helenius 2011).

Figure 2. Forced Targeting of Raptor to the LEL is Sufficient to Activate mTOR Signaling

Electroporation was used to co-transfect mouse Tibialis Anterior muscles with 30 μ g of plasmid DNA encoding various forms of HA-tagged Raptor and 2 μ g of Myc-tagged p70^{S6k} (Goodman et al. 2013; Sancak et al. 2010). At 7 days post transfection, the muscles were collected, homogenized, and then the Myc-tagged p70^{S6k} was immunopurified and subjected to western blot analysis for p70^{S6k} phosphorylated on the Threonine 389 residue [P-p70(T389)] and total p70^{S6k} as previously described (Goodman et al. 2010). The values at the top of the blot represent the ratio of P-p70(T389) to total p70^{S6k} (a marker of mTOR signaling) and were expressed relative to the values obtained in the WT-Raptor group. * Significantly different from WT-Raptor, $P < 0.05$.

Figure 3. Insulin Abolishes the Association of TSC2 with the LEL

Mice were given an intraperitoneal injection of 20 U/kg insulin or the vehicle as a control condition and then collected 30 min later. (A–F) Cross-sections of the Tibialis Anterior muscles from control (A–C), or insulin (D–F), stimulated mice were then subjected to immunohistochemistry for LAMP2 and TSC2 as previously described (Jacobs et al. 2013). (B, E) Grayscale images of the signals for LAMP2. (C, F) Grayscale images of the signals for TSC2. Scale bars in B and E represent 10 μm. (G) Colocalization of the large and intense TSC2 positive puncta with LAMP2 was quantified as detailed previously (Jacobs et al. 2013). Values in the graph represent the means + SEM and were obtained from n = 14–46 randomly selected images per group. * Significantly different from control, P = 0.05.

Figure 4. Mechanical Stimulation Induces an Increase in the Number of LAMP2 and mTOR Positive Puncta

The Tibialis Anterior (TA) muscles from mice were subjected to mechanical stimulation (MCH) via a bout of eccentric contractions as previously described (Jacobs et al. 2013). (A–F) Cross-sections from control (A–C), or MCH (D–F) muscles were then subjected to immunohistochemistry for LAMP2, mTOR, and type 2b myosin heavy chain (MHC2b), as previously described (Jacobs et al. 2013; Goodman et al. 2012). (B, E) Grayscale images of the signals for LAMP2. (C, F) Grayscale images of the signals for mTOR. Scale bars in C and F represent 20 μm . (G) The number of LAMP2 and mTOR puncta/ μm^2 in randomly selected MHC2b positive fibers were manually counted by an investigator that was blind of the sample identifications. Values in the graph represents the mean + SEM and were obtained from a total of $n = 126$ –133 fibers per group. * Significantly different from control, $P < 0.05$.

Figure 5. Conceptual Model of How Mechanical Stimuli Could Regulate mTOR Signaling at the LEL

In this model, the LEL in skeletal muscles serve as a major regulatory center for controlling mTOR signaling. In response to the mechanically-induced signaling events (shown with arrows), mTOR signaling transitions to its active state. See the summary section for details. Adapted and reprinted from (Jacobs et al. 2013) with permission from the publisher (John Wiley and Sons).