Myoblast Models of Skeletal Muscle Hypertrophy and Atrophy

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Abstract

Purpose of Review: To highlight recent breakthroughs and controversies in the use of myoblast models to uncover cellular and molecular mechanisms regulating skeletal muscle hypertrophy and atrophy.

Main Findings: Myoblast cultures provide key mechanistic models of the signalling and molecular pathways potentially employed by skeletal muscle *in-vivo* to regulate hypertrophy and atrophy. Recently the controversy as to whether IGF-I is important in hypertrophy following mechanical stimuli vs. alternative pathways has been hotly debated and is discussed. The role of myostatin in myoblast models of atrophy, and interactions between protein synthetic pathways including Akt/mTOR and the 'atrogenes' are explored.

Summary: Targeted *in-vivo* experimentation directed by skeletal muscle cell culture and bioengineering (3-dimensonal skeletal muscle cell culture models) will provide key biomimetic and mechanistic data regarding hypertrophy and atrophy and thus enable the development of important strategies for tackling muscle wasting associated with ageing and disease processes.

Keywords: muscle precursor cell, satellite cell, IGF-I, myostatin, 3D muscle constructs.

Introduction: The use of Myoblast Models to Investigate Mechanisms of Skeletal Muscle Size Regulation

Adult skeletal muscle fibre number is set *in-utero* and adult fibres are terminally differentiated or incapable of division. Despite these phenomena, adult skeletal muscle is highly adaptable, responding to the soluble and biophysical cues that it encounters on a daily basis. Skeletal muscle undergoes rapid growth (hypertrophy) during development, exercise, stretch and mechanical loading (weight bearing) and also severe loss (atrophy) with ageing, disuse and disease. It is also capable of repair and regeneration following injury. Much of this adaptability (growth and repair) is achieved via resident adult stem cells, termed satellite or muscle precursor cells (MPC) that have mitotic potential. Recent data suggest that blocking myostatin (a negative regulator of muscle mass), enables hypertrophy not only via satellite cell accretion but also via increased modulation of the synthesis and turnover of structural proteins within a myotube/fibre (1)*.

Satellite cells are fundamental to muscle adaptation; they are specialised cells, normally residing in a quiescent state beneath the basal lamina of myofibres. Following physiological, bio-mechanical or pathological cues, they are activated to myoblasts and become committed to the muscle/mesenchymal linage, adopting not only a muscle phenotype, but given relevant cues, also neural (2), osteoblast (3, 4), chrondocyte (5) and adipocyte (6) linages. In order to elicit reparative responses, satellite cells must **i.** be activated, **ii.** increase their numbers (proliferation), **iii.** migrate to the site of injury and **iv.** fuse (differentiate) with the damaged fibre (7). Because of their fundamental roles in muscle maintenance and adaptation, myoblasts are frequently studied as *in-vitro* models of growth, migration, differentiation and death. Basal, hypertrophic or atrophic conditions are used and provide relevant models of muscle growth (exercise and puberty) or wasting in catabolic conditions (e.g. cancer, AIDS, congestive heart failure, sepsis, COPD, rheumatoid arthritis and sarcopenia (muscle wasting associated with ageing)). These myoblast models use either primary skeletal muscle cells derived from human or animal biopsies, or, more frequently,

established cell lines such as the mouse C_2, C_2C_{12} (a clonal derivative and daughter of the C_2 cells), or rat L6 cells. Advantages of cell lines vs. primary human culture include: Availability, ethics, reduced cost, access to cells, speed of growth and pure myogenic populations. Disadvantages of muscle cultures vs. *in-vivo* models include: Reduced differentiation capacity with passage, an inability to sustain myotubes for extended periods in culture and the environment of 2-D monolayer that lacks bio-mimicity, thus making direct comparisons with muscle *in-vivo* difficult. Development and manipulation of models using both collagen and fibrin gels to incorporate a 3-Dimensional (3-D) structure for myoblasts in culture (8)**(9) will potentially enable more physiological experimentation and analyses of the regulators of hypertrophy and atrophy.

This review will therefore highlight the most recent breakthroughs in the use of myoblasts as models to investigate and manipulate cellular and molecular regulators of hypertrophy and atrophy. Given the nature of this review, *in-vivo* data will be reported only to portray the relevance of the *in-vitro* findings.

Myoblast Models of Hypertrophy: Insulin-like Growth-Factors (IGFs) versus Mechano-Transduction.

Insulin-like Growth Factors (IGFs) influence hypertrophy of skeletal muscle primary and cell line cultures, enhancing proliferation, differentiation (reviewed in (10)), survival (11), satellite cell recruitment (12) and myofibrillar protein accretion (13). Despite high levels of systemic liver-derived IGF, a recent *in-vivo* study suggests that local production of IGF-I by skeletal muscle is imperative in hypertrophy. Where liver deficient IGF-I mice demonstrate similar strength gains to controls with larger increases in IGF-I receptor tyrosine phosphorylation (14) and associated PI3K/Akt/mTOR induced protein synthesis (14). Despite these compelling data, the importance of IGF-I in mechanical load (weight bearing)

induced hypertrophy has recently been debated (15, 16)**. Never the less, the withdrawal of serum *in-vitro* is sufficient to induce myoblast differentiation in both cell lines and primary cultures which are able to produce endogenous IGF-I that facilitates the fusion process (17)**, furthermore, supplementation with IGF-I augments hypertrophy compared with basal conditions (18-20). Validation of the mechanisms pertaining to IGF, hypertrophy and mechanical load *in-vivo* therefore continues and would be facilitated by an *in-vitro* model that incorporates mechanical load. A recent study, using stretch, illustrated that primary chick myotubes undergo significant hypertrophy following activation of the PI3K/Akt/mTOR pathways (which lie downstream of IGF-I/IGF-IR activation). Hypertrophy was also prevented using pharmacological inhibitors of PI3K and mTOR (21), however, the authors did not measure endogenous production of IGF-I. Therefore, explicit confirmation of the role of IGF in these models is awaited.

Mechano-Transduction Signalling

The mammalian target of rapamycin, mTOR, can be activated via signals independent of IGF-I, via a pathway involving phospholipase D (PLD), phosphatidic acid and a downstream regulator Rheb (ras homologue enriched brain) (see (22-24). Furthermore, mTOR can be stimulated via amino acids, particularly leucine (25, 26), potentially via Rheb binding and interaction with amino acid sensitive phosphatidylinositol 3-kinase/Vps34 (24) or MAP4K3 (27). The stress response gene Redd 2, may also be important in inhibiting mTOR via the tuberous sclerosis 1 (TSC1) and 2 (TSC2) complex (28), and following mechanical overload Redd 2 is reduced to enable mTOR to initiate p70S6K expression, which is involved in protein synthesis and hypertrophy (29) (see Figure 1). To substantiate that mechanical signals can operate independently of IGF-I signalling; i*n-vivo* evidence from Spangenburg *et al.* (30)** suggests that mice overexpressing a dominant negative form of IGF-IR (MKR mice), elicited similar hypertrophic responses, following synergistic ablation of the plantaris muscle compared to wild-type mice. Suggesting that IGF-I is not required for load-induced

hypertrophy. By contrast, Heron-Milhavet *et al.* (31)** also using the MKR mouse showed IGF-I to be fundamental in myoblast fusion, with primary MKR myoblasts showing impaired differentiation, versus wild-type controls, following damage (31). Interestingly, MKR-derived muscle cells had equivalent levels of myogenin (a myogenic regulatory factor fundamental to lineage and hypertrophy) positive cells to wild type. However, the ability of the myogenin positive cells to fuse into multinucleated myotubes was significantly lower in MKR vs. wild-type-derived myoblasts. Indeed, a significantly greater proportion of fusion-hampered MKR-derived myoblasts compared with control cells (31), suggests that IGF-I does play an influential role in differentiation and hypertrophy but that other factors enable hyperplasia. Although data from Spangenburg *et al.* (30) appear to contradict the observations by Heron-Milhavet *et al.* (31), different modes of hypertrophy were being examined. Spangenburg *et al.* (30) performed no cellular or histological analyses, thus questioning whether the increase in muscle mass observed in MKR mice (similar to controls) corresponded to a true hypertrophic vs. hyperplastic responses.

A Biphasic Role for IGF-I?

Utilising an *in-vitro* model of hypertrophy and atrophy (comparing younger phenotypes of clonally derived daughter C_2C_{12} vs. parental C_2 cells), we have recently published that IGF-I is important for the greater differentiation potential of C_2C_{12} vs. C_2 cells (17)**. Importantly, IGF-I expression was similar at 48 hrs following initiation of differentiation in both cell types, despite greater morphological differentiation in the C_2C_{12} cells. By 72 hrs, however, IGF-I expression was significantly greater in C_2C_{12} vs. C_2 cells as were morphological and biochemical differentiation. These data indicate a potential biphasic role for IGF in underpinning the temporal complexity of differentiating myoblasts. Despite similar levels of IGF-I expression at 48 hrs, reductions in myoD and myogenin were evident in the C_2 vs. C_2C_{12} cells and this may underpin the reduced potential for differentiation of these cells. Finally, an inverse expression pattern of IGF binding protein-2 (IGFBP2) was evident in the

two cell types. The role for IGFBP2 warrants further investigation as it may be crucial in modulating IGF-induced differentiation especially with age (17)**.

Other Potential Hypertrophic Mechanisms

A recent study, using a single fibre approach, suggested that extracellular matrix and fibroblasts are fundamental for muscle hypertrophy, enabling increases in hepatocyte growth factor (HGF) expression. HGF binds to the c-met receptor on the cell membrane of skeletal muscle, thus enabling satellite cell activation (32). Importantly, however, high levels of HGF are not only associated with satellite cell activation, but also the up-regulation of myostatin (discussed below) mRNA, the product of which leads to satellite cell quiescence. These data suggest a fine regulatory role for HGF, distinct from IGF/mTOR signalling, in hypertrophy vs. self-renewal of skeletal muscle cells (33)*. Nitric oxide (NO) is also reportedly increased following mechanical stretch and leads to the up-regulation of matrix metalloproteinase activity, enabling matrix remodelling required to support hypertrophy (32). Indeed, overexpression of MMP-9 in C_2C_{12} cell clones (C2M9) improves their migration *in-vitro* and their engraftment *in-vivo*, both of which are required for hypertrophy and regeneration (34)*. β-catenin/c-Myc-signaling, important in ribosomal biogenesis, also increases following mechanical overload (load on the muscles, which leads to failure), with inactivation of β-catenin completely preventing hypertrophy in response to mechanical overload in mice (35). Indeed, hypertrophy induced in C_2C_{12} myoblasts using both IGF-IEa and MGF increased nuclear β-catenin *in-vitro* (36) implicating a role for this molecule in potentially linking hypertrophy following IGF signalling and/or following a mechanical stimulus.

MicroRNAs at the Cutting Edge

The class of approximately 22 nucleotide noncoding RNAs (microRNAs) that regulate gene expression at the post-transcriptional level may play fundamental roles in skeletal muscle hypertrophy. Recently, both miR-1 and miR-206 have been implicated in skeletal muscle cell differentiation. Overexpression in C_2C_{12} myoblasts reduced proliferation and induced differentiation *in-vitro* (37)*. These miRNAs also function to control among other regulators, Pax7, which is required for appropriate satellite cell survival, proliferation, and differentiation. The role of miRNAs in a myoblast model of mechanical load requires further investigation**.**

Summary: Myoblast Models of Hypertrophy

Overall, the convergence of mechanical, endocrine, autocrine and pancrine signals results in activation of PI3K, Akt, mTOR leading to protein synthesis and hypertrophy via proliferation and differentiation of myoblasts, as well as corresponding ribosomal biogenesis through β-catenin/c-Myc-signaling. However, the relative contribution of each parameter, especially following mechanical load remains to be determined and has implications for therapeutic interventions aimed at improving hypertrophy during disease, ageing and following exercise. Finally, the importance of the implementation of 3-D myoblast models to study the integration of skeletal myoblasts with the ECM *in-vitro*, and to apply to situations of mechanical load/overload or stretch *in-vivo* are important for future developments in the field.

Muscle Atrophy: The Problem

Skeletal muscle atrophy occurs when proteolysis overwhelms protein synthesis. Increased protein degradation may occur as a consequence of many factors, including changes in anabolic hormones e.g. IGF-I, GH, testosterone, glucocorticoids; and increases in TGF-β, myostatin, and cytokines such as TNF-α, TWEAK and IL-6. Oxidative stress and reduced amino acid availability can also tip the balance in favour of atrophy. Muscle wasting can occur as a consequence of: mechanical unloading, a reduction in use/exercise (disuse atrophy), chronic catabolic disease (cachexia) and ageing (sarcopenia). Even though resistance exercise may slow the atrophy process, many patients are too old, ill or simply unable (frail or obese) to exercise. Furthermore, resistance exercise has to be continually undertaken to be of long-term benefit, meaning high cost of skilled trainers and high drop-out due to its demanding physical nature. It is therefore important to also develop pharmalogical therapies to treat muscle atrophy.

Myostatin and Atrophy

It is beyond the scope of this review to discuss all factors that contribute to muscle atrophy (for excellent current reviews see (7, 38, 39)). However, recent *in-vitro* myoblast research has focussed on myostatin (growth differentiation factor- 8/GDF-8). Myostatin is a member of the Transforming Growth Factor-Beta (TGF-β) family of proteins and a negative regulator of skeletal muscle growth. Pioneering work by McPherron and collegues (40, 41) using knock out technologies, demonstrated the important inhibitory role of myostatin in skeletal muscle of mice and also reported that the 'double muscling' phenotype in Belgian Blue and Piedmontese cattle occurred as a result of mutations in the myostatin gene (41). As a consequence, this inhibitory growth factor has received a lot of attention as a potential therapeutic target to combat muscle wasting. Myostatin−/− mice that are crossed with follistatin transgenic mice display even larger muscle phenotypes as a result of blocking other inhibitory TGF-β family members such as GDF-11 and activins (42). However, the first human trial using low dose anti-myostatin antibodies in muscular dystrophy patients did not

enable hypertrophy (43). However, this is a complex disease, where the underlying disorder is due to a lack of dystrophin rather than an increase in myostatin and it may not be the best model (44).

Myostatin Signalling

As a consequence of these data, mouse and rat myoblasts have been utilised to investigate the molecular mechanisms of myostatin in muscle. Myostatin reportedly blocks differentiation of myoblasts into myotubes (45) by reducing myoD (46), myogenin and protein synthetic pathways via Akt in C_2C_{12} myotubes (47) and via Akt/TORC1/p70S6K in human skeletal myoblasts (48)**. Myostatin signals via the type IIb activin receptor that enables interactions with activin receptor-like-kinase 4 (ALK4) or ALK5 (both type I receptors- see Figure 1) (49). As a consequence of the association of these myostatin/receptor complexes, phosphorylation of transcription factors Smad2 and Smad3 occurs followed by their translocation to the nucleus (50) where they alter gene transcription. Trendelenburg *et al.* (48)**, demonstrated that follistatin (myostatin inhibitor) and type I ALK receptor inhibitors increased both the size and number of human skeletal myoblasts in culture and, in the presence of exogenous myostatin were able to restore differentiation capacity. Furthermore, siRNAs for Smad 2 or 3 reduced the effect of myostatin on differentiation, with both in combination eliciting an additive effect. Interestingly, there was a 50% reduction in phosphorylated Akt and p70S6K in the presence of myostatin in differentiating myoblasts and exogenous IGF-I could rescue this effect. However, IGF-I did not change Smad2/3 reporter activity indicating that IGF-I did not oppose myostatin actions via Smad, but via Akt and the induction of protein synthesis via p70S6K. Overall, therefore the IGF-I/Akt/protein synthesis signalling seems dominant over the myostatin/Smad inhibition. Conversely however, Smad 2 or 3 siRNAs restored Akt activation in the presence of myostatin, suggesting Smad2/3 do regulate Akt function but distinctly to IGF-I (48)**.

A recent study by Satori *et al.* (51)** published simultaneously with that of Trendelenburg *et al.* (48)** showed that activation of the Smad 2 and 3 pathway using electroporation to introduce genes encoding active forms of ALK4 or 5 and TGF-β itself, induced myofibre atrophy. This effect could be reversed using small hairpin RNAs (shRNAs) blocking Smad2 and Smad3. Importantly, constitutive overexpression of Akt prevented the muscle fibre atrophy induced by Smad2/3 activation (electroporation for ALK4 or 5 mentioned above), further co-borating the *in-vitro* role of Akt in reducing the impact of myostatin.

Myostatin: Protein Synthesis or Protein Degradation?

Some controversy remains over whether myostatin functions via traditional expression of "atrogenes" that promote protein degradation via E3 ubiquitin ligases such as MuRF1 and MAFbx. Early work strongly suggested that myostatin increased levels of FOXO1 that in turn up-regulated MAFbx (47) that leads to protein degradation of cytoskeletal proteins such as desmin and titin. Similarly in C_2C_{12} myoblasts the addition of myostatin increased MAFbx, but not MuRF1. Data were confirmed in murine models where myostatin increased MAFbx but not MuRF1 expression (51)**. By contrast, the study by Trendelenburg *et al.* (48)** reported a decrease in both MuRF1 and MAFbx mRNA. However, Welle (52) reviewed that neither publication included a direct measure of proteolysis, however, that the vast majority of evidence suggests that changes in protein synthesis rather than degradation are key. Although compelling *in-vitro/in-vivo* signalling evidence suggests reduced protein synthesis, unresolved studies regarding the protein degradation remain. Indeed, very recent findings suggesting that the *in-vivo* murine or *in-vitro* myoblast knockdown of MAFbx, using shRNAs, supresses myostatin expression and muscle atrophy (53)**, suggesting a feed-forward loop whereby increased MAFbx influences the local production and hence action of myostatin.

Myostatin and Premature Ageing?

Although myostain inhibitor studies have shown some success in reducing wasting in rodents (54, 55), myostatin inhibitors in human studies should be approached with care when considering regeneration with age. McFarlane *et al.* (56) showed that blocking myostatin, causes high Pax7 expression resulting in increased self-renewal of C_2C_{12} myoblasts followed by quiescence. However, they also reported that over expressing Pax7 in C_2C_{12} cells conferred increased self-renewal but reduced myogenic proliferation and differentiation. Therefore, blocking myostatin in adults may be advantageous in the short-term; however, high expression of Pax7 would influence self-renewal and differentiation and potentially affect subsequent regeneration in later life. This may further compound ageing where myostatin levels are already higher than in younger individuals (57). Indeed, myostatin knock out animals, although displaying larger muscle mass, are not proportionally stronger (58), this too would be detrimental, i.e. increased weight, but not strength to lift in older people.

Conclusion

Myoblast models have paved the way for understanding the convergence of key mechanisms involved in hypertrophy and atrophy of skeletal muscle; some of the most pertinent recent findings have been discussed in this review. However, future development of myoblast models must incorporate engineering strategies to make the models more reflective of the *in-vivo* situation and evolve the current 3-D models already available (8)**(9). In this way, cell-based models in a dish can be utilised to address key *in-vitro* questions, which can then be focussed to address more challenging *in-vivo* questions.

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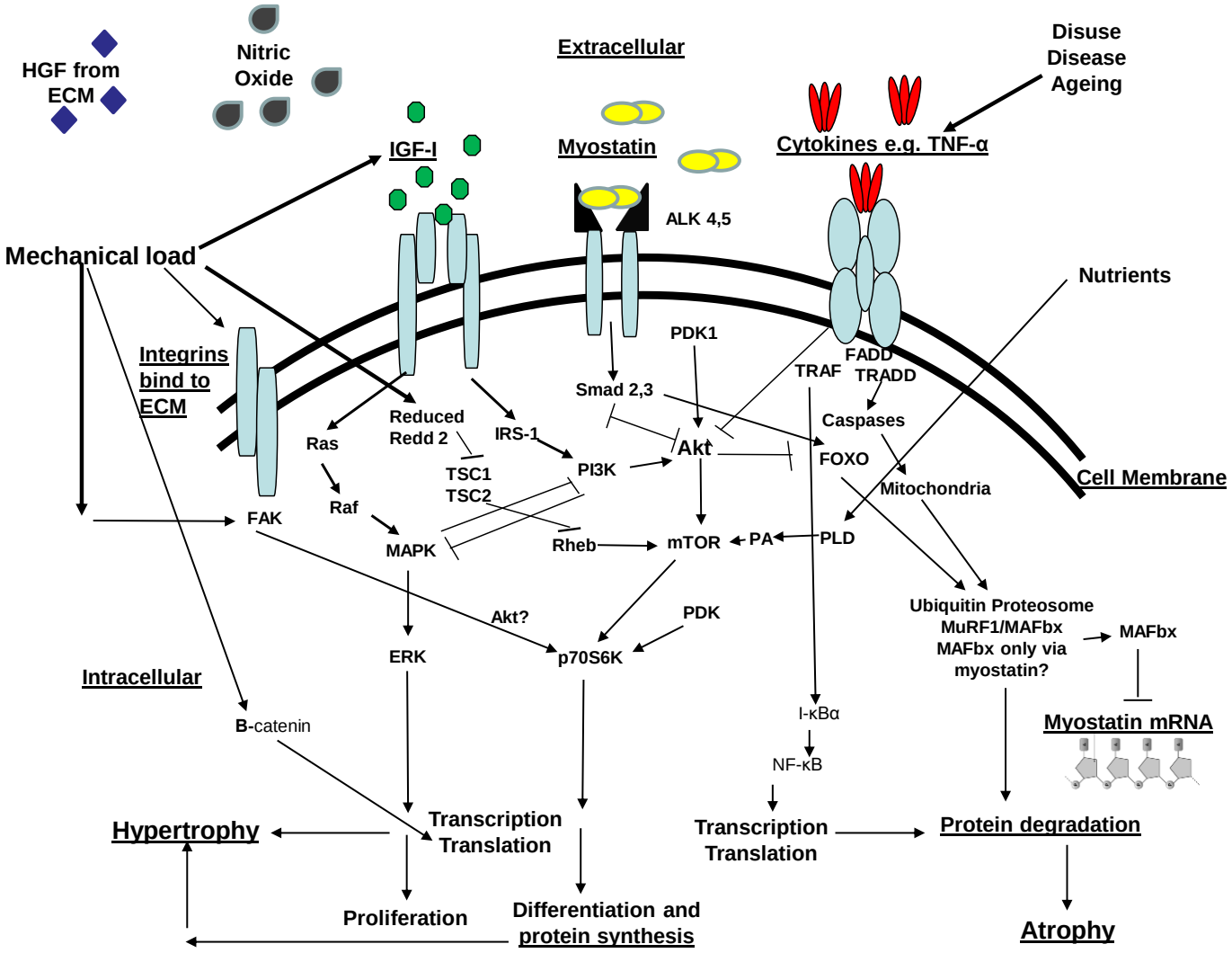

Figure Legend

Figure 1. The regulation of protein synthesis and muscle hypertrophy vs. protein degradation and muscle atrophy.

IGF-I; Insulin-like growth factor-I, IRS-1; Insulin Receptor Substrate-1, TNF-α; Tumour Necrosis Factor-Alpha, ALK 4, 5; Activin Receptor-Like-Kinase 4 and Activin Receptor-Like-Kinase 5, ECM; Extracellular Matrix, HGF; Hepatocyte Growth Factor, TRADD; TNF Receptor 1 Associated Death Domain, TRAFF; TNF receptor Associated Factor, FAK; Focal Adhesion Kinase, FADD; Fas Associated Death Domain, FOXO; Forkhead Homeobox Type O, PDK-1; Phosphoinositide-Dependent Kinase-1, Akt; Protein Kinase B, MAPK; Mitogen Activated Protein Kinase, mTOR; Mammalian Target of Rapamycin, PI3K; Phosphatidylinositol 3-kinase, ERK; Extra Cellular Signal Regulated Kinase, Ras; Ras Protein, Raf; MAP Kinase Kinase Kinase (MAP3K), p70S6K; P70S6 Kinase- a serine/threonine kinase- Phosphorylation of S6 induces protein synthesis at the ribosome, TSC1, TSC2; Tuberous Sclerosis Protein 1, Tuberous Sclerosis Protein 2, Redd 2; Regulated in Development and DNA Damage Response 2, Rheb; Ras Homolog Enriched in Brain, PLD; Phospholipase D, PA; Phosphatidic acid PA, I-κBα; Inhibitor-Kappa B Alpha, NF-κB; Nuclear Factor-Kappa B, β-Catenin; Beta-Catenin.