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Signaling Pathways: The Benefits of Good Communication

Dispatch

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Recent studies show that hyperactivated mTOR, the 'target of rapamycin' that senses nutrient availability in eukaryotic cells, inhibits signaling by insulin receptor substrates. This crosstalk reveals how hyperactivated mTOR may suppress metastasis locally, while causing systemic insulin resistance that can progress to diabetes.

This story begins with a rare genetic disease known as tuberous sclerosis, which causes benign tumors in various organs and results from mutations in one or the other of two identified genes *TSC1* and *TSC2*. Tuberous sclerosis afflicts some people severely, causing developmental delay, mental retardation and autism, while others are so mildly affected that the condition goes undiagnosed. Regardless of the clinical presentation, the tumors in tuberous sclerosis patients, also known as hamartomas, are rarely malignant.

The TSC1 and TSC2 proteins form a heterodimer which acts as a GTPase activating protein (GAP) that links upstream growth factor signaling cascades to the 'target of rapamycin', mTOR, a serine/threonine kinase that regulates protein synthesis through downstream effectors such as ribosomal S6 kinase (S6K) and the translation factor inhibitor 4EBP1. This pathway is critical for proper cell growth and regulation of organ size [1]. Growth factor signals, such as insulin, insulin-like growth factor (IGF), platelet-derived growth factor and others, activate mTOR when nutrients are available and intracellular energy levels are high; mTOR is inactivated during cellular starvation [2]. The mutations that cause tuberous sclerosis inactivate the GAP activity of the TSC1–TSC2 heterodimer leading to uncontrolled hyperactivation of mTOR.

The insulin and IGF1 receptors are tyrosine kinases which phosphorylate the insulin receptor substrate (IRS) upon ligand binding. Phosphorylated IRS, in turn, acts as a protein scaffold that activates the phosphatidylinositol (PI) 3-kinase/Akt cascade [3]. The production of PIP_3 by PI 3-kinase recruits the serine/threonine kinases PDK1 and Akt to the plasma membrane, where Akt is activated by PDK1-mediated phosphorylation [4]. Akt phosphorylates many proteins with important physiological roles, including TSC2, inhibiting its GAP activity toward the small G-protein Rheb [5]. The accumulation of GTP-bound Rheb leads to activation of mTOR through an as yet unknown mechanism (Figure 1).

As mentioned above, mutations in TSC1 or TSC2 that promote activation of mTOR cause benign hamartomas that rarely metastasize. By contrast, activation of mTOR by mutations that stimulate Akt causes malignant tumors. An explanation for this difference is revealed, at least in part, by two recent studies [6,7] which use mouse embryo fibroblasts deficient in either *TSC1* or *TSC2* to dissect the pathway components altered in tuberous sclerosis. Both groups [6,7] found the PI 3-kinase/Akt cascade in *TSC1*$^{-/-}$ and *TSC2*$^{-/-}$ mouse embryo fibroblasts is insensitive to insulin or IGF1, whereas other growth factors stimulate the pathway normally. Insulin-stimulated Akt activation is restored in these mutant cells upon inhibition of mTOR or S6K activity. Shah *et al*. [6] report that over-expression of Rheb in HEK293 cells mimics *TSC1* or *TSC2* deficiency. These data indicate that hyperactivation of mTOR/S6K signaling blocks the activation of PI 3-kinase/Akt during insulin or IGF stimulation.

The IRS proteins are unique elements in the insulin and IGF signaling cascade and thus likely sites of mTOR-mediated inhibition. Earlier work showed that many kinases, including rapamycin-sensitive enzymes, promote serine/threonine phosphorylation of IRS1 and IRS2 that inhibits their function and promotes their degradation [8,9]. Shah *et al.* [6] found that IRS1 and IRS2 protein levels are reduced in *TSC1*$^{-/-}$ and *TSC2*$^{-/-}$ cells as a result of their decreased half-life and lower transcript levels. Harrington *et al.* [7] found specific loss of IRS1 at the mRNA and protein level in *TSC2*$^{-/-}$ cells.

Regardless of these differences, both groups [6,7] found that insulin or IGF stimulated Akt phosphorylation could be rescued by rapamycin treatment, coincident with restored IRS protein levels. The rapamycin-mediated rescue was blunted by reducing *IRS1* or *IRS2* expression with specific siRNAs, and rendered unnecessary by overexpression of *IRS1*. Thus, chronic hyperactivation of mTOR by inactivation of TSC1–TSC2 stimulates components of the protein synthesis pathway, while inhibiting the IRS branch of the insulin/IGF signaling cascade [6,7].

Dysregulation of IRS1 or IRS2 upstream of the PI 3-kinase/Akt signaling pathway may be critical to the benign nature of tumors in tuberous sclerosis. *TSC2*$^{-/-}$ cells display no chemotaxis toward IGF1, but retain the ability to move towards epidermal growth factor (EGF) [7]. Moreover, *TSC1*$^{-/-}$ and *TSC2*$^{-/-}$ cells are more sensitive to apoptotic stimuli such as serum withdrawal or etoposides, consistent with the view that IGF1 signaling is inactive in these mutant cells [6,7]. Rapamycin treatment restores both cell survival and the chemotactic response to IGF1, raising a caution about the indiscriminant use of rapamycin in cancer therapy [6,7].

Nagle *et al.* [10] recently reported that IRS2 acts to promote lung metastases in murine primary breast cancer caused by mammary-specific expression of transforming middle T antigen. Thus, depletion of IRS proteins by the hyperactivated mTOR cascade could explain, at least in part, the infrequent malignancy of tuberous sclerosis tumors. A second mutation that

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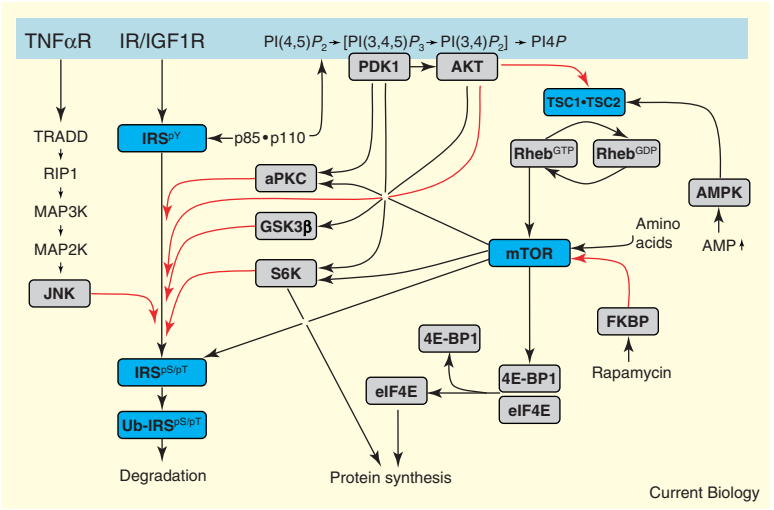

Figure 1. Growth factors and nutrient signaling regulate the mTOR serine/threonine kinase and its downstream effectors S6K and 4E-BP1.

When activated by ligand binding, the insulin receptor kinase recruits IRS molecules and phosphorylates them on tyrosine residues that serve as docking sites for downstream signaling molecules such as PI 3-kinase. Activated PI 3-kinase triggers a ubiquitous lipid and protein kinase cascade; the signal propagates through Akt to TSC1–TSC2 heterodimers and the small G protein Rheb, leading to mTOR activation. Nutrients such as glucose and amino acids are required for complete activation of mTOR and S6K. Rheb and TSC1–TSC2 are also sensitive to nutrient availability. Rapamycin is a bacterial product that forms a complex with a protein known as FKBP that binds to mTOR and inhibits its kinase activity. Phosphorylation of mTOR effector proteins, such as the serine/threonine kinase S6K and 4E-BP1, is critical for protein synthesis and cell proliferation. Several kinases are known to promote the phosphorylation of IRS, as shown. Rapamycin-sensitive ubiquitinylation of IRS molecules leads to their uncoupling from the insulin receptor and degradation. Black arrows indicate stimulatory events and red arrows indicate inhibitory events.

activates PI 3-kinase/Akt signaling, such as one that hyperactivates the EGF receptor or inactivates PTEN, might promote malignant tumor formation in tuberous sclerosis patients. Regardless, strategies aimed at inhibiting IRS protein signaling may provide a rational way of targeting IGF-dependent malignant tumors of mammary, colon or prostate origin.

The mechanisms directed by mTOR that inhibit IRS protein function are difficult to resolve. Harrington *et al.* [7] explored the role of IRS serine/threonine phosphorylation in *TSC2*$^{-/-}$ cells. IRS1 and IRS2 each contain over 40 serine/threonine phosphorylation sites, many of which appear to inhibit insulin signaling (White lab's unpublished data). One site in IRS1, serine 302, was found to be hyperphosphorylated in *TSC2*$^{-/-}$ cells, but returns to the wild-type state upon treatment with rapamycin or specific siRNAs against S6K1 and S6K2 [7]. *In vitro* assays have shown that phosphorylation of serine 302 interferes with the association between IRS1 and the insulin receptor, as proposed for serine 307 phosphorylation in IRS1 [11]. Harrington *et al.* [7] conclude that phosphorylation of serine 302 is a key step to inhibiting IRS-protein functions.

The mechanisms that regulate IRS function are likely to be more complicated, as the mTOR cascade also has a permissive role when nutrients are available. Starvation for amino acids or glucose inhibits insulin/IGF1-stimulated tyrosine phosphorylation of IRS1, while diminishing serine 302 phosphorylation. Replenishing amino acids restores serine 302 phosphorylation and insulin-stimulated tyrosine phosphorylation of IRS1; however, mutation of serine 302 to alanine inhibits insulin-stimulated tyrosine phosphorylation of IRS1 even when amino acids are present [12]. Thus, mTOR might be a dual checkpoint enzyme: under ordinary conditions, mTOR signaling promotes the interaction between IRS and the insulin/IGF1 receptor; but when mTOR is hyperactivated, either during chronic nutrient excess or in *TSC1*$^{-/-}$ or *TSC2*$^{-/-}$ cells, it promotes hyperphosphorylation of IRS1 that inhibits insulin/IGF signaling (Figure 2).

Physiological conditions that lead to constitutive activation of the mTOR cascade, such as the hyperinsulinemia that accompanies chronic nutrient excess and obesity, may exacerbate the insulin resistance that causes metabolic disease and type 2 diabetes. Long-term treatment of mouse embryo fibroblasts with insulin or IGF1 promotes rapamycin-sensitive degradation of IRS2 by an ubiquitin-mediated and proteosome-dependent process. This cellular state causes inhibition of insulin/IGF-stimulated Akt activation [9]. Perhaps specific phosphoserine sites on IRS proteins promote ubiquitinylation and proteosome-mediated degradation. Regardless of the exact mechanism by which mTOR is hyperactivated, cellular insulin resistance appears to be an inevitable consequence.

Can hyperactivation of the mTOR/S6K cascade contribute to systemic insulin resistance? A recent analysis [13] of *S6K1*$^{-/-}$ mice supports the view that S6K1 has an important role in the inhibition of IRS1 signaling by diet or obesity. Wild-type mice fed a high fat diet display increased S6K phosphorylation, indicative of active kinase, and reduced Akt phosphorylation upon acute insulin stimulation in adipose, liver and muscle [13]. Moreover, IRS1 from obese wild-type mice is hyperphosphorylated on serine 307 and serines 636/639 in adipose tissue; phosphorylation at the latter sites inhibits IRS1 and PI 3-kinase binding [8]. By contrast, insulin-stimulated Akt phosphorylation and IRS1 serine phosphorylation were found to be nearly normal in *S6K1*$^{-/-}$ mice fed a high fat diet [13]. Um *et al.* [13] conclude that excess nutrients resulting from an inappropriate diet or chronic obesity may cause insulin resistance through hyperactivation of the mTOR/S6K cascade, which disrupts IRS function via serine phosphorylation events.

Simple organisms like yeast or cultured eukaryotic cells rely upon mTOR as a checkpoint to integrate

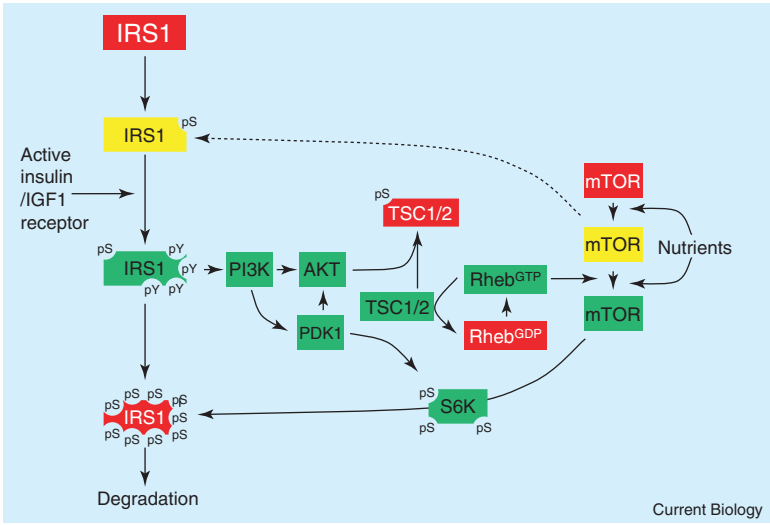

Figure 2. A model in which mTOR acts as a dual checkpoint regulating IRS signaling.

Inactive or hyperactivated mTOR inhibits insulin-stimulated tyrosine phosphorylation of IRS proteins. IRS-1 is primed to interact with activated insulin or IGF1 receptors by a rapamycin-sensitive serine phosphorylation, probably involving nutrient-activated mTOR. Primed IRS1 is strongly tyrosine phosphorylated during insulin stimulation, which leads to activation of downstream effectors, such as the PI 3-kinase/Akt cascade, that stimulate mTOR/S6K signaling. The second phase of mTOR regulation of IRS1, possibly involving S6K and many other kinases, promotes extensive phosphorylation of multiple serine and threonine residues throughout IRS1. Hyperphosphorylated IRS1 is uncoupled from receptor tyrosine kinases, interacts poorly with downstream effectors and is targeted for degradation. This model can explain how severe starvation can inhibit insulin signaling, while excess calories and chronic hyperinsulinemia can elevate mTOR activity sufficiently to contribute to peripheral insulin resistance. Inactivation of TSC1–TSC2 dimers leads to hyperactivation of mTOR and complete inhibition of IRS signaling. By contrast, dysregulation of S6K in cells or deletion of the *S6K1* gene in mice reduces serine phosphorylation of IRS proteins, stabilizes the protein at levels that promote insulin signaling [13].

cellular growth and function with available nutrients and intracellular energy stores. As complex animals, including worms, flies and humans, emerged, insulin signaling evolved to provide system-wide integration, extending to the peripheral tissues and the central nervous system. Mice lacking *Irs1* are small and insulin resistant, while those lacking *Irs2* develop type 2 diabetes mellitus as a result of peripheral insulin resistance and pancreatic β cell failure [14]. The discovery that IRS is inhibited by mTOR/S6K cascades has revealed an elusive connection between nutrient homeostasis and normal growth, and tumor formation and metastasis. In the central nervous system, this mechanism might inhibit Irs2 signaling, promoting hyperphagia that exacerbates nutrient excess [15]. By contrast, exercise-induced activation of the AMP kinase that down-regulates mTOR activity might promote IRS–protein signaling in peripheral insulin-sensitive tissues that prevents obesity and diabetes.

A number of important questions remain unanswered. Does the low incidence of malignancy in tuberous scleroris arise from the depletion of IRS proteins, and could this model be generally applied in cancer treatment? Might tissue-specific inhibition of mTOR be a treatment for metabolic diseases and type 2 diabetes, especially as IRS2 is critical at multiple levels for nutrient homeostasis [15,16]? Might the protection of IRS protein levels in β cells explain why rapamycin is the best immunosuppressive drug for islet transplant recipients? Understanding the crosstalk between IRS and the mTOR cascade should provide answers to these questions, while revealing strategies to achieve a healthy balance between nutrient homeostasis and dysregulated cell growth.

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