Rictor Acetylation Promotes mTORC2-Mediated Akt Phosphorylation

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Abstract

The Ser/Thr protein kinase mTOR is a critical regulator of cell growth, survival and metabolism in response to growth factors. mTOR functions in at least two distinct multiprotein complexes known as mTOR complex (mTORC) -1 and -2. mTORC2 phosphorylates Akt at S473, an event required for increased Akt kinase activity. Although it is known that the mTORC2 components Rictor and mSin1 are required for the stability and activity of mTORC2, little is known about post-translational modifications that regulate mTORC2. Work presented in this thesis identifies Rictor acetylation as a positive regulator of mTORC2-mediated phosphorylation of Akt at S473. Inhibition of deacetylases, including the NAD^+-dependent Sirtuins, promotes Rictor acetylation and IGF1-mediated Akt phosphorylation at S473. While mapping the major acetylated regions contained within Rictor we identified two important regions: 1) a region critical for interaction with mSin1.1 and LST8 that subsequently promotes mTORC2 stabilization, and 2) an adjacent acetylated region localized between amino acids 1041 and 1137. Analysis of this acetylated region in Rictor identified 9 lysines that could potentially be post-translationally modified, each of which we divided into 4 distinct groups or modules (M1-M4). Site-directed mutagenesis (lysine to arginine) of each of the lysines located within two of the modules (M2 and M4) reduced Rictor acetylation and IGF1-dependent mTORC2 kinase activity. These results indicate that multiple-site acetylation of Rictor signals for increased activation of mTORC2 in response to IGF1 stimulation. To further examine

whether other post-translational modifications contribute to Rictor acetylation, evidence provided here indicates that the O-linked N-acetylglucosamine (O-GlcNAc) moiety potentiates Rictor acetylation. This is biologically significant because heightened O-GlcNAcylation of Rictor not only increased acetylation, but promoted mTORC2-mediated phosphorylation of Akt at S473. Since Akt is known to promote metabolic pathways that generate metabolite signaling molecules required for acetylation and O-GlcNAc modification, post-translational modifications that target Rictor may comprise a positive feedback loop during conditions of nutrient abundance.

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Chapter 1: Introduction

1.1: IGF1 signaling

Insulin-like growth factor 1 (IGF1) was first identified over 50 years ago as a peptide hormone that mediates the effects of growth hormone (GH) (Daughaday, 2006). It was initially named sulfation factor and later was renamed somatomedin C for its promotion of sulfate uptake in the synthesis of cartilage and bone in response to GH (Salmon and Daughaday, 1957; Daughaday *et al.*, 1972). Upon purification of the peptide and analysis of its sequence in 1978, it was again renamed for its similarity to insulin (Rinderknecht and Humbel, 1978). IGF1 shares approximately 40% sequence similarity with proinsulin (Ohlsson *et al.*, 2009). Growth signaling from IGF1 is significantly distinguished from insulin signaling by the fact that all cell types harbor its physiological receptor, the type I IGF receptor (IGF1R; Clemmons, 2009). This allows IGF1 to transmit growth signals to tissues throughout the body. The synthesis of IGF1 occurs predominantly in the liver in response to GH. However, IGF1 can also be synthesized in peripheral tissues where it can exert local or paracrine functions, such as tissue repair (Perrini *et al.*, 2010). Secreted IGF1 is regulated by IGF1 binding proteins that regulate growth factor activity and half-life in circulation.

IGF1R is a heterotetramer consisting of two ligand-binding subunits and two receptor tyrosine kinase subunits (Rosenzweig and Atreya, 2010; Clemmons, 2009). Upon IGF1 binding, IGF1R tyrosine kinase autophosphorylates key Tyr residues, creating binding sites for Src homology 2 (Sh2)-domain containing proteins. In canonical IGF1R activation, insulin-receptor substrates-1 and -2 (IRS-1 and -2) bind to the activated receptor and

are subsequently phosphorylated (White, 2002). IRS phosphorylation creates binding sites for the 85 kDa regulatory subunit of phosphatidylinositol-3-kinase (PI3K), which tethers the 110 kDa catalytic PI3K subunit. PI3K is also activated downstream of other receptor tyrosine kinases, such as the insulin receptor, epidermal growth factor receptor, and platelet-derived growth factor receptor. PI3K phosphorylates membrane lipids phosphatidylinositol (4,5) diphosphate (PIP_2), generating phosphatidylinositol (3,4,5) triphosphate (PIP_3). This 3′-phosphorylation can be reversed by the tumor suppressor protein phosphatase and tensin homolog (PTEN; Maehama and Dixon, 1998; Zhang and Yu, 2010). The balance between the activities of PI3K and PTEN therefore determines the level of PIP_3 in the membrane. PIP_3 initiates key signaling cascades for cell growth, proliferation, survival and nutrient uptake. One of the critical mediators of these signaling pathways is protein kinase B (PKB), or Akt.

1.2: Akt is activated by membrane recruitment and phosphorylation

Akt is a Ser/Thr kinase with key roles in cell metabolism, survival, growth, and proliferation. Its importance in normal physiology and aberrant function in cancer and diabetes have made it the subject of intense research since its discovery in 1991 as the proto-oncogene of the retrovirus *Akt8* (Bellacosa *et al.*, 1991; Manning and Cantley, 2007). The cellular Akt homolog regulates numerous biological processes including cell growth, metabolism, cell cycle, survival, and nutrient uptake (Manning and Cantley, 2007; Robey and Hay, 2009; Hanada *et al.*, 2004).

There are three mammalian isoforms of Akt encoded by different genes and known as Akt1, Akt2, and Akt3. All three isoforms are highly homologous, possessing over 80% sequence identity and equivalent *in vitro* kinase activity (Walker *et al.*, 1998; Robey and Hay, 2009). However, they demonstrate tissue-specific differences in their relative expression and isoform-specific knockouts have identified a few distinct physiological functions (Dummler and Hemmings, 2007; Bhaskar and Hay, 2007). Akt1 is the predominant isoform in most tissues, is important for growth in development and contributes to cancer progression. Akt2 is emerging as the isoform most critical to metabolic signaling and is the predominant isoform expressed in muscle and adipose. For example, Akt2 was found to be more important than Akt1 for insulin-stimulated glucose uptake in adipocytes (Katome *et al.*, 2003). Akt3 is important in brain development, and is highly expressed in testes. The mechanism for the observed differences in the function of Akt isoforms is not yet known, but may involve differences in substrate preference or subcellular localization (Bhaskar and Hay, 2007). Akt1 and Akt2 double knockout mice die immediately after birth and display defects in muscle, skin, bone, and adipose development (Peng *et al.*, 2003). These defects mirror insulin receptor and IGF1 receptor null mice, suggesting that Akt1 and Akt2 are the primary mediators of insulin/IGF1 signaling during development (Bhaskar and Hay, 2007).

Akt is a member of the AGC (related to AMP/GMP and protein kinase C) kinase family (Jacinto and Lorberg, 2008; Bozulic and Hemmings, 2009). Other members of this family include protein kinase A (PKA), protein kinase C (PKC),

3′-phosphoinositide dependent kinase-1 (PDK1), p70 S6 kinase (S6K), serum- and glucocorticoid-induced protein kinase (SGK), and p90 ribosomal S6 kinase (RSK). The domain structure of Akt consists of an N-terminal pleckstrin homology (PH) domain, a catalytic domain conserved among all AGC kinases, and a C-terminal hydrophobic motif (HM), also conserved among most AGC kinases (Jacinto and Lorberg, 2008). The HM consists of hydrophobic patch of amino acids containing a phosphorylation site ($FPQFS_{473}$*YSASG in Akt1). Activation of Akt requires phosphorylation of the activation, or T loop in the catalytic domain and of the HM (Bellacosa *et al.*, 2004; Manning and Cantley, 2007). The domain organization of Akt1, including the T loop and HM, is shown in Figure 1.

Akt activation is initiated by binding of its PH domain to PI3K-generated PIP_3, which causes Akt to translocate to the membrane. Membrane translocation stimulates phosphorylation of Akt1 at T308 and S473, events required for increased kinase activity (Bellacosa *et al.*, 1998; Alessi *et al.*, 1996). The AGC kinase PDK1 is also recruited to the membrane by binding of its PH domain to PIP_3 and phosphorylates Akt at T308, upon co-localization to the membrane (Alessi *et al.*, 1997). A diagram of these events is shown in Figure 2.

The identity of the Akt S473 kinase was unknown for nearly a decade and was referred to as “3′-phosphoinositide dependent kinase-2 (PDK2).” Many PDK2 candidates have been proposed and shown to positively regulate Akt S473 phosphorylation, including Akt itself, integrin-linked kinase (ILK), and DNA-

Figure 1: Domain Organization of Akt1. The pleckstrin homology (PH) domain and kinase domain (KD) are shown, with conserved T-loop containing PDK1 phosphorylation site T308. The hydrophobic motif (HM) lies in the C-terminus and contains S473, a site phosphorylated by mTOR Complex 2.

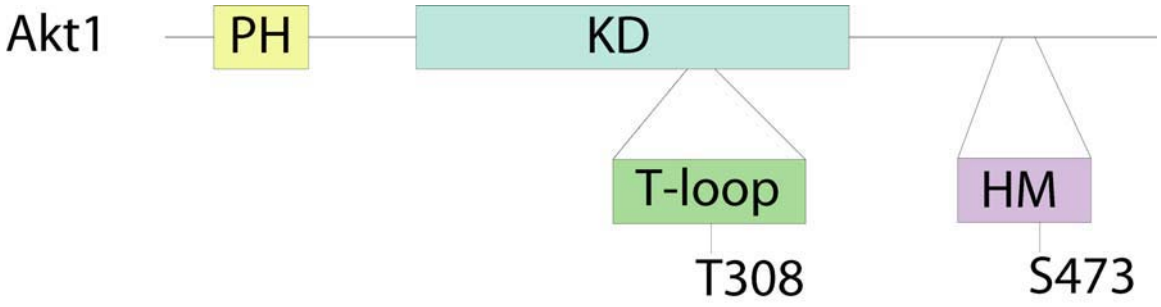
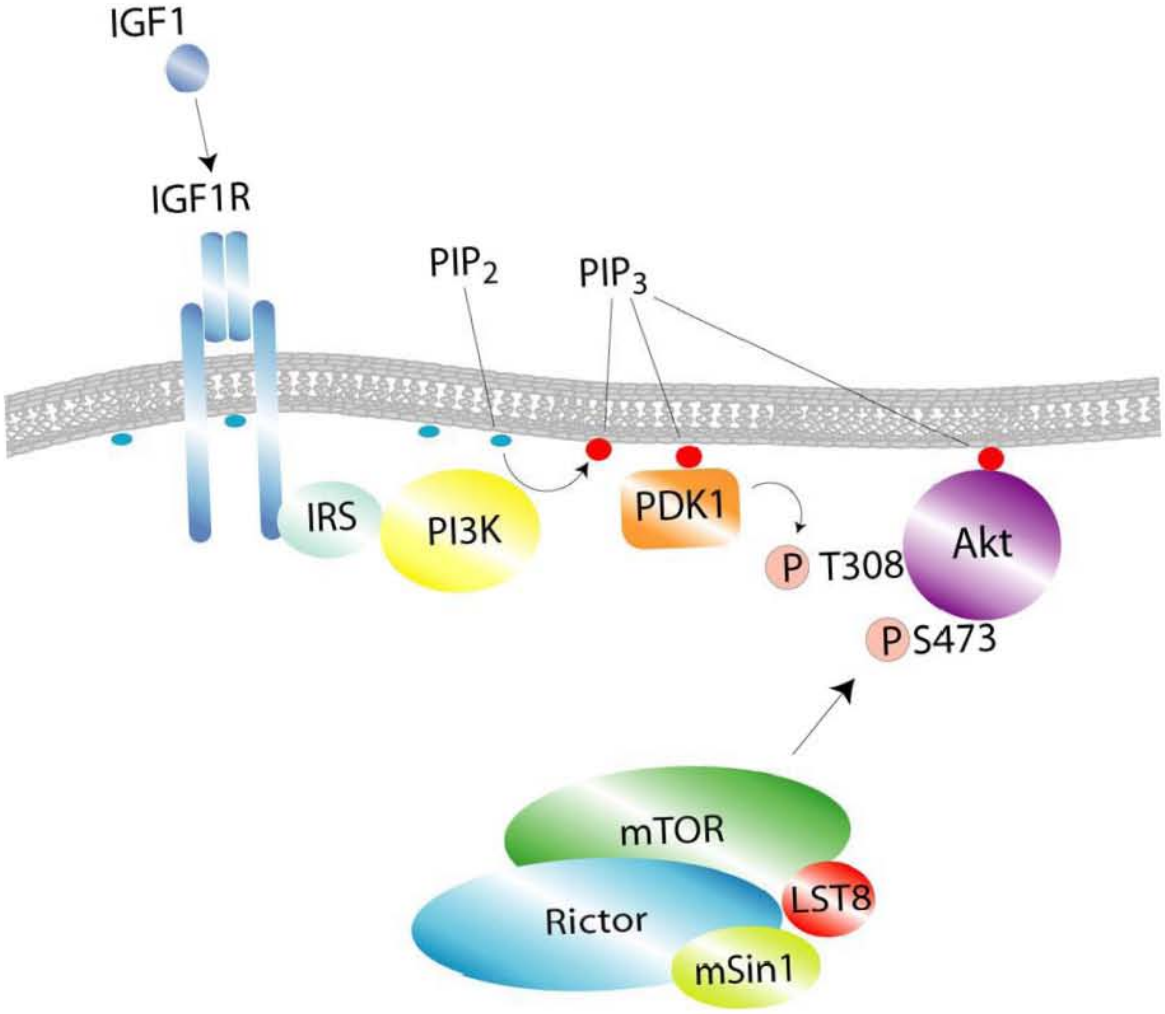

Akt1
PH
KD
T-loop
HM
T308
S473

Figure 2: IGF1 activation of Akt. Insulin-like growth factor (IGF1) binding to the Type I IGF1 receptor (IGFIR) recruits insulin receptor substrate (IRS) scaffolding proteins. IRS binds phosphatidylinositol-3-kinase (PI3K), which phosphorylates membrane lipids PIP_2 (small blue circles) to generate PIP_3 (small red circles). The kinases PDK1 and Akt are bound to the membrane by interaction with PIP_3, which stimulates PDK1 to phosphorylate Akt on T308. Concomitantly, Akt is phosphorylated on S473 by mTOR Complex 2.


IGF1
IGF1R
PIP2
PIP3
IRS
PI3K
PDK1
P T308
Akt
P S473
mTOR
LST8
Rictor
mSin1

dependent protein kinase (DNA-PK; Toker and Newton , 2000; Feng *et al.*, 2004; Persad *et al.*, 2001). However, the evidence demonstrating that these kinases played a direct, physiological role in Akt S473 phosphorylation remained a debate for several years (Lynch *et al.*, 1999; Hill *et al.*, 2001). Of these candidates, DNA-PK is distinguished in that it has been shown to phosphorylate Akt at S473 specifically in response to DNA damage (Feng *et al.*, 2004; Park *et al.*, 2009). It was not until 2005, fourteen years after the initial discovery of Akt that the growth factor responsive PDK2 was identified. The Sabatini laboratory demonstrated that the mammalian target of rapamycin complex 2 (mTORC2) directly phosphorylates Akt at S473 (Sarbassov *et al.*, 2005). mTORC2 consists of the protein kinase mTOR and required subunits rapamycin-insensitive companion of mTOR (Rictor), mammalian stress-activated protein kinase interacting protein (mSin1), and the mammalian lethal with Sec13 protein 8 (LST8). This complex will be described in greater detail in Chapter 1.4. Sabatini's group demonstrated that Akt S473 phosphorylation was dependent on the expression of mTOR and the mTORC2-specific component Rictor. Additionally, Akt S473 was phosphorylated *in vitro* by mTORC2 purified from cells and this kinase activity was stimulated by serum treatment (Sarbassov *et al.*, 2005). The importance of mTORC2 as the primary S473 kinase that signals from PI3K has been substantiated by multiple investigators (Hresko and Mueckler, 2005; Shiota *et al.*, 2006; Frias *et al.*, 2006; Jacinto *et al.*, 2006; Guertin *et al.*, 2006; Kumar *et al.*, 2008; Shu and Houghton, 2009; Kumar *et al.*, 2010).

Phosphorylation of both T308 and S473 results in a 1000-fold increase in Akt catalytic activity (Alessi *et al.*, 1996). Although estimates of the relative contribution of each phosphorylation vary, it is clear that T308 phosphorylation is absolutely required for activity, while S473 substantially augments catalytic activity (Alessi *et al.*, 1996; Bellacosa, *et al.*, 1998; Yang *et al.*, 2002a; Guertin *et al.*, 2006). In response to a growth factor stimulus, Akt phosphorylation at S473 and T308 occur with similar kinetics. Examination of the order of occurrence of the two events has therefore focused on the relative dependence of one site on the other. Studies using site-directed mutants, where either S473 or T308 were mutated to alanine, have found that T308 can be phosphorylated without S473 phosphorylation and vice versa (Alessi *et al.*, 1996). However, Ser473 phosphorylated Akt is a better PDK1 substrate than unphosphorylated Akt *in vitro* (Alessi *et al.*, 1996; Sarbassov *et al.*, 2005). Altogether, these studies have indicated that while phosphorylation of these sites can occur independently, S473 phosphorylation may prime Akt for T308 phosphorylation under physiological conditions.

Over the last 15 years, structural studies have provided insights into the regulation of Akt kinase activity by phosphorylation. These studies provide a rationale for the apparent cooperativity between the two sites. The structure of the Akt catalytic domain, like other conventional protein kinases, consists of an amino-terminal lobe (N lobe) and a carboxyl-terminal lobe (C lobe) connected by an activation, or T loop. T308 lies in the T loop and its phosphorylation facilitates binding of substrate and catalysis (Yang *et al.*, 2002b). Although

monophosphorylated Akt2 at T309 (equivalent to Akt1 T308) is catalytically active, the crystal structure revealed that the enzyme resides in equilibrium between inactive and active conformations (Yang *et al.*, 2002a). Thus, phosphorylation of S473 is thought to cause an allosteric intermolecular interaction that shifts the equilibrium towards the active enzyme conformation. In the structure of Akt2 monophosphorylated at T309, the N-lobe alphaC helix, the N-lobe alphaB helix, the T loop, and the HM are unstructured (Yang *et al.*, 2002a). Addition of peptides mimicking phosphorylation within the HM or use of phospho-mimics of S474 phosphorylation (equivalent to Akt1 S473), results in ordering of the alphaB and alphaC helices, the HM, and the T loop (Yang *et al.*, 2002a, Yang *et al.*, 2002b). The ordering of these domains results in a reorientation of the N and C lobes, stabilizing the nucleotide binding site in a catalytically active structure that is nearly identical to the catalytically active structure of PKA (Yang *et al.*, 2002a). In the active conformation, His-196 of the alphaC helix forms a salt bridge with the phosphate group of phosphorylated T309, further stabilizing the structure (Yang *et al.*, 2002b). By analogy, Akt1 phosphorylation at S473 results in allosteric stabilization of the catalytically active kinase structure, which is also dependent on phosphorylation at T308.

Interestingly, the specific activity of PDK1 is increased by a phosphorylated Akt HM, which may create a PDK1 docking site and potentiate PDK1 activation (Pearl and Barford, 2002; Scheid and Woodgett, 2003). The Akt HM is thought to act as an allosteric activator for PDK1, which lacks its own HM (Bellacosa, *et al.*, 2005). Thus Akt S473 phosphorylation stabilizes the active

kinase structure of Akt and promotes PDK1-mediated phosphorylation of Akt at T308.

Dual phosphorylation of Akt is reversed by the action of phosphatases. Akt activity is negatively regulated by protein phosphatase 2A (PP2A) (Ugi *et al.*, 2004), the primary kinase phosphatase in the cell (Millward *et al.*, 1999). Akt S473 phosphorylation is specifically removed by PH domain leucine rich repeat protein phosphatase (PHLPP; Gao *et al.*, 2005). Although the primary target of PHLPP in the cell is believed to be Akt, PHLPP also dephosphorylates the AGC kinase PKCα (Gao *et al.*, 2005; Jackson *et al.*, 2010). The existence of a PH domain within PHLPP suggests that it may be regulated by PI3K, however, the PH domain of PHLPPα is dispensable for Akt S473 dephosphorylation (Gao *et al.*, 2005; Brognard and Newton, 2008).

1.3: Akt regulates cell survival, cell cycle and metabolism

Akt is a critical hub in the regulation of cell growth and cell fate. Tens of Akt substrates have been identified and over a dozen have been confirmed to directly regulate cell biology (Manning and Cantley, 2007; Robey and Hay, 2009). I will describe a few of the most significant Akt substrates in regulation of metabolism, survival, and growth.

Akt regulates metabolic functions at a physiological level, such as glucose homeostasis and at the cellular level, such as by promoting ATP production. Glucose homeostasis is the result of balancing glucose uptake and utilization by peripheral tissues with glucose production by the liver. A specific loss of glucose

homeostasis, referred to as insulin resistance, is defined as decreased clearance of plasma glucose in response to insulin. Akt2 plays a critical role in glucose homeostasis, since mice with a germline deletion of Akt2 develop insulin resistance (Cho *et al.*, 2001). Akt regulates insulin-stimulated glucose uptake by promoting translocation of the Glut4 glucose transporter to the membrane in skeletal muscle and adipose. Upon insulin treatment, Akt directly phosphorylates AS160 at T642, a GTPase-activating protein for the small GTPase Rab (Kane *et al.*, 2002). Phosphorylation of AS160 stimulates Rab-mediated translocation of the Glut4 receptor to the cell surface, where it facilitates glucose uptake.

Glucose utilization, another important component of glucose homeostasis, involves both catabolic and anabolic pathways that are regulated by Akt. Akt activation is associated with an elevation of oxygen consumption and increases in total cellular ATP content, while Akt deficiency has the opposite effect (Gottlob *et al.*, 2001; Nogueira *et al.*, 2008; Hahn-Windgassen *et al.*, 2005). Akt is thought to promote energy production by promoting glycolysis and the coupling of glycolysis to oxidative phosphorylation. The primary intermediaries of these Akt functions are the mitochondrial hexokinases, which catalyze the first committed step in glycolysis. Akt promotes the expression of hexokinase II and the mitochondrial association of hexokinases I and II (Robey and Hay, 2009). Akt may also activate phosphofructokinase-1, an important rate-limiting enzyme involved in glycolysis, by directly activating phosphofructokinase-2 (Deprez *et al.*, 1997; Robey and Hay, 2009). The ability of Akt to regulate hexokinase is thought

to contribute not only to ATP production, but to Akt-mediated cell survival (Gottlob *et al.*, 2001).

Insulin-stimulated glycogen synthesis is a primary mechanism of glucose utilization. Akt regulates glycogen synthesis through two targets, glycogen synthase kinase 3 (GSK3) and protein phosphatase 1 (PP1). Both GSK3 and PP1 regulate the activity of glycogen synthase (GS) by modulating the phosphorylation status of the enzyme. In muscle and liver cells, GSK3 phosphorylates and inhibits GS during starvation. Insulin stimulates Akt-mediated phosphorylation of GSK3, releasing GS from GSK3-mediated inhibition (Cross *et al.*, 1995; Roach, 2002). PP1 promotes glycogen synthase activity by dephosphorylating the enzyme, an activity that is promoted upon phosphorylation of PP1 by Akt (Parker *et al.*, 1983). Thus Akt-mediated inactivation of GSK3 and activation of PP1 promotes glucose utilization by promoting glycogen synthesis.

In addition to decreasing plasma glucose by increasing glucose uptake and utilization, Akt negatively regulates gluconeogenesis. During fasting, the forkhead box O (FOXO) transcription factors, in particular FOXO1, promote expression of gluconeogenic enzymes in hepatocytes (Zhang *et al.*, 2006). In the fed state, Akt activity inhibits gluconeogenesis through phosphorylation of FOXO1 on T24 and S256 (Rena *et al.,* 1999). Site-specific phosphorylation of FOXO1 facilitates 14-3-3 binding, which triggers nuclear export of FOXO1 away from gluconeogenic target genes (Tran *et al.*, 2003; Brunet *et al.*, 2002). FOXO proteins also suppress the expression of glycolytic genes involved in promoting a metabolic switch from carbohydrate to fatty acid catabolism during starvation

(Robey and Hay, 2009). Therefore, by phosphorylating and inactivating FOXO transcriptional activity, Akt promotes the expression of glycolytic genes favoring glucose utilization. To summarize the effects of Akt on cell metabolism, the activation of Akt by insulin or growth factors promotes the uptake, utilization, and storage of glucose, while repressing glucose production by the liver. The Akt-regulated uptake and catabolism of glucose contribute to growth-factor dependent cell survival (Gottlob *et al.*, 2001).

Akt also promotes cell survival by direct and indirect inhibition of pro-apoptotic proteins. Some of the important metabolic substrates of Akt, such as FOXO and GSK3, have additional roles in regulating pro-apoptotic B-cell CLL/lymphoma 2 (BCL-2) homology domain 3 (BH3)-only proteins. BH3-only proteins bind and inactivate antiapoptotic BCL-2 proteins. Akt-mediated phosphorylation of FOXO proteins results in reduced expression of BH3-only proteins Noxa and Bim (Dijkers *et al.*, 2000; Stahl *et al.*, 2002; Obexer *et al.*, 2007). The pro-apoptotic function of GSK3 occurs by inhibiting the BCL-2 family member myeloid cell leukemia sequence 1 (MCL-1), ultimately promoting caspase-9 processing. Phosphorylation of GSK3α and GSK3β by Akt results in its inhibition and stabilizes MCL-1 anti-apoptotic function (Cross *et al.*, 1995; Zhao *et al.*, 2007).

Two other key Akt substrates include BCL2-associated agonist of cell death (BAD) and the E3 ubiquitin-protein ligase MDM2/HDM2. The BH3-only protein BAD heterodimerizes with pro-survival BCL-2 or BCL-X_L proteins within the mitochondrial outer membrane. The formation of these BAD heterodimers

sensitizes the mitochondria to release cytochrome c and subsequently induce apoptosis. Following stimulation by PI3K-activating growth factors or cytokines, Akt phosphorylates BAD at S136, inhibiting its apoptotic function (Datta *et al.*, 2000). p53 induces apoptosis through transcription of BH3-only proteins Puma and Noxa (Villunger *et al.*, 2003). Akt opposes this activity by phosphorylating the p53 ubiquitin ligase, MDM2/HDM2, to promote p53 degradation (Mayo and Donner, 2001; Ogawara *et al.*, 2002).

Activation of Akt by growth factors not only promotes cell metabolism and survival, but also growth and proliferation. One of the primary Akt targets in promoting cell growth is the tuberous sclerosis complex (TSC). Through TSC, Akt activates the mTOR complex 1 (mTORC1), a primary regulator of cell biomass accumulation, through the regulation of translation and ribosome biogenesis. A key mTORC1 target in this pathway is S6K. The regulation and function of mTORC1 will be described in greater detail in sections 1.4-1.6. TSC is a heterodimeric complex composed of TSC1 and TSC2 with GTPase activating protein (GAP) capability toward the small GTPase Rheb (Ras homology enriched in brain). The GTP-bound form of Rheb is a potent activator of mTORC1. Akt phosphorylates TSC2, inactivating the TSC1-TSC2 complex and thus promoting the activity of Rheb and mTORC1. Several sites of Akt phosphorylation on TSC2 have been identified, including S939 and T1462. Growth factor-stimulated mTORC1 phosphorylation of S6K can be blocked by the overexpression of TSC2 S939A/T1462A mutants that no longer can be phosphorylated by Akt (Inoki *et al.*, 2002; Manning *et al.*, 2002). These

observations, together with the importance of mTORC1 in regulation of translation and growth, demonstrate the key role of TSC2 in Akt-mediated growth control downstream of PI3K.

PRAS40 is another negative regulator of mTORC1 that is inhibited by direct Akt phosphorylation at T246 (Kovacina *et al.*, 2003; Vander Haar *et al.*, 2007; Sancak *et al.*, 2007; Wang *et al.*, 2007a). In addition to this phosphorylation site, PRAS40 is also phosphorylated at S183 and S221 by mTORC1 itself, creating a positive feedback loop (Wang *et al.*, 2008; Fonseca *et al.*, 2007; Oshiro *et al.*, 2007). Moreover, it was recently reported that Akt-mediated phosphorylation of PRAS40 T246 promotes S183 phosphorylation by mTORC1. Thus, Akt may promote mTORC1 positive feedback under growth-promoting conditions via PRAS40.

Akt activity is intimately tied to mTOR. Akt activation is mediated by phosphorylation of S473 by mTORC2. Akt then phosphorylates key regulators of growth, survival and metabolism. One of the primary downstream mediators of Akt-growth promoting signals is mTORC1. In the following sections, I will describe the mTOR complexes and their relationship to Akt in greater detail.

1.4: mTOR exists in two distinct complexes: mTORC1 and mTORC2

The Ser/Thr protein kinase mTOR regulates cell growth, nutrient uptake and survival in response to multiple growth factors (LaPlante and Sabatini, 2009; Guertin and Sabatini, 2007; Corradetti and Guan, 2006; Bhaskar and Hay, 2007). The mTOR kinase is conserved in all eukaryotes. In *S. cerevisiae*, it is encoded

by two genes, *tor1* and *tor2*, while metazoans contain a single gene encoding TOR. TOR is the cellular target of the anti-proliferative and immunosuppressive bacterial macrolide rapamycin (Sabers *et al.*, 1995). Rapamycin is produced by a species of *Streptomyces hygroscopis* isolated from the soil of the island Rapa Nui (Easter Island), which gave rapamycin its name (Vezina *et al.*, 2005). In eukaryotic cells, rapamycin forms a complex with the FK506-binding protein of 12 kDa (FKBP12) and this complex directly inhibits TOR kinase activity (Heitman *et al.*, 1991; Sabatini *et al.*, 1994; Brown *et al.*, 1994). However, in the mid-1990s, studies into the functions of the *S. cerevisiae* TOR1 and TOR2 identified distinct rapamycin-sensitive and rapamycin-insensitive functions (Zheng *et al.*, 1995; Schmidt *et al.*, 1997). The rapamycin-sensitive role in growth is required for the G1-S phase transition, while the essential rapamycin-insensitive role regulates spatial control of growth, involving cell polarization and cytoskeleton organization. Two distinct, TOR-containing multiprotein complexes distinguishing these TOR functions were identified by the Hall lab in 2002 (Loewith *et al.*, 2002). In *S. cerevisiae*, TORC1 consists of one of the two TOR isoforms, TOR1 or TOR2, and the conserved proteins controller of growth 1 (KOG1) and lethal with Sec13 protein 8 (LST8). TORC2 contains TOR2 and the conserved proteins LST8, AVO1 and AVO3 (Jacinto and Lorberg, 2008; Inoki and Guan, 2006). TORC1 is sensitive to rapamycin and regulates cell growth, while TORC2 is insensitive to rapamycin and regulates the actin cytoskeleton (Jacinto and Lorberg, 2008).

Distinct TOR complex 1 (TORC1) and TOR complex 2 (TORC2) functions have been identified and avidly studied in model organisms including yeast, slime

mold, flies, worms, and mice, as well as human cell lines (Jacinto and Lorberg, 2008; Zoncu *et al.*, 2011). Studies in mammals have elucidated mTOR as a key regulator of the AGC family of kinases, including S6K, Akt, SGK and PKC, which together regulate essential cellular processes through substrate-selective mTOR complexes (Jacinto and Lorberg, 2008).

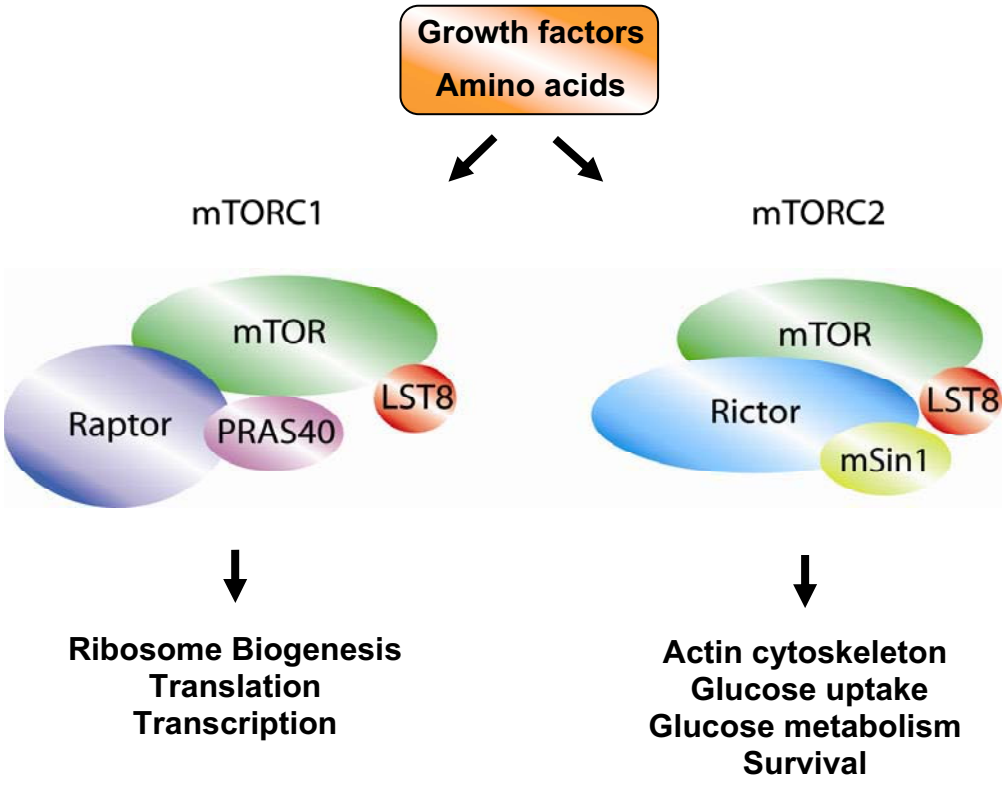
As in yeast, mTORC1 is inhibited by rapamycin (Sabers *et al.*, 1995; Kim *et al.*, 2002; Hara *et al.*, 2002). In the context of mTORC2, mTOR is insensitive to acute rapamycin treatment. However, in some cell types, prolonged rapamycin treatment can inhibit mTORC2 (Loewith *et al.*, 2002; Jacinto *et al.*, 2004; Sarbassov *et al.*, 2006). FKBP12-rapamycin does not bind mTORC2, but it can inhibit mTORC2 assembly after 24 hours of treatment, possibly by depleting the pool of available mTOR (Sarbassov *et al.*, 2006).

mTOR is a member of the phosphoinositide-3-kinase related protein kinase (PIKK) family, which also includes DNA-PK, ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3 related (ATR; Corradetti and Guan, 2006; Bhaskar and Hay, 2007). mTOR is a large protein, consisting of 2549 amino acids, with a molecular weight of approximately 289 kDa. The domain organization of mTOR consists of 20 HEAT repeats, a focal adhesion targeting (FAT) domain, an FKBP12-rapamycin binding (FRB) domain, a kinase domain and a focal adhesion targeting C-terminal domain (FATC). The HEAT repeats, comprised of two tandem α-helices, are putative protein-protein interaction domains that enable mTOR to form large multiprotein complexes. Gel filtration

chromatography estimates the size of mTOR complexes at 1.5-2 MDa (Kim *et al.*, 2002; Bader and Vogt, 2004).

mTORC1 and mTORC2 phosphorylate distinct substrates (LaPlante and Sabatini, 2009; Guertin and Sabatini, 2007; Corradetti and Guan, 2006). mTOR substrate selectivity is regulated by adaptor proteins contained within large multiprotein complexes. The composition of mTORC1 and mTORC2 is shown in Figure 3. mTORC1 consists of mTOR, the regulatory-associated protein of mTOR (Raptor; a KOG1 ortholog), LST8, and PRAS40 (Adami *et al.*, 2007; Vander haar *et al.*, 2007; Sancak *et al.*, 2007; Dunlop *et al.*, 2009; Jacinto and Lorberg, 2008; Inoki and Guan, 2006). Raptor determines mTORC1 specificity and is thought to direct substrate to the mTOR catalytic domain (Hara *et al.*, 2002; Kim *et al.*, 2002). Although the physiological function of LST8 is unclear, it has been shown to potently activate mTORC1 activity *in vitro*. However, LST8 is dispensable for mTORC1-mediated phosphorylation of substrates in embryonic development (Kim *et al.*, 2003; Guertin *et al.*, 2006). As described in the previous section, PRAS40 interaction inhibits mTORC1 and is regulated by Akt and mTORC1 itself. A second inhibitory protein, DEP-domain containing mTOR-interacting protein (Deptor), has recently been identified that inhibits both mTORC1 and mTORC2 activity (Peterson *et al.*, 2009). Interestingly, mTORC1 and mTORC2 feedback to inhibit Deptor expression (Peterson *et al.*, 2009).

Figure 3: mTOR Complex 1 and mTOR Complex 2. The two mTOR Complexes (mTORCs) are both activated by growth factors and amino acids, but regulated distinct cellular processes. Each mTOR Complex (mTORC) contains two common subunits and two unique subunits. mTORC1 consists of mTOR, Raptor, PRAS40 and LST8. mTORC2 consists of mTOR, Rictor, mSin1 and LST8.


Growth factors
Amino acids
mTORC1
mTORC2
mTOR
Raptor
PRAS40
LST8
mTOR
Rictor
LST8
mSin1
Ribosome Biogenesis
Translation
Transcription
Actin cytoskeleton
Glucose uptake
Glucose metabolism
Survival

The subunit composition of mTORC2 (introduced in section 1.2) consists of mTOR, LST8, Rictor (an AVO3 ortholog), and mSin1 (an AVO1 ortholog; Jacinto and Lorberg, 2008; Inoki and Guan, 2006). Although LST8 is a component of both mTOR complexes, it is essential to mTORC2 but not mTORC1 function (Guertin *et al.*, 2006). mSin1 functions as a scaffold protein that recruits substrates to the catalytic domain of mTOR (Jacinto *et al.*, 2006). Rictor is required for both mSin1 stability and mTORC2 activity, and together, Rictor and mSin1 are thought to form the structural foundation of mTORC2 (Frias *et al.*, 2006; Jacinto *et al.*, 2006; Yang *et al.*, 2006). Another Rictor-interacting protein has been identified, called the protein observed with Rictor-1 (Protor-1), however, the importance of this interaction has not been fully elucidated (Thedieck *et al.*, 2007; Woo *et al.*, 2007; Pearce *et al.*, 2007).

1.5: mTORC1 and mTORC2 phosphorylate distinct substrates

mTORC1 regulates translation, lipid synthesis, mitochondrial metabolism and biogenesis, and autophagy. Positive regulation of anabolic processes by mTORC1 promotes cell growth and proliferation. The primary mTORC1 targets in regulating protein synthesis are S6K and eukaryotic initiation factor 4E (eIF4E) binding protein 1 (4EBP1). S6K is activated by mTORC1-mediated phosphorylation at T389, which liberates it from the eukaryotic initiation factor 3 (eIF3) translation initiation complex. S6K then phosphorylates the ribosomal protein S6, stimulating translation of mRNAs (Ruvinsky *et al.*, 2005). mTORC1 stimulates translation of mRNAs with 7-methyl guanine caps though

phosphorylation and inhibition of 4EBP1. 4EBP1 is an inhibitor of the cap-dependent translation initiation factor eIF4E. mTORC1 positively regulates translation by increasing cell mass and by upregulating the synthesis of growth-promoting proteins, including c-Myc and cyclin D1 (Mamane *et al.*, 2004).

The first identified function of mTORC2 was in the regulation of actin cytoskeleton organization (Jacinto *et al.*, 2004; Sarbassov *et al.*, 2004). Knockdown of mTORC2 components alters cell morphology and adhesion, in part by perturbing actin polymerization (Jacinto *et al.*, 2004; Sarbassov *et al.*, 2004). These cytoskeletal defects in mTOR-, Rictor-, or LST8-depleted cells were found to be mediated by reduced phosphorylation of PKCα and paxillin and reduced GTP-loading of the small GTPases RhoA and Rac1 (Jacinto *et al.*, 2004; Sarbassov *et al.,* 2004). Expression of active Rac rescued the cytoskeletal defects caused by TORC2 depletion, indicating that Rac mediates TORC2-dependent cytoskeletal organization (Jacinto *et al.*, 2004). Studies have confirmed the *in vivo* regulation PKCα by mTORC2, since loss of any one of the components, Rictor, Sin1 or LST8 decreases phosphorylation of PKCα within the conserved HM site (Ikenoue *et al.*, 2008a). PKC also possesses a turn motif (TM), which is phosphorylated during protein maturation and important for stability of the protein. mTORC2 is required for phosphorylation at the TM of both typical PKCα and atypical PKCε .(Facchinetti *et al.*, 2008; Ikenoue *et al.*, 2008a). However, it is not known whether mTORC2 phosphorylates PKCs at the HM or TM directly or whether these events are mediated by another kinase.

mTORC2 and mTORC1 have been shown to phosphorylate the Akt paralog SGK on the conserved HM site, S422 (Garcia-Martinez and Alessi, 2008; Hong *et al.*, 2008). One group demonstrated that Rictor, but not Raptor, knockdown reduced S422 phosphorylation in mTOR-overexpressing cells (Hong *et al.*, 2008). This group went on to show that immunoprecipitated mTOR phosphorylated SGK *in vitro* in a manner that was sensitive to acute rapamycin treatment, further implicating mTORC1 (Hong *et al.*, 2008). In contrast, another group observed that in mouse embryonic fibroblasts lacking the mTORC2 components Rictor, Sin1 or LST8, SGK was not phosphorylated at S422 (Garcia-Martinez and Alessi). Furthermore, they show that in *in vitro* immunokinase assays, mTORC2 phosphorylated SGK S422 and that mTORC1 phosphorylated S6K, but not SGK S422 (Garcia-Martinez and Alessi, 2008). These studies suggest that either mTORC1 or mTORC2 can phosphorylate SGK S422, but it is not yet clear which complex predominates physiological SGK activation.

The best-characterized mTORC2 substrate is S473 of Akt (Sarbassov *et al.*, 2005; Hresko and Mueckler, 2005; Shiota *et al.*, 2006). Genetic disruption of either Rictor or mSin1 has demonstrated the importance of mTORC2 for phosphorylation of Akt at S473. In many processes, including embryonic development, insulin signaling, growth factor signaling and differentiation, mTORC2 is essential for Akt S473 phosphorylation (Hresko and Mueckler, 2005; Shiota *et al.*, 2006; Frias *et al.*, 2006; Jacinto *et al.*, 2006; Guertin *et al.*, 2006; Kumar *et al.*, 2008; Shu *et al.*, 2009; Kumar *et al.*, 2010). *In vitro* biochemical studies have demonstrated that mTORC2 phosphorylation of Akt S473 is

responsive to growth factor stimuli (Sarbassov at al, 2005; Yang *et al.*, 2006). Additionally, the mTORC2 component mSin1 has been shown to co-immunoprecipitate Akt (Jacinto *et al.*, 2006). Collectively, these studies demonstrate the importance of mTORC2 for Akt phosphorylation at S473 in response to growth factor stimuli.

mTORC2 also phosphorylates Akt at T450 within the turn motif during Akt translation to prevent its cotranslational ubiquitination (Oh *et al.*, 2010).

Although S473 phosphorylation of Akt increases kinase activity, surprisingly, only a subset of Akt substrates involved in metabolism and cell survival require mTORC2. These studies utilized cells in which mTORC2 components, Rictor or Sin1, were either genetically knocked out or knocked down by small-interfering RNAs, resulting in a loss of Akt phosphorylation at S473. Rictor and Sin1 are required for Akt-mediated phosphorylation of FOXO1 and FOXO3a at T24/T32 (Guertin *et al.*, 2006; Jacinto *et al.*, 2006; Kumar *et al.*, 2008). Loss of mTORC2 activity has also been shown to reduce AS160 phosphorylation in muscle and fat cells, suggesting that Akt phosphorylation at S473 is important for insulin-stimulated glucose uptake (Kumar *et al.*, 2008; Kumar *et al.*, 2010). On the other hand, mTORC2 components that control Akt phosphorylation at S473 are dispensable for Akt-mediated phosphorylation of TSC2 and GSK3 (Guertin *et al.*, 2006; Jacinto *et al.*, 2006; Kumar *et al.*, 2008). Significantly, this rules out the possibility that mTORC2 positively regulates mTORC1 through TSC2. Muscle cells lacking Rictor demonstrated increased glycogen synthase activity, an effect that seemed to be mediated by an increase

in PP1 activity rather than by GSK3 inactivation (Kumar *et al.*, 2008). In investigations of the physiological role of mTORC2, adult mice with muscle-specific Rictor deletion displayed defects in glucose tolerance (Kumar *et al.*, 2008). Adipocyte-specific Rictor deletion also perturbs glucose tolerance and results in insulin-resistant liver and skeletal muscle (Kumar *et al.*, 2010). Collectively, these observations suggest that mTORC2 directs Akt activity toward activation of substrates involved in metabolism and survival, suggesting a specialized function for S473-phosphorylated Akt. Many Akt substrates have not yet been tested for the effect of mTORC2 ablation and therefore it will be important to examine all known Akt substrates. Tissue-specific knockouts will continue to be valuable to understanding the physiological importance of mTORC2, since Rictor- and Sin-null mouse embryos are not viable.

The mechanisms by which Akt S473 phosphorylation influences only certain substrates are not understood. However, it has been proposed that S473 phosphorylation may provide a mechanism by which Akt switches substrate preference. An alternative hypothesis is that some substrates require the increased Akt catalytic activity provided by T308 and S473 phosphorylation (Jacinto *et al.*, 2006; Manning and Cantley, 2007).

Based on the investigation of the Akt S473 phosphatase, PHLPP, one should use caution when interpreting mTORC2 ablation studies that investigate the biological function of Akt S473 phosphorylation. PHLPP knockdown results in increased phosphorylation of many Akt substrates, including GSK3 and TSC2 (Brognard *et al.*, 2007). Moreover, both isoforms PHLPP1 and PHLPP2

demonstrate substrate specificity for different Akt family members. For example, knockdown of PHLPP1 increased S473 phosphorylation of Akt2 (but not Akt1), increasing phosphorylation of MDM2/HDM2 and GSK3α (Brognard *et al.*, 2007). In contrast, PHLPP2 selectively regulates Akt3 to control the cyclin-dependent kinase inhibitor p27 (Brognard *et al.*, 2007). These results suggest that Akt isoforms can be specifically targeted to different substrates through regulation of S473 phosphorylation and dephosphorylation. Therefore, it will be important to examine the role of mTORC2-mediated phosphorylation of different Akt family members before concluding that mTORC2 regulates only a confined number of Akt substrates.

1.6: mTOR complexes are regulated by PI3K and interacting proteins

The influx of nutrients into mammalian cells is not only controlled by food intake, but physiologically regulated by insulin- and growth factor-mediated regulation of nutrient uptake. mTOR integrates signals from both growth factors and nutrients such that it is activated when energy sources are abundant and inhibited by scarcity. The existence of the two distinct mTOR complexes has raised speculation as to whether certain nutrient conditions would alter the balance of activity between mTORC1 and mTORC2. In this section, I will briefly review the regulation of mTORC1, mTORC2 and the interplay between the two complexes.

mTORC1 is activated by growth factors and amino acids and inhibited by ATP depletion. Experimental evidence demonstrates that Akt is a growth factor-stimulated activator of mTORC1. The ability of Akt to activate mTORC1 was

demonstrated by overexpression of a constitutively active Akt mutant that increased the phosphorylation of mTORC1 targets S6K and 4EBP1 (Gingras *et al.*, 1998; Kohn *et al.*, 1998). Conversely, S6K and 4EBP1 phosphorylation are inhibited by overexpression of a kinase-dead Akt (Gingras *et al.*, 1998).

As described in section 1.3, TSC2 is phosphorylated by Akt, providing a primary mechanism for mTORC1 activation downstream of PI3K (Manning *et al.*, 2002; Inoki *et al.*, 2002; Potter *et al.*, 2002). Akt-mediated phosphorylation of TSC2 at S939 and T1462 inhibits the Rheb-GAP activity of TSC1-TSC2 (Manning *et al.*, 2002; Potter *et al.*, 2002). The reduction in TSC1-TSC2 activity favors the active GTP-bound form of Rheb, which activates mTOR. Rheb has been shown to directly interact with mTORC1 and stimulate its kinase activity *in vitro*, although the precise mechanism of this activation is unknown (Long *et al.*, 2005a).

In addition to its activation downstream of growth factors, mTORC1 initiates a negative feedback loop on PI3K activation by insulin- and IGFI-receptors. The mTORC1 substrate S6K phosphorylates IRS-1 on S302 to inhibit PI3K activity (Harrington *et al.*, 2005). This negative feedback loop is prominent when mTORC1 is hyperactivated, such as in TSC1/2 mutant cells. In insulin resistance, continual oversupply of nutrients chronically activates mTORC1 until the S6K-IRS negative feedback mechanism represses PI3K activity. The resulting PI3K inhibition restricts Akt signaling, including Akt-mediated glucose uptake and is thought to contribute to insulin resistance in peripheral tissues

(Harrington *et al.*, 2005). Thus, mTORC1 is activated by insulin/IGF1 signaling and a key regulator of glucose homeostasis.

mTORC1 activity is significantly inhibited during times of extreme caloric restriction associated with starvation. Inhibition of mTORC1 reduces its regulated anabolic processes and can eventually lead to growth arrest and autophagy. Autophagy is the process by which, under extreme caloric restriction, cells degrade organelles and catabolize their contents to generate energy. Depletion of cellular energy is associated with a rise in the AMP/ATP ratio, which activates AMP-activated protein kinase (AMPK). AMPK inhibits mTORC1 by two distinct mechanisms. AMPK phosphorylates TSC2 on T1227 and S1345, which are distinct from the Akt phosphorylation sites (Inoki *et al.*, 2003). Phosphorylation of TSC2 by AMPK increases TSC1-TSC2 Rheb-GAP activity, thus reducing the GTP-bound form of Rheb, leading to mTORC1 inhibition (Inoki *et al.*, 2003). The second mechanism of inhibition involves AMPK-mediated phosphorylation of Raptor to abolish mTORC1 activity (Gwinn *et al.*, 2008). Collectively, these studies demonstrate the inhibition of mTORC1 by AMPK when cellular energy is depleted.

Withdrawal of amino acids inhibits mTORC1 activity, even in the presence of growth factors (Laplante and Sabatini, 2009). Depletion of amino acids inhibits the interaction between mTOR and Rheb through a mechanism independent of TSC1/2 (Long *et al.*, 2005b; Nobukuni *et al.*, 2005). Conversely, the availability of amino acids stimulates mTORC1 activity through molecular mechanisms that were recently elucidated. Amino acids initiate translocation of mTORC1 to

lysosomal membranes, where it is activated by colocalization with Rheb (Sancak *et al.*, 2010; Abraham, 2010). The localization of mTORC1 to lysosomal membranes requires the Rag GTPases and a trimeric scaffolding complex called the Ragulator (Sancak *et al.*, 2008; Kim *et al.*, 2008; Sancak *et al.*, 2010). The amino acid-regulated step involves GTP-GDP exchange of the Rag proteins. Targeting mTORC1 to lysosomal membranes is sufficient for its activation by Rheb, which is independently localized to this endomembrane compartment (Sancak *et al.*, 2010). Although this mechanism elucidates the amino acid requirement of mTORC1, active mTORC1 is not reported to be restricted to the lysosomal membrane. Rather, mTORC1 also localizes to other compartments, including the periphery of the ER and Golgi, mitochondria and the nucleus (Drenan *et al.*, 2003; Desai *et al.*, 2002; Zhang *et al.*, 2002). Therefore, in response to amino acid signaling, mTORC1 activation involves multiple translocation steps, first to the lysosome and then to substrates, or alternatively to the Rag-Ragulator complex to allow targeting of mTORC1 to Rheb.

mTORC1 is negatively regulated by Deptor and PRAS40. PRAS40-mediated inhibition is opposed by Akt and mTORC1 (as described in greater detail in section 1.4). Deptor inhibits mTOR kinase activity by binding N-terminal to the mTOR kinase domain (Peterson *et al.*, 2009). Deptor expression is negatively regulated by mTORC1 and mTORC2 (Peterson *et al.*, 2009), however, little is known about post-translational modifications that control Deptor-mTOR interaction.

In contrast to what is known for mTORC1, the regulation of mTORC2 is poorly understood. It is thought to be activated in a PI3K-dependent manner, requiring TSC1/2 and the availability of free amino acids. Similar, to mTORC1, mTORC2 is negatively regulated by its interaction with Deptor.

The primary target of mTORC2, Akt S473, is phosphorylated upon growth factor stimulation in a manner sensitive to PI3K-inhibition and to the depletion of Rictor or Sin1 (Sarbassov *et al.*, 2005 Hresko and Mueckler, 2005; Shiota *et al.*, 2006; Frias *et al.*, 2006; Jacinto *et al.*, 2006; Guertin *et al.*, 2006; Kumar *et al.*, 2008; Shu *et al.*, 2009; Kumar *et al.*, 2010). However, it is not clear whether PI3K is required only for recruitment of Akt to the membrane via its PH domain or whether PI3K is also required for stimulation of mTORC2 activity. Artificial targeting of Akt to the membrane by engineering a myristylated-Akt fusion is sufficient for Akt to be phosphorylated at T308 and S473 (Brazil and Hemmings, 2001), indicating that once Akt is localized to the membrane both mTORC2 and PDK1 are able to effectively phosphorylate Akt. However, additional studies examining *in vitro* kinase activity demonstrate that serum or insulin treatment stimulates mTORC2 activity (Sarbassov *et al.*, 2005; Frias *et al.*, 2006).

Unlike mTORC1, mTORC2 is not activated by Rheb, therefore its PI3K activation must involve distinct mechanisms. It has been proposed that mTORC2 may be recruited to the membrane by PIP_3 through its subunit mSin1 (Laplante and Sabatini, 2009). mSin1 possesses a putative PH domain that binds lipids (Schroder *et al.*, 2007). In support of this hypothesis, mSin1 isoforms produced by alternative splicing form distinct mTORC2 complexes that vary in

response to insulin (Frias *et al.*, 2006). The mSin1.5 isoform, which lacks the PH domain, has been shown to form mTORC2 complexes that are unique in that they are not responsive to serum or insulin stimulation (Frias *et al.*, 2006). Therefore, it is plausible that PH domain-containing mSin1.1 and mSin1.2 provide a mechanism for direct activation of mTORC2 by PIP_3.

Although mTORC2 is not regulated by Rheb, TSC1-TSC2 is required for PI3K-dependent activation of mTORC2 (Huang *et al.*, 2008). The TSC1-TSC2 complex was found to specifically interact with Rictor-containing mTORC2 and not Raptor-containing mTORC1. The mechanism of TSC1-TSC2 activation of mTORC2 is unknown, but is independent of its GAP activity (Huang *et al.*, 2008).

An alternative mechanism for mTORC2 activation is via phosphatidic acid (PA). PA is a phospholipid second messenger produced by phospholipase D (PLD), a growth factor responsive enzyme. Inhibition of PLD has been shown to inhibit insulin-stimulated mTORC1 and mTORC2 activity (Toschi *et al.*, 2009). PLD was specifically required for the interaction between mTOR and Rictor (in mTORC2) and mTOR and Raptor (in mTORC1; Toschi *et al.*, 2009). Future work is necessary to determine the contribution of PLD to growth factor-mediated mTORC2 activation.

A recent study found that mTORC2 is activated by amino acids through PI3K (Tato *et al.*, 2010). The *in vitro* kinase activity of mTORC2 isolated from cells was stimulated by treatment of the cells with amino acids (Tato *et al.*, 2010). Although the mechanism of this activation is not known, amino acids most likely

activate mTORC2 through a distinct mechanism from mTORC1 activation, since mTORC2 is not regulated by Rheb.

1.7: mTOR complexes are regulated by post-translational modifications

With the emergence of the histone and multisite (multiple site) protein code hypotheses, the study of post-translational modification of important proteins has expanded (Gardner *et al.*, 2011; Yang, 2005). Many non-histone proteins, such as p53, FOXO and c-myc, are subject to multiple modifications that have been found to act sequentially or combinatorially to modulate protein function (Yang and Seto, 2008b). Diverse multisite modifications, including methylation, acetylation, ubiquitination, sumoylation and phosphorylation, have been found on histone tails and are now recognized to play essential functions in transcriptional activation and repression (Gardner *et al.*, 2011). It has since become evident that many important non-histone proteins are also regulated by multi-site modifications (Yang, 2005; Yang and Seto, 2008b).

While the regulation of mTOR by PI3K and nutrients has been the subject of extensive research, far less is known about the regulation of mTOR complexes by direct post-translational modification. Unlike Akt, mTOR activation does not require phosphorylation of the T loop connecting the N and C lobes of the kinase domain. As with other atypical protein kinases, this segment contains a Glu residue (E2363) that mimics the phosphorylated state (Jacinto, 2008). Proteomics screens have identified 20 phosphorylation sites within the mTOR sequence (Daub *et al.*, 2008; Dephoure *et al.*, 2008; Opperman *et al.*, 2009),

however, only four of these have been characterized. Two of the phosphorylation sites, T2446 and S2448, are located within a repressor domain (Sekulic *et al.*, 2000). Both sites are phosphorylated by S6K (Chiang and Abraham, 2005; Holz and Blenis, 2005) and T2446 can be phosphorylated by AMPK (Cheng *et al.*, 2004). Deletion of the repressor domain increases mTORC1 activity (Sekulic *et al.*, 2000), but the physiological importance of the phosphorylation sites is not known. A third phosphorylation site, S2481, is autophosphorylated and thus correlates with mTOR activity (Peterson, *et al.*, 2000; Soliman *et al.*, 2010). The most recently identified site, S1261, has been shown to positively regulate mTORC1-mediated phosphorylation of S6K and 4EBP1 in an amino acid and PI3K-dependent manner (Acosta-Jaquez *et al.*, 2009).

mTORC1 activity can be regulated by post-translational modification of its adaptor protein, Raptor. Under energy stress AMPK phosphorylates Raptor on S722 and S792, inhibiting mTORC1, resulting in cell cycle arrest (Gwinn *et al.*, 2008). Additionally, multisite phosphorylation of Raptor (C-terminal to the AMPK sites) was shown to positively regulate mTORC1 kinase activity (Foster *et al.*, 2010). These examples demonstrate that post-translational modifications involving phosphorylation of the mTORC1 adaptor protein, Raptor, can modulate the kinase activity of mTOR.

The mTORC2 adaptor, Rictor, is also post-translationally modified. Rictor is phosphorylated on T1135 by S6K1, which promotes association with 14-3-3ε (Julien *et al.*, 2009; Dibble *et al.*, 2009; Treins *et al.*, 2009). Expression of a

Rictor T1135A mutant that can no longer be phosphorylated reduces cellular levels of Akt phosphorylated at S473, however, the mechanism of mTORC2 inhibition is unknown. Several groups have shown that phosphorylation of Rictor at T1135 does not affect mTORC2 kinase activity, localization or assembly (Julien *et al.*, 2009; Dibble *et al.*, 2009, Treins *et al.*, 2009, Boulbes *et al.*, 2010).

Proteomic screens have identified many other Rictor phosphorylation sites that are not yet characterized, as well as a single acetylated lysine, K582 (Choudhary *et al.*, 2009). Lysine acetylation is a dynamic modification tied to cellular metabolism, which makes it an intriguing candidate for regulation of mTORC2.

1.8: Acetylation is a dynamic post-translational modification

Lysine acetylation is a dynamic, reversible, and functionally important regulatory post-translational modification analogous to phosphorylation (Kouzarides, 2000; Spange *et al.*, 2009). First discovered as a histone modification over four decades ago (Gershey *et al.*, 1968), acetylation has since been found to regulate numerous non-histone proteins in the nucleus, cytoplasm and mitochondria (Spange *et al.*, 2009; Yang and Seto, 2008b; Norvell and McMahon, 2010). The effects of acetylation on the function of its substrates include modulation of protein-protein interactions, protein-DNA interactions, subcellular localization, and protein stability (Glozak *et al.*, 2005; Yang, 2005). Metabolic pathways not only regulate acetylation of target proteins, but many of the downstream

substrates are key components of nutrient sensing and signaling pathways (Bao and Sack, 2010; Wellen and Thompson, 2010; Norvell and McMahon, 2010).

Acetylation occurs through the activity of acetyltransferases, commonly called histone acetyltransferases (HATs). HATs catalyze the addition of an acetyl group at the ε-amino position of lysine on polypeptides. Lysine acetylation is reversed by the action of histone deacetylases (HDACs), which remove the acetyl moiety, regenerating unmodified lysine on the protein target. Since acetylation of lysine residues neutralizes the positive charge of the lysine ε-amino group, acetylation can significantly alter intra- or intermolecular interactions. For example, acetylation of the transcription factor Stat1 on K410 and K413 promotes binding to the p65 subunit of NF-κB, resulting in decreased nuclear localization of NF-κB and subsequent loss of transcription (Krämer *et al.*, 2006). Conversely, acetylation can inhibit protein-protein interactions. For example, acetylation of the pro-survival protein Ku70 disrupts its interaction with Bax, releasing Bax to the mitochondria to perform its pro-apoptotic function (Cohen *et al.*, 2004). In addition to altering protein-protein interactions, acetylated lysines act as recognition modules for bromodomain-containing acetyltransferases or key modulating proteins. Like phosphorylation, acetylation can be regulated by physiological and environmental stimuli, such as growth factors, cytokines, cellular stress and metabolic status (Cohen *et al.*, 2004; Nusinzon *et al.*, 2005; Wellen *et al.*, 2009; Huang and Chen, 2005).

Reversible acetylation is tied to cellular metabolism both by the regulatory nature of its substrates and by the mechanism of acetylation and deacetylation

(Bao and Sack, 2010; Wellen and Thompson, 2010). In acetylation and deacetylation, metabolites that are utilized in catabolic or anabolic reactions can have additional roles as metabolite signaling molecules. For example, acetyl CoA is produced in the mitochondria from pyruvate as part of the TCA cycle. It is then converted to citrate, which either continues on the TCA cycle or is transported into the cytosol. In the cytosol, the ATP citrate lyase (ACL) converts citrate back to acetyl CoA. The pool of acetyl CoA generated by ACL is used as a substrate in the synthesis of lipids and as the acetyl group donor for HATs in the nucleus and cytosol (Wellen and Thompson, 2010; Bao and Sack, 2010). Therefore, the synthesis of acetyl CoA connects HAT enzymatic activity with cell metabolism. In support of this hypothesis, ACL was recently shown to be required for growth factor-induced increases in global histone acetylation (Wellen *et al.*, 2009). Gene expression, including transcription of the glucose transporter Glut4, was positively regulated by the availability of glucose in an ACL-dependent manner (Wellen *et al.*, 2009). Collectively, these results suggest that glucose and growth factors positively regulate histone acetylation and gene transcription through the availability of acetyl-CoA.

HATs are a diverse group of proteins that often function in large protein complexes (Yang and Seto, 2007). They are conserved from yeast to human, with over 30 HATs identified in mammals (Spange *et al.*, 2009). HATs are classified into three major families: 1) GNAT (general control non-derepressible 5 [Gcn5]-related N-acetyltransferases); 2) MYST (monocytic leukemia zinc finger protein [MOZ], Ybf2/Sas3, something about silencing 2 [Sas2] and HIV Tat-

interactive protein of 60 kDa [TIP60]); and 3) p300 (adenoviral E1A-associated protein of 300 kDa)/CBP (CREB-binding protein) (Roth *et al.*, 2001; Yang and Seto, 2007). Of these, p300/CBP has the broadest range of substrates, acetylating many non-histone proteins in addition to histones (Kimura *et al.*, 2005). p300 is activated by autoacetylation of 12 sites which are clustered in a trypsin sensitive loop (Thompson *et al.*, 2004). Interestingly, p300 catalytic activity is also stimulated by phosphorylation S1834 by Akt resulting in increased histone acetylation and gene expression (Huang and Chen, 2005; Liu *et al.*, 2006). These studies further support the hypothesis that PI3K/Akt activation in the fed state positively regulates acetylation.

Reversal of lysine acetylation is carried out by histone deacetylases (HDACs). Mammals have two families of HDACs with distinct catalytic mechanisms: 1) Rpd3/Hda1, which is subdivided into class I, II, and IV HDACs, and 2) the Sirtuins, which comprise the Class III HDACs (Yang and Seto, 2008a; Bao and Sack, 2010). Class I/II/IV HDACs use zinc as a cofactor in hydrolysis of acetyl-lysine, while Sirtuins utilize nicotinic adenine dinucleotide (NAD^+) (Yang and Seto, 2008a). The Class I HDACs, orthologs of the yeast HDAC Rpd3, consist of HDAC1, HDAC2, HDAC3 and HDAC8. HDAC4-7 and HDAC10 comprise Class II HDACs and are yeast Hda1 orthologs. HDAC11 comprises Class IV, as it has equal homology to Rpd3 and Hda1.

The yeast silencing information regulator 2 (Sir2) is the founding member of the Sirtuin family of HDACs (Imai, 2000). Sir2 regulates chromatin silencing at several loci, including telomeres. Though it is the subject of debate, Sir2 has

been implicated in the regulation of aging, particularly in lifespan extension induced by calorie restriction (Guarente and Picard, 2005). Similarly, the mammalian Sirtuins have been shown to protect cells from stress and extend lifespan of certain cell types, including neurons (Haigis and Guarente, 2006). There are seven Sirtuins in mammals, SIRT1-7, and all seven Sirtuins except SIRT4 demonstrate deacetylase activity (Bao and Sack, 2010). Two of the Sirtuins, namely SIRT4 and SIRT6, demonstrate ADP-ribosyltransferase activity. SIRT1 localizes predominantly in the nucleus, though it also translocates to the cytosol (North and Verdin, 2004; Tanno *et al.*, 2007; Li *et al.*, 2008). SIRT6 and SIRT7 localize to the nucleus, SIRT2 is predominantly cytosolic and SIRT3 and SIRT5 localize to the mitochondria (North and Verdin, 2004; Bao and Sack, 2010).

Sirtuin deacetylase activity is uniquely coupled to cell metabolism. In Sirtuin-mediated protein deacetylation, NAD^+ is consumed, producing the unacetylated protein, O-acetyl ADP ribose and nicotinamide (NAM; Bao and Sack, 2010; North and Verdin, 2004). The canonical role of NAD^+ in the cell is as an oxidized electron carrier produced in oxidative phosphorylation, thus its availability depends on the metabolic status of the cell. Sirtuins are activated in response to nutrient depletion due to a rise in the intracellular levels of NAD^+ and feedback inhibited by the noncompetitive inhibitor NAM (Lin *et al.*, 2000; Anderson *et al.*, 2002; Dioum *et al.*, 2009).

Sirtuins are known to regulate important substrates involved in metabolism and cell survival. SIRT1 deacetylates peroxisome proliferator-activated receptor-

gamma (PPARγ) co-activator 1 (PGC-1α), increasing its activity. SIRT1-mediated deacetylation PGC1α promotes gluconeogenesis in the liver, antagonizing insulin signaling (Rodgers *et al.*, 2005). SIRT1 is also implicated in the regulation of cholesterol, fatty acid and glucose metabolism in the muscle through the action of PGC1α (Nemoto *et al.*, 2005; Gerhart-Hines *et al.*, 2007; Rodgers and Puigserver, 2007). SIRT1 also directly deacetylates PPARγ, repressing its activity and promoting fat mobilization in white adipocytes (Picard *et al.*, 2004). Collectively, these studies support a role for Sirtuins in regulating key metabolic processes important for weight control and may oppose insulin signaling.

Previous work has shown that signaling proteins in the insulin/IGF1 pathway upstream and downstream of Akt are acetylated. For example, FOXO transcription factors are targets of both SIRT1 and SIRT2 (Calnan and Brunet, 2008; Wang *et al.*, 2007b). Oxidative stress promotes SIRT1-dependent deacetylation of FOXO3, inhibiting transcription of pro-apoptotic genes and promoting transcription of genes involved in stress response and cell cycle arrest (Brunet *et al.*, 2004). In this instance, SIRT1 and IGF1 signaling may work in concert to promote cell survival. Alternatively, SIRT2 signaling to FOXO promotes apoptosis through increased Bim expression under severe oxidative stress (Wang *et al.*, 2007b). Thus, in response to the degree of stress, SIRT1 and SIRT2 can either promote or protect cells from apoptosis by controlling FOXO gene expression.

Upstream of Akt, the IRS-1/2 proteins and PTEN are acetylated substrates. IRS-1 deacetylation by HDAC2 has been implicated in compromised insulin signaling (Kaiser and James, 2004). In contrast, IRS-2 deacetylation by SIRT1 has been reported to promote insulin signaling (Li *et al.*, 2008). PTEN is acetylated by PCAF at K125 and K128 and deacetylated at these sites by HDAC1 and HDAC2 (Okumura *et al.*, 2006). PCAF-mediated acetylation of PTEN inhibits its enzymatic activity. Interestingly, growth factors are required for PCAF to acetylate PTEN, creating a positive feedback loop in the PI3K/Akt pathway. Additionally, PTEN is acetylated at K401 within the PDZ motif, a protein-protein interaction domain, however, the physiological significance of this modification is not known (Ikenoue *et al.*, 2008b). PTEN K401 is primarily acetylated by CBP and deacetylated by SIRT1 (Ikenoue *et al.*, 2008b).

Collectively, these studies highlight the ability of acetylation to promote (as well as inhibit) the insulin/IGF1 signaling pathway to FOXO. Like phosphorylation, dynamic regulation of key acetylated targets plays a nuanced role in growth factor signaling depending on substrate- and site-specific modification in response to diverse cellular conditions. Since mTORC2 is a key growth factor-responsive regulator of Akt, we hypothesized that the activity of this kinase complex would be regulated by dynamic acetylation.

To summarize this section, acetyl CoA and NAD^+ are metabolite signaling molecules that couple nutrient availability to metabolic and anti-apoptotic signaling pathways through dynamic protein acetylation and deacetylation. Since mTORC2 regulates glucose metabolism through Akt, my original hypothesis was

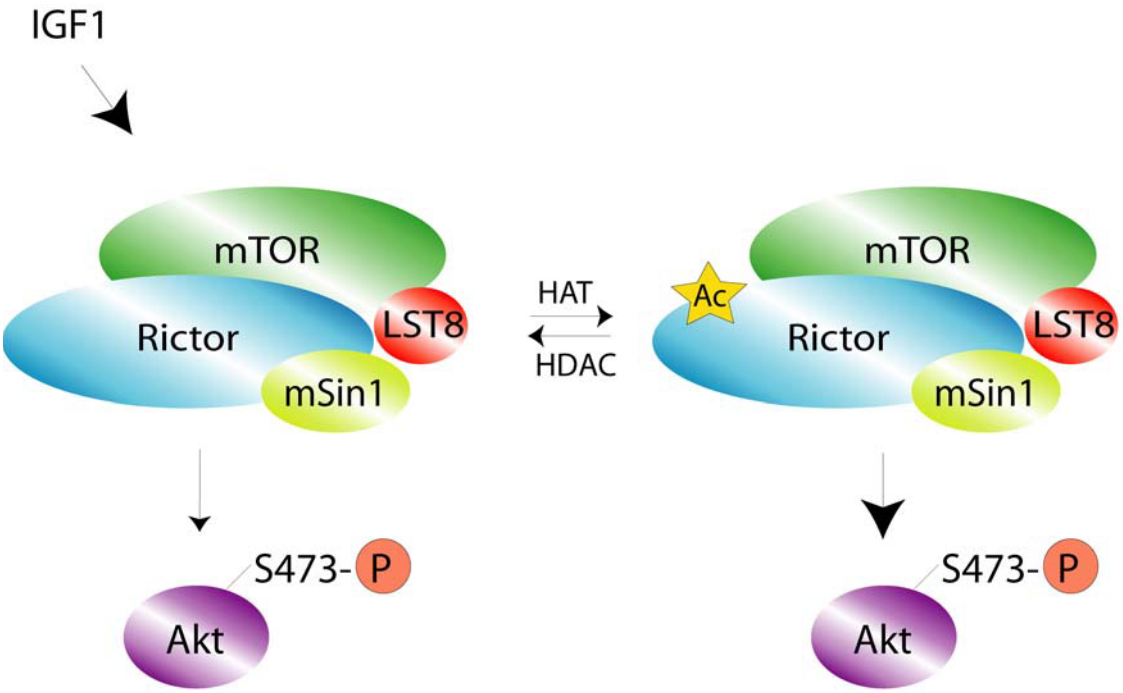
that mTORC2 activity is positively regulated by acetylation and inhibited by Sirtuin-mediated deacetylation, as shown in Figure 4. The evidence collected in investigation of this hypothesis comprises the contents of Chapter 2.

1.9: O-GlcNAcylation is a glucose-responsive post-translational modification

The importance of mTOR and Akt in regulation of cell metabolism led to further review of the literature for dynamic, nutrient-sensitive post-translational modifications other than acetylation. This search yielded a sugar modification of Ser/Thr which is specifically responsive to glycolytic flux, O-linked N-acetylglucosamine (O-GlcNAc). In this process, termed GlcNAcylation, a single GlcNAc moiety is added to Ser or Thr residues in a reversible manner. Since its discovery in ^{3}H-galactose labeling experiments in the 1980s (Torres and Hart, 1984), O-GlcNAc has been found to modify approximately one thousand nuclear and cytoplasmic proteins (Butkinaree *et al.*, 2010). Global protein GlcNAcylation is modulated by flux through the hexosamine biosynthesis pathway (HBP), a minor branch in glycolysis.

HBP terminates in the production of uridine 5'diphosphate N-acetylglucosamine (UDP-GlcNAc), a critical donor molecule for protein glycosylation. The synthesis of UDP-GlcNAc is a branch of glycolysis that begins with fructose 6-phosphate and requires several anabolic reactions to generate UDP-GlcNAc (Hanover *et al.*, 2010). It is estimated that 2-5% of the glucose that is consumed in glycolysis is shunted to the HBP (Walgren *et al.*, 2003; Housley

Figure 4: mTORC2 activity is positively regulated by acetylation and inhibited by Sirtuin-mediated deacetylation. We hypothesized that mTORC2 components are directly targeted by acetyltransferases, resulting in increased mTORC2 -mediated phosphorylation of Akt at S473 in the presence of IGF1. Upon depletion of cellular nutrients, a rise in NAD^+/NADH levels stimulates Sirtuin-mediated deacetylation of mTORC2 components, resulting in decreased IGF1-stimulated mTORC2 activity.


IGF1
mTOR
Rictor
LST8
mSin1
S473-
P
Akt
HAT
HDAC
Ac
mTOR
Rictor
LST8
mSin1
S473-
P
Akt

et al., 2008; Kang *et al.*, 2008). This pathway also utilizes glutamine, the lipid precursor acetyl CoA and the high energy molecule uridine 5' triphosphate (UTP; Teo *et al.*, 2010). Thus, the generation of UDP-GlcNAc via HBP is a significant consumer of cellular resources. The UDP-GlcNAc generated by HBP is utilized in the synthesis of oligosaccharides for N-linked and O-linked protein glycosylation, glycolipids, proteoglycans, glycosaminoglycans, glycosylphosphatidylinositol (GPI)-anchored proteins (Teo *et al.*, 2010).

The O-GlcNAc transferase (OGT) utilizes UDP-GlcNAc as the donor GlcNAc for O-linked monosaccharide modification of Ser or Thr residues of polypeptides (Kreppel *et al.*, 1997; Lubas *et al.*, 1997). O-GlcNacylation is reversed by the activity of the β-D-N-acetylglucosaminidase (O-GlcNAcase; Dong and Hart, 1994; Gao *et al.*, 2001). Both OGT and O-GlcNAcase are well conserved from *C. elegans* to humans and the *ogt* gene is essential in mouse embryonic stem cells, indicating that O-GlcNAcylation is an important modification in higher eukaryotes (Wells *et al.*, 2001; Shafi *et al.*, 2000).

GlcNAcylation is implicated as a nutrient sensor via the HBP pathway. The K_m of OGT is close to the physiological level of UDP-GlcNAc, rendering its activity responsive to changes in UDP-GlcNAc levels within the physiological range (Kreppel and Hart, 1999). In support of this type of regulation, HBP flux has been correlated to global protein O-GlcNAc levels (Boehmelt *et al.*, 2000; Vosseler *et al.*, 2002; Hazel *et al.*, 2004; Park *et al.*, 2005).

O-GlcNAcylation regulates nuclear and cytoplasmic proteins involved in diverse processes, including transcription, signaling, nuclear import, and control

of biosynthetic enzymes (Butkinaree *et al.*, 2010). One of the primary mechanisms by which O-GlcNAc regulates protein targets is cross-talk with phosphorylation (Butkinaree *et al.*, 2010). O-GlcNAcylation has been shown to directly compete with phosphorylation of Ser and Thr sites, or to inhibit phosphorylation indirectly. Through inhibition of phosphorylation, elevated O-GlcNAc inhibits multiple targets in the insulin/IGF1 pathway, including IRS, PDK1, and Akt (Park *et al.*, 2005; Whelan *et al.*, 2010). Elevation of protein O-GlcNAcylation has been shown to inhibit insulin signaling and promote insulin resistance (Marshall *et al.*, 1991; Vosseller *et al.*, 2002).

Several proteins in the insulin/IGF1 signaling pathway are GlcNAcylated and may contribute to insulin resistance. O-GlcNAcylation of IRS-1 inhibits phosphorylation of Y605, an important event in PI3K p85 recruitment (Whelan *et al.*, 2010). Akt can be GlcNAcylated at S473, the site phosphorylated by mTORC2 (Park *et al.*, 2005; Gandy *et al.*, 2006; Kang *et al.*, 2008). Increased GlcNAcylation of Akt at S473 correlates negatively with its phosphorylation and is associated with induction of apoptosis in mouse pancreatic beta cells (Kang *et al.*, 2008). Akt GlcNAcylation has been implicated in both the development of insulin resistance and pancreatic beta cell death, leading to Type II diabetes (Gandy *et al.*, 2006; Kang *et al.*, 2008). Downstream of Akt, FOXO1 is GlcNAcylated at T317, an event that increases its transcriptional activity and is enhanced under hyperglycemic conditions. Collectively, these studies support O-GlcNAc as a modulator of the IGF1/Akt pathway, resulting in increased activity of FOXO proteins.

The increased GlcNAcylation of Akt does not always induce insulin resistance in insulin-responsive cells (Macauley *et al.*, 2008; Macauley *et al.*, 2010). In these instances, phosphorylation of IRS and Akt were not affected by increased GlcNAcylation. This suggests that there may be an alternative role of GlcNAcylation in the IGF1/insulin pathway. In addition to regulating phosphorylation, O-GlcNAc modification has been shown to regulate nuclear localization, protein-protein interactions, and protein stability (Zachara and Hart, 2004). For example, Akt1 has been shown to increase nuclear localization in response to O-GlcNAcase inhibition (Gandy *et al.*, 2006).

The majority of studies of O-GlcNAc in this pathway have focused on elevated O-GlcNAc levels and compromised insulin signaling. We hypothesized that GlcNAcylation and acetylation directly promote mTORC2 activity and subsequent Akt phosphorylation at S473. In this model, GlcNAcylation and acetylation would signal the availability of adequate nutrients to promote cell survival and metabolism through Akt. The study of GlcNAcylation of mTORC2 is presented in Chapter 3.

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Chapter 2: Rictor Multiple Site Acetylation Stimulates mTORC2 Activity to Regulate Akt Phosphorylation

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This chapter is based on work in preparation for submission to the European Molecular Biology Organization Journal.

Emily Glidden is the sole first author.

Lisa Gray assisted in the generation of Rictor site-directed mutants. Suneil Vemuru assisted in the generation of a Rictor deletion construct.

2.1: Project Rationale

A basic research interest of the Mayo laboratory has been to study the molecular mechanisms by which post-translational modification involving acetylation impacts biological function within cells. One of our particular interests has been in the regulation of protein function by the nutrient-sensitive Sirtuin family of deacetylases. When I joined the laboratory, they had recently published a paper demonstrating the ability of SIRT1 to directly deacetylate the p65 subunit of the transcription factor NF-κB. Following this publication, a postdoctoral fellow in the lab, Dr. Fan Yeung, began investigating how acetylation MEK1 regulated its kinase activity. Dr. Yeung went on to show that acetylated MEK1 was specifically regulated by the SIRT1 deacetylase. Based on these initial observations, I was interested in studying Sirtuin-mediated regulation of critical kinases involved in cell metabolism. At the time I initiated my graduate studies, the Sabatini lab had discovered that Rictor-containing mTOR complex 2 (mTORC2) was the long sought after "PDK2" kinase responsible for phosphorylating Akt at S473. We hypothesized that mTORC2 is regulated by acetylation and that distinct subunits within the complex were directly deacetylated by the nutrient-sensing Sirtuins. My work investigating the regulation of mTORC2 by dynamic acetylation of Rictor and its affect on Akt phosphorylation at S473 led to the content presented in Chapter 2.

2.2: Abstract

The Ser/Thr kinase Akt is a critical mediator of cell survival, growth and metabolism in response to growth factors. A key step in Akt activation is phosphorylation at S473 by the mammalian target of rapamycin (mTOR) complex 2 (mTORC2). Although it is known that the mTORC2 component Rictor is required for the stability and activity of mTORC2, little is known about post-translational modifications that regulate mTORC2. We have identified Rictor acetylation as a positive regulator of mTORC2-mediated phosphorylation of Akt at S473. Inhibition of deacetylases, including the NAD^+-dependent Sirtuins, promotes Rictor acetylation and IGF1-mediated Akt phosphorylation at S473. We mapped two regions within Rictor important to mTORC2 function: 1) a region critical for interaction with mTORC2 components mSin1.1 and LST8, and 2) an adjacent acetylated region localized between amino acids 1041 and 1137. Site-directed mutagenesis of multiple lysines within the acetylated region to arginines reduced Rictor acetylation and IGF1-dependent mTORC2 kinase activity. These results indicate that multiple-site acetylation of Rictor signals for increased activation of mTORC2 and may provide a critical link between nutrient-sensitive deacetylases and mTORC2 signaling to Akt.

2.3: Introduction

The serine/threonine protein kinase mammalian target of rapamycin (mTOR) regulates cell growth, nutrient uptake, and survival, in response to multiple growth signals. mTOR, a member of the phosphoinositide-3-kinase related protein kinase (PIKK) family, functions in at least two multiprotein complexes, mTOR complex 1 (mTORC1), and mTOR complex 2 (mTORC2; LaPlante and Sabatini, 2009; Guertin and Sabatini, 2007; Corradetti and Guan, 2006). mTORC1 and mTORC2 regulate cellular processes that are essential for life (Gangloff *et al.*, 2004; Murakami *et al.*, 2004; Guertin *et al.*, 2006; Jacinto *et al.*, 2006; Shiota *et al.*, 2006; Yang *et al.*, 2006). mTORC1 regulates translation, cell growth and proliferation, primarily by phosphorylating p70 ribosomal S6 kinase (S6K1) and eukaryotic initiation factor 4E binding protein 1 (4EBP1) (Ruvinsky *et al.*, 2005; Mamane *et al.*, 2004). mTORC2 was initially identified as a regulator of actin cytoskeleton organization, an effect mediated by phosphorylation of PKCα, paxillin and activation of the small G proteins RhoA and Rac1 (Jacinto *et al.*, 2004; Sarbassov *et al.,* 2004). However, the best characterized mTORC2 substrate is S473 of the Ser/Thr kinase Akt, also called protein kinase B (PKB) (Sarbassov *et al.,* 2005; Hresko and Mueckler, 2005; Shiota *et al.*, 2006).

mTORC2 consists of the catalytic subunit, mTOR, the mammalian lethal with Sec13 protein 8 (LST8), the rapamycin-insensitive companion of mTOR (Rictor) and mammalian stress-activated protein kinase interacting protein (mSin1). Rictor is required for mSin1 stability and mTORC2 activity (Sarbassov *et al.*, 2005; Frias *et al.*, 2006; Jacinto *et al.*, 2006; Yang *et al.*, 2006). Together,

Rictor and mSin1 are thought to form the structural foundation of mTORC2, while mSin1 recruits substrates to the catalytic domain of mTOR (LaPlante and Sabatini, 2009; Jacinto *et al.*, 2006). The protein observed with Rictor-1 (Protor-1) also interacts with mTORC2, however the contribution of this interaction to mTORC2 function is not well understood (Thedieck *et al.*, 2007; Woo *et al.*, 2007; Pearce *et al.*, 2007).

mTORC2 and Akt are activated downstream of growth factors such as insulin-like growth factor (IGF1), upon binding to its cognate receptor tyrosine kinase (Clemmons, 2009; Rosenzweig and Atreya, 2010). Activated Type I IGF1 receptor (IGF1R) phosphorylates insulin receptor substrate proteins (IRS)-1 or -2, which recruit the lipid kinase phosphatidylinosital 3-kinase (PI3K) (White, 2002). PI3K activity generates phosphatidylinositol-3,4,5-triphosphate (PIP_3), which recruits Akt and 3′-phosphoinositide dependent kinase-1 (PDK1) to the membrane through their respective pleckstrin homology (PH) domains. Membrane co-localization stimulates PDK1 to phosphorylate Akt at T308 and mTORC2 phosphorylates Akt at S473 (Alessi *et al.*, 1997; Bellacosa *et al.*, 1998; Sarbassov *et al.*, 2005). Although DNA-PK and autophosphorylation by Akt have been shown to phosphorylate S473 in response to select stimuli (Toker *et al.*, 2000; Feng, *et al.,* 2004), genetic disruption of either Rictor or mSin1 has demonstrated the importance of mTORC2 in phosphorylation of S473 in response to growth factors (Sarbassov *et al.*, 2005; Yang *et al.*, 2006; Hresko and Mueckler, 2005; Shiota *et al.*, 2006; Frias *et al.*, 2006; Jacinto *et al.*, 2006; Guertin *et al.*, 2006; Kumar *et al.*, 2008; Shu *et al.*, 2009; Kumar *et al.*, 2010).

Akt kinase activity *in vitro* increases with phosphorylation of both T308 and S473 (Alessi *et al.*, 1996; Sarbassov *et al.*, 2005). Upon phosphorylation within its activation T-loop at T308, Akt undergoes a conformational change that facilitates substrate binding and catalysis (Yang *et al.*, 2002; Bellacosa *et al.*, 2005). Phosphorylation of Akt within the C-terminal hydrophobic motif (HM) at S473 allows the HM to act as an allosteric activator, potentiating kinase activity (Yang *et al.,* 2002; Alessi *et al.*, 1996). S473 phosphorylation is critical for a subset Akt substrates regulating cell survival and metabolism (Frias *et al.*, 2006; Guertin *et al.*, 2006; Jacinto *et al.*, 2006; Kumar *et al.*, 2008; Kumar *et al.*, 2010). For example, mTORC2-driven phosphorylation of Foxo1/3a at Thr24/32 by Akt results in cytosolic sequestration of these transcription factors, promoting resistance to stress-induced apoptosis and altering expression of metabolic enzymes (Dijkers *et al.*, 2000; Stahl *et al.*, 2002; Zhang *et al.*, 2006). Despite the importance of mTORC2 for Akt phosphorylation, little is known about post-translational modifications that link mTORC2 activity with cell metabolism.

Acetylation is a reversible post-translational modification that regulates key signaling molecules in response to growth factor stimulation (Li *et al.*, 2008; Zhang, 2007; Song *et al.*, 2011). Acetyltransferases, commonly called histone acetyltransferases (HAT), utilize the metabolite signaling molecule acetyl-CoA to form N-ε-acetyl lysine on polypeptides. The generation of nuclear/cytoplasmic acetyl-CoA has recently been linked to cellular glucose levels and to the level of histone acetylation (Wellen *et al.*, 2009). Reversal of lysine acetylation is carried out by histone deacetylases (HDACs). Mammals have two families of HDACs

with distinct catalytic mechanisms: Rpda3/Hda1, which is subdivided into class I, II, and IV HDACs, and the Sirtuins, which comprise the Class III HDACs. Class I/II/IV HDACs use zinc as a cofactor in hydrolysis of acetyl-lysine (Yang and Seto, 2008a). Sirtuins utilize nicotinic adenine dinucleotide (NAD^+) as a cosubstrate to deacetylate protein targets (Bao and Sack, 2010; North and Verdin, 2004). Sirtuins are activated in response to nutrient depletion due to a rise in the intracellular levels of NAD^+, and feedback inhibited by the noncompetitive inhibitor nicotinamide (NAM; Lin *et al.*, 2000; Anderson *et al.*, 2002; Dioum *et al.*, 2009).

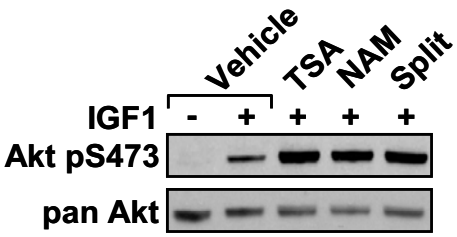
Since acetylation regulates critical enzymes involved in signaling and metabolism (Li *et al.*, 2008; Spange *et al.*, 2009; Lan *et al.*, 2008; Yuan *et al.*, 2010; Zhao *et al.*, 2010; Zhong *et al.*, 2010), we sought to determine whether dynamic acetylation of mTORC2 components modulated kinase activity. In this body of work, we provide evidence that acetylation of Rictor positively upregulates the ability of mTORC2 to phosphorylate Akt S473 in response to IGF1 stimulation. Acetylation of Rictor is regulated by HDACs, including the NAD^+-dependent deacetylases, Sirtuins. Acetylation of Rictor increases mTORC2 activity, since mutant Rictor proteins that cannot be acetylated significantly reduce mTORC2-mediated phosphorylation of Akt at S473 in response to IGF1. Our findings identify Rictor acetylation as a nutrient-sensitive signal important for activation of mTORC2 in phosphorylation of the master metabolic regulator, Akt.

2.4: The mTORC2 adaptor protein Rictor is acetylated.

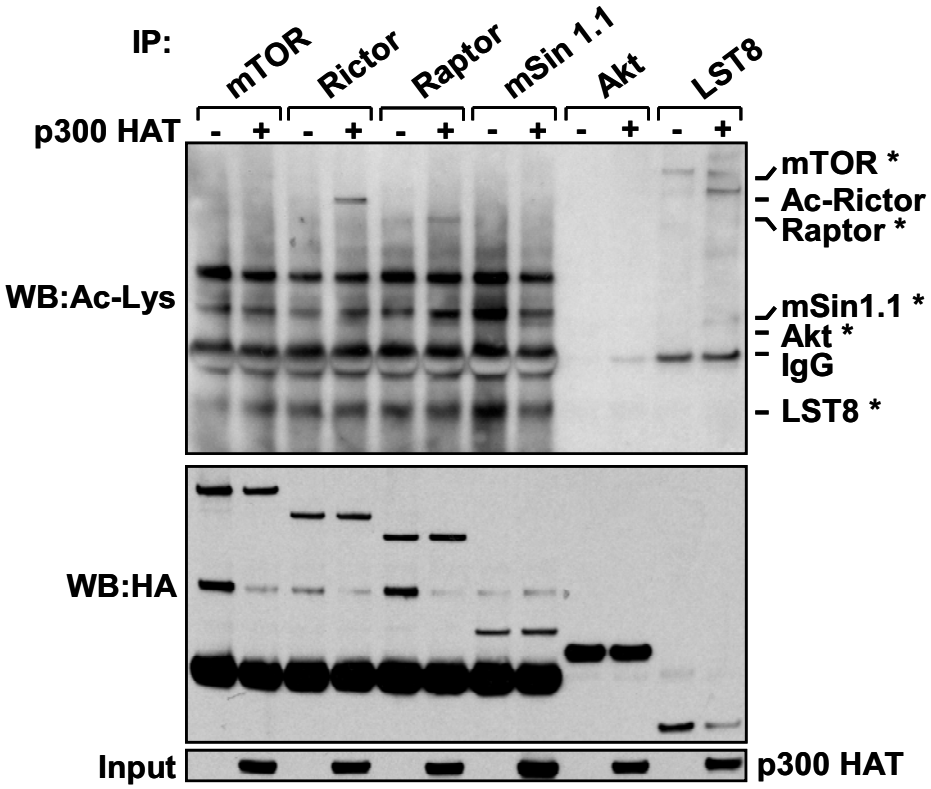
Since the mTOR pathway is intimately tied to cellular metabolism, we examined whether inhibition of HDAC activity results in increased Akt phosphorylation. Treatment of HeLa cells with the Class I/II/IV HDAC inhibitor trichostatin (TSA), or Class III HDAC inhibitors nicotinamide (NAM) and splitomicin, increased IGF1-mediated Akt phosphorylation, compared to vehicle control (Fig. 5A). To determine whether Akt or components of the mTOR complexes were directly post-translationally modified, in-cell-acetylation assays were performed (Chen *et al.*, 2002). Co-expression of p300 effectively acetylated Rictor, but not other members of mTORC2, namely mTOR, mSin 1.1, or LST8 (Fig. 5B). In our hands, p300 was unable to directly acetylate Akt. p300 reproducibly acetylates Raptor, however, this effect was significantly less robust than p300-mediated acetylation of Rictor (Fig. 5B). To examine whether Rictor acetylation is sensitive to deacetylase inhibitors, in-cell-acetylation assays were repeated and cells were treated with TSA, NAM or with both compounds. Cells stimulated with IGF1 and treated with TSA or NAM alone showed increased p300-mediated acetylation of Rictor, compared to vehicle control (Fig. 5C). Cells treated with both TSA and NAM displayed more acetylated Rictor, suggesting that Rictor is regulated by both Class I/II/IV and Class III HDACs. Since SIRT1 is known to regulate nutrient sensing pathways, *in vitro* deacetylation assays were performed. SIRT1 deacetylated Rictor *in vitro* in an NAD^+-dependent manner, suggesting that Rictor was susceptible to direct regulation by the nutrient sensing deacetylase (Fig. 5D).

Figure 5: Acetylation of Rictor and Akt phosphorylation at S473 are regulated by NAD+-dependent deacetylases. A) Inhibition of both NAD+-independent and NAD+-dependent deacetylases increases Akt S473 phosphorylation. Hela cells were starved treated with HDAC inhibitors TSA, NAM, Splitomicin (Split) or vehicle control prior to stimulation with IGF1. Cell lysates were analyzed by immunoblotting with α-phospho-Ser473 Akt antibodies (pS473 Akt) or α-pan Akt antibodies. B) Rictor is an acetylated protein. mTOR, Rictor, Raptor, mSin1.1, LST8, or Akt were expressed in an in-cell-acetylation assay with or without p300. Target proteins were immunoprecipitated and analyzed by immunoblotting with α-pan acetyl-Lys (Ac-Lys) or α-HA antibodies to detect acetylated proteins or total protein, respectively. Asterisks indicate expected position of proteins not detected by Ac-Lys. C) Rictor acetylation is regulated by deacetylases. HA- and FLAG-tagged Rictor (HA/FLAG-Rictor) or vector control were co-transfected with p300 in an in-cell-acetylation assay. Cells were treated with TSA, NAM, or TSA and NAM together, prior to stimulation with IGF1. Rictor proteins were immunoprecipitated with α-FLAG agarose and analyzed for acetylated and total Rictor as in B. D) SIRT1 deacetylates Rictor *in vitro*. HA/FLAG-Rictor was expressed in 293T cells with or without p300 HAT. Rictor was immunoprecipitated from cell lysates using α-FLAG antibodies and mixed with recombinant SIRT1 enzyme, with or without NAD^+. Detection of acetylated and total Rictor was as in B.

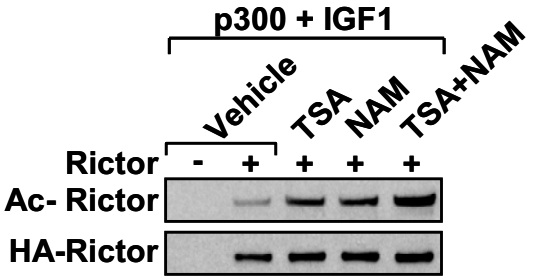
A.


Vehicle
TSA
NAM
Split
IGF1
- + + + +
Akt pS473
pan Akt

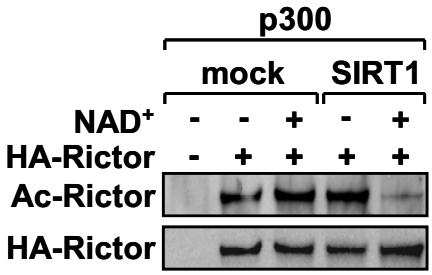
B.


IP:
mTOR
Rictor
Raptor
mSin 1.1
Akt
LST8
p300 HAT
- + - + - + - + - + - +
WB:Ac-Lys
mTOR *
Ac-Rictor
Raptor *
mSin1.1 *
Akt *
IgG
LST8 *
WB:HA
Input
p300 HAT

C.


p300 + IGF1
Vehicle
TSA
NAM
TSA+NAM
Rictor - + + + +
Ac- Rictor
HA-Rictor

D.


p300
mock
SIRT1
NAD+ - - + - +
HA-Rictor - + + + +
Ac-Rictor
HA-Rictor

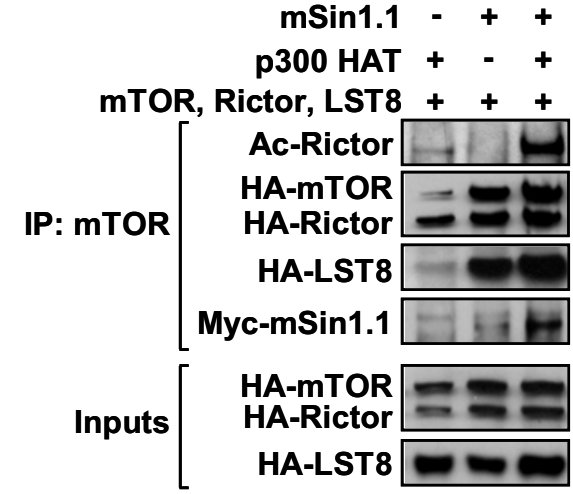
Experiments shown in Figure 5 identify Rictor as an acetylated protein susceptible to regulation by the Class I/II/IV and Class III deacetylases.

2.5: Rictor acetylation potentiates mTORC2 activity resulting in increased Akt S473 phosphorylation.

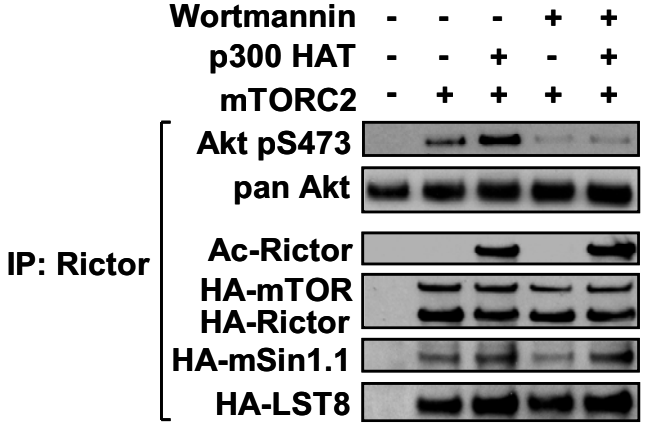
Since Rictor is a critical member of mTORC2 required for Akt S473 phosphorylation, we sought to test whether the intact mTORC2 complex potentiated Rictor acetylation. As previously reported (Frias *et al.*, 2006; Yang *et al.,* 2006; Jacinto *et al.*, 2006), mSin1.1 expression stabilized mTORC2 (Fig. 6A). This effect could be observed following immunoprecipitation where mSin1.1 expression resulted in a more efficient pulldown of mTOR, Rictor, and LST8. Interestingly, expression of mSin1.1 along with other members of mTORC2 greatly increased the level of acetylated Rictor detected in the in-cell-acetylation assays (Fig. 6A). Additionally, more LST8 and mSin1.1 protein were detected in the immunoprecipitants in the presence of p300-mediated acetylation of Rictor. These results indicate that Rictor is more effectively acetylated in the context of mTORC2, suggesting that this post-translational modification might be important for controlling mTORC2 activity. To test this, immunokinase assays were performed by expressing mTORC2, immunoprecipitating Rictor-containing mTORC2, and mixing protein complexes with recombinant Akt and ATP. Cells expressing p300 significantly increased mTORC2 activity, as detected by the ability of mTORC2 to phosphorylate recombinant Akt *in vitro* (Fig. 6B). Akt was specifically phosphorylated by mTORC2 since the addition of the PIKK-inhibitor

Figure 6: Acetylation of Rictor is associated with increased mTORC2 kinase activity. A) mSin1.1 promotes mTORC2 complex integrity and Rictor acetylation. 293T cells were transfected with HA-tagged Rictor and LST8, and HA- and AU1-tagged mTOR, with or without myc-tagged mSin1.1 and p300 HAT. α-AU1 antibodies were used to precipitate mTOR complexes from cell lysates. Acetylated proteins and total protein levels were assayed as in Fig. 2B. B) p300 HAT increases mTORC2 kinase activity. 293T cells were transfected with or without mTORC2 (HA-tagged mTOR, LST8 and mSin1.1, HA/FLAG-Rictor), and p300 HAT. Rictor-containing mTORC2 complexes were immunoprecipitated and mixed with recombinant Akt and ATP, with or without the mTOR inhibitor Wortmannin. Reactions were analyzed by immunoblotting with the indicated antibodies.

A.


mSin1.1 - + +
p300 HAT + - +
mTOR, Rictor, LST8 + + +
IP: mTOR
Ac-Rictor
HA-mTOR
HA-Rictor
HA-LST8
Myc-mSin1.1
Inputs
HA-mTOR
HA-Rictor
HA-LST8

B.


Wortmannin - - - + +
p300 HAT - - + - +
mTORC2 - + + + +
IP: Rictor
Akt pS473
pan Akt
Ac-Rictor
HA-mTOR
HA-Rictor
HA-mSin1.1
HA-LST8

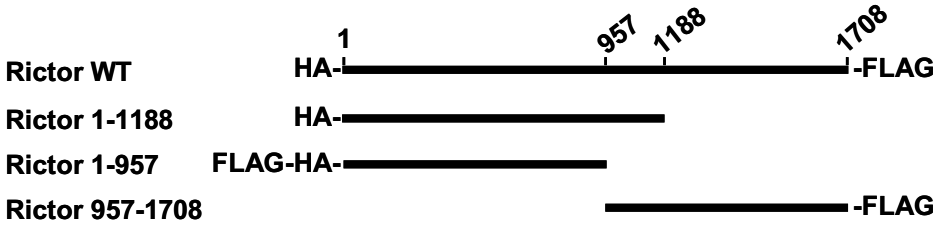
wortmannin to the kinase reaction resulted in inhibition. Results shown in Figure 6 indicate that the mTORC2 complex promotes Rictor acetylation, an effect that promotes mTORC2-mediated phosphorylation of Akt at S473.

2.6: The central region encompassing residues 957-1188 of Rictor contains the major sites of acetylation.

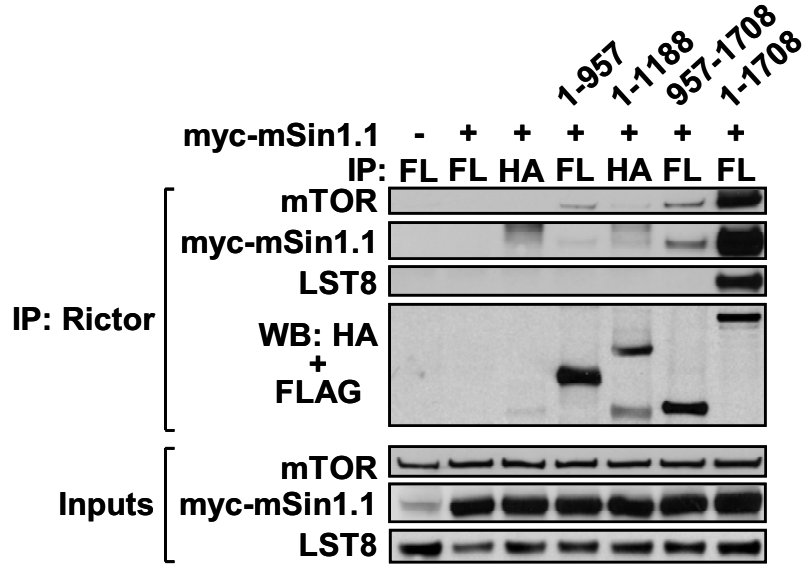
Before initiating experiments to identify acetylated regions within Rictor, we first tested a series of N-terminal and C-terminal Rictor deletions for interaction with the mTORC2 components (Fig. 7A). Although full length Rictor and N-terminal and C-terminal Rictor proteins were effectively immunoprecipitated using either anti-HA or -Flag antibodies, only wild-type Rictor interacted with mTOR, mSin1.1, and LST8 (Fig. 7B). Constructs lacking either the N-terminus or C-terminus were unable to effectively co-immunoprecipitate mTORC2 components. Since it was not feasible to examine Rictor deletion constructs in the context of the mTORC2, we simply examined whether p300 could acetylate N- and C-terminal Rictor truncations. Expression of p300 effectively acetylated wild-type (1-1708), as well as constructs encoding amino acid residues 957-1708 and 1-1188 (Fig. 7C). However, the Rictor C-terminal truncation consisting of amino acids 1-957 was not acetylated by p300. Since Rictor 1-1188 or 957-1708 proteins were both acetylated by p300, our results highlight an overlapping central region (residues 957 to 1188) as a possible acetylated region within Rictor. This region is distinct from the previously identified Rictor acetylation site, K582, of unknown biological function (Choudhary *et al.*, 2009).

Figure 7: The N-terminus of Rictor is dispensable for acetylation but both termini are required for mTORC2 integrity. A) Diagram of Rictor truncations used to map regions required for acetylation and mTORC2 formation. B) Rictor co-immunoprecipitation of mTOR, mSin1.1 and LST8 requires both termini. 293T cells were transfected with or without myc-mSin1.1 and various Rictor proteins. Rictor proteins were immunoprecipitated agarose conjugated α-FLAG (FL) antibodies or α-HA antibodies as indicated. Coimmunoprecipitants and Rictor proteins were analyzed by immunoblotting with the indicated antibodies. C) Acetylation of overlapping Rictor truncations highlights central region containing residues 957-1188. 293T cells were transfected with p300 HAT and various Rictor proteins. Proteins were immunoprecipitated with as in B and immunoprecipitants were analyzed by immunoblotting with the indicated antibodies.

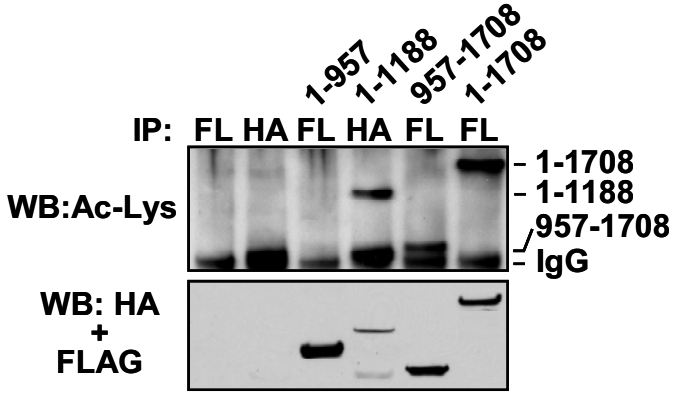
A.


1
957
1188
1708
Rictor WT
HA-
-FLAG
Rictor 1-1188
HA-
Rictor 1-957
FLAG-HA-
Rictor 957-1708
-FLAG

B.


1-957
1-1188
957-1708
1-1708
myc-mSin1.1 - + + + + + +
IP: FL FL HA FL HA FL FL
IP: Rictor
mTOR
myc-mSin1.1
LST8
WB: HA + FLAG
Inputs
mTOR
myc-mSin1.1
LST8

C.

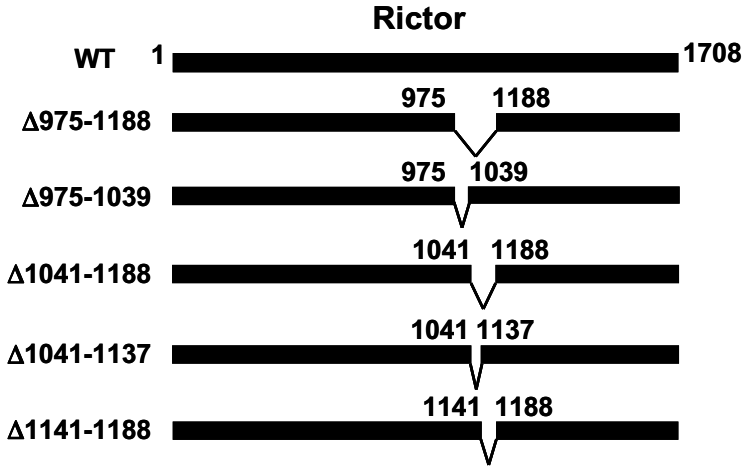

1-957
1-1188
957-1708
1-1708
IP: FL HA FL HA FL FL
WB:Ac-Lys
1-1708
1-1188
957-1708
IgG
WB: HA + FLAG

2.7: The acetylated region in Rictor is adjacent to an mTORC2 stabilization region.

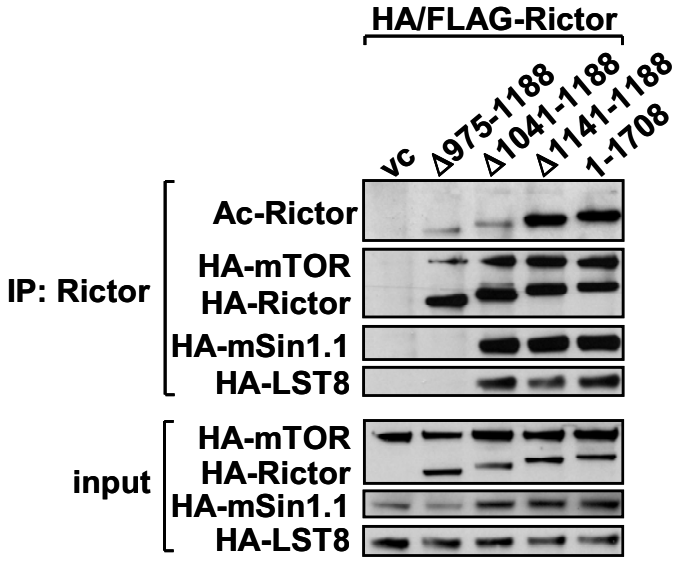
To elucidate the importance of residues 957-1188 within Rictor for acetylation, a series of Rictor deletion constructs were generated (Fig. 8A). The largest deletion, Rictor Δ975-1188, showed a loss of acetylation (Fig. 8B). However, this same construct was unable to interact with mSin1.1 and LST8, and showed reduced binding to mTOR. Since mSin1.1 is required for stabilization of mTORC2, we could not determine whether the loss of mSin1.1 and/or LST8 binding contributed to our inability to detect Rictor acetylation. However, since truncated Rictor proteins can be directly acetylated by p300 in the absence of the mTORC2 complex (Fig. 7C), we hypothesized that the sites of acetylation most likely resided between residues 975-1188. Primary sequence analysis of this region identified six lysine-rich regions located between residues 975 and 1188. A smaller deletion construct, Rictor Δ1041-1188, retained its ability to interact with mSin1.1 and LST8, but was no longer effectively acetylated by p300 (Fig. 8B). Rictor Δ1141-1188 was acetylated to the same extent as full length Rictor, and as expected, retained its ability to interact with mSin1.1 and LST8 (Fig. 8B). Collectively, results from deletion analysis suggested that Rictor was acetylated between residues 1041-1141. To address this, in-cell-acetylation assays were performed using the Rictor Δ1041-1137 construct. Similar to Rictor Δ1041-1188, Rictor Δ1041-1137 interacted with each of the mTORC2 members, but was no longer acetylated by p300 (Fig. 8C). These results suggest that this region contained the major sites of acetylation (Fig. 8C).

Figure 8: Identification of adjacent mSin1.1 binding and acetylated regions within Rictor. A) Diagram of Rictor internal deletion constructions and Rictor WT B) Rictor residues 1041-1188 are required for acetylation. In-cell-acetylation assay was performed with HA/FLAG-Rictor proteins with internal amino acid deletions as indicated, or WT (1-1708), co-expressed with mTORC2 components. Rictor-containing mTORC2 complexes were immunoprecipitated using agarose-conjugated α-FLAG antibodies and analyzed by immunoblotting with the indicated antibodies. C) Rictor Δ975-1039 does not coimmunoprecipitate mSin1.1 or LST8 but is weakly acetylated. In-cell-acetylation assay was performed as in A with indicated Rictor proteins. Rictor-containing mTORC2 complexes were immunoprecipitated and analyzed as in Fig. 5A. D) Rictor Δ1041-1137 is not acetylated but does form mTORC2. Rictor internal deletions were assayed for acetylation and mTOR, mSin1.1 and LST8 coimmunoprecipitation as in A. E) Chart summarizing findings from acetylation and coimmunoprecipitation assays with Rictor internal deletion proteins.

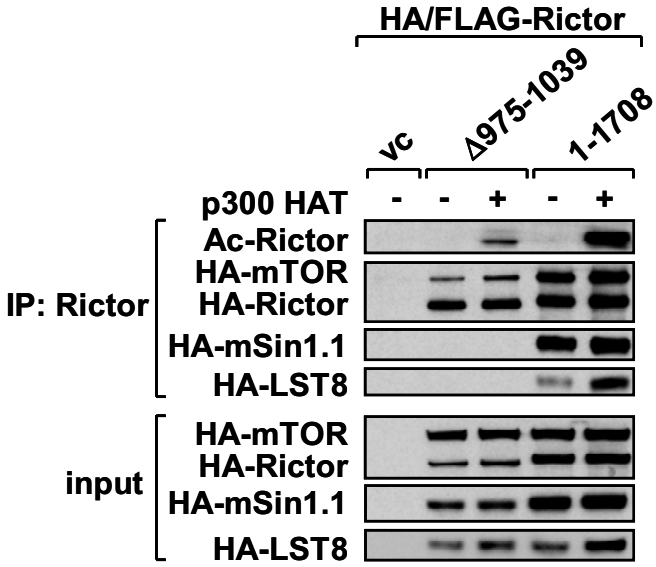
A.


Rictor
WT
1
1708
Δ975-1188
975
1188
Δ975-1039
975
1039
Δ1041-1188
1041
1188
Δ1041-1137
1041
1137
Δ1141-1188
1141
1188

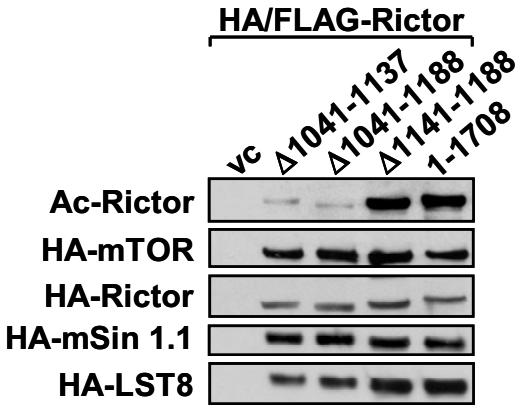
B.


HA/FLAG-Rictor
vc
Δ975-1188
Δ1041-1188
Δ1141-1188
1-1708
IP: Rictor
Ac-Rictor
HA-mTOR
HA-Rictor
HA-mSin1.1
HA-LST8
input
HA-mTOR
HA-Rictor
HA-mSin1.1
HA-LST8

C.


HA/FLAG-Rictor
vc
Δ975-1039
1-1708
p300 HAT
- - + - +
IP: Rictor
Ac-Rictor
HA-mTOR
HA-Rictor
HA-mSin1.1
HA-LST8
input
HA-mTOR
HA-Rictor
HA-mSin1.1
HA-LST8

D.


HA/FLAG-Rictor
vc
Δ1041-1137
Δ1041-1188
Δ1141-1188
1-1708
Ac-Rictor
HA-mTOR
HA-Rictor
HA-mSin 1.1
HA-LST8

E.

Rictor	mTOR	LST8	mSin1.1	Acetylated
1-1708	+++	+	+	+++++
Δ975-1188	+	-	-	-
Δ975-1039	+	-	-	++
Δ1041-1188	+++	+	+	-
Δ1041-1137	+++	+	+	-
Δ1141-1188	+++	+	+	+++++

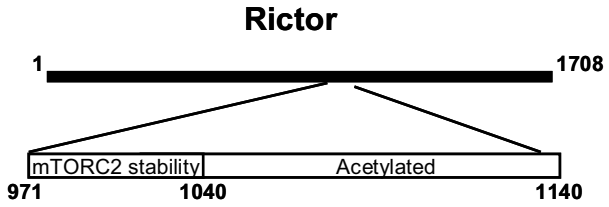
Since regions within Rictor responsible for mSin1.1-mediated stabilization have not been previously identified, we examined whether Rictor Δ975-1039 contained an mSin1.1 stabilization region and whether deletion of this region impacted Rictor acetylation. Unlike wild-type Rictor, the Rictor Δ975-1039 protein was unable to interact with either mSin1.1 or LST8 and weakly interacted with mTOR (Fig. 8D). Rictor Δ975-1039 also showed reduced acetylation, compared to wild-type Rictor. Since p300-directed acetylation of Rictor is greatly increased in the context of the mSin1.1 containing mTORC2 (Fig. 6A), the loss of Rictor acetylation observed in Figure 8D is most likely due to the inability of the Rictor Δ975-1039 to interact with mSin1.1. Collectively, results from Figure 8 indicate that Rictor contains an mTORC2 stabilization region between 975-1039 which resides adjacent to the putative acetylated region (1041-1137, Fig. 8E).

2.8: Rictor residues 1040-1140 contain the acetylation regions responsive to deacetylase inhibitors.

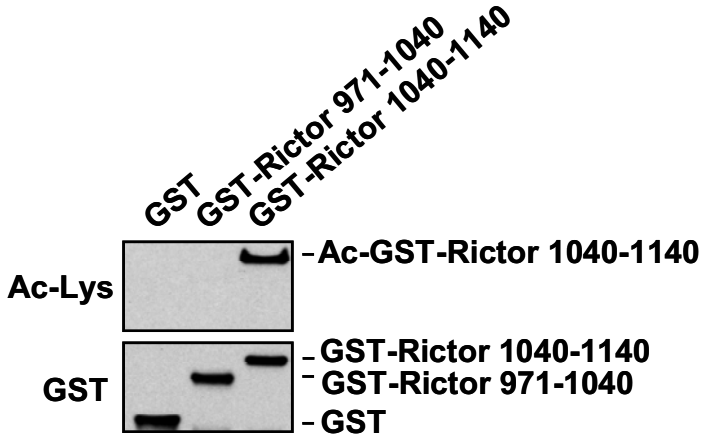
To determine whether the central region of Rictor is sufficient to be recognized and acetylated by p300, GST-Rictor fusion constructs for mammalian expression were generated that contained either the mTORC2 stabilization region (971-1040) or the putative acetylated regions (1040-1140). As shown in Figure 9A, Rictor residues 1040-1140 contain four lysine-rich motifs (M1-4) composed of a total of 9 lysine residues. GST-Rictor 1040-1140 was efficiently acetylated by p300, while GST-Rictor 971-1040 or GST alone could not be acetylated (Fig. 9B). The differences in acetylation between the GST-Rictor constructions were

Figure 9: Rictor residues 1040-1140 contain acetylated lysines that are regulated by deacetylases. A) Chart depicting regions used to create GST-Rictor fusions 971-1040 (mTORC2 stabilization) and 1040-1140 (putative acetylated region). B) Rictor residues 1040-1140 contain acetylated lysines. GST, GST-971-1040 or GST-1040-1140 were expressed in 293T cells for in-cell-acetylation. GST proteins were precipitated with glutathione-sepharose and analyzed for acetylation and total GST protein by immunoblotting. C) Rictor 1040-1140 acetylation is regulated by deacetylases. In-cell-acetylation was performed with GST-Rictor 1040-1140. Prior to lysis, cells were pre-treated with TSA and NAM and stimulated with IGF1. GST-Rictor 1040-1140 was assayed for relative acetylation and total protein as in B.

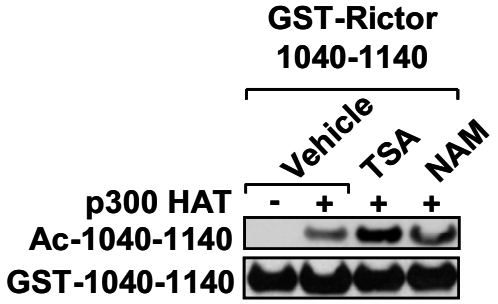
A.


Rictor
1
1708
mTORC2 stability
Acetylated
971
1040
1140

B.


GST
GST-Rictor 971-1040
GST-Rictor 1040-1140
Ac-Lys
Ac-GST-Rictor 1040-1140
GST
GST-Rictor 1040-1140
GST-Rictor 971-1040
GST

C.


GST-Rictor
1040-1140
Vehicle
TSA
NAM
p300 HAT
-
+
+
+
Ac-1040-1140
GST-1040-1140

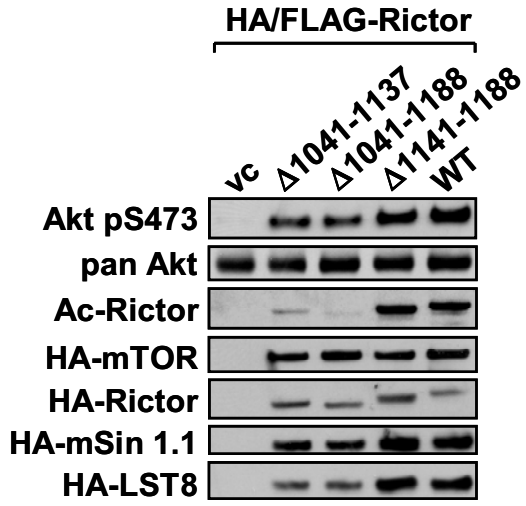
not due to uneven protein expression or loading. Since our studies indicate that mTORC2 signaling is sensitive to deacetylase inhibitors (Fig. 5), we repeated in-cell-acetylation assays using the GST-Rictor 1040-1140 in the presence or absence of TSA or NAM. Treatment of cells with either Class I/II/IV or Class III deacetylase inhibitors further potentiated p300-mediated acetylation of GST-Rictor 1040-1140. These results indicate that this region of Rictor contains lysine residues that are responsive to p300 directed acetylation and deacetylase inhibition.

2.9: Identification of Rictor acetylation modules that contribute to mTORC2 kinase activity.

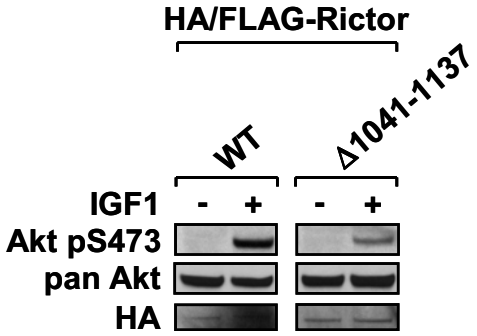
Since p300 expression potentiates mTORC2 kinase activity (Fig. 6B), immunokinase assays were performed to evaluate whether the Rictor acetylated region was required to potentiate mTORC2 kinase activity. To accomplish this, immunoprecipitated mTORC2 was pulled down using the HA/FLAG-tagged Rictor, complexes were incubated with recombinant Akt, and Akt phosphorylation at S473 was detected by Western. Deletion constructs encoding Rictor Δ1041-1137 and Δ1041-1188 showed reduced mTORC2 kinase activity relative to wild-type Rictor (Fig. 10A). However, Rictor Δ1141-1188, which contains a deletion outside the acetylated region, retained mTORC2 kinase activity similar to that observed for wild-type Rictor. Rictor Δ1041-1137 and Δ1041-1188 retained the ability to interact with mSin1.1 and LST8, but were less effective in mTORC2

Figure 10: Acetylation of Rictor Lys within M1-M4 increases mTORC2 kinase activity. A) Hypoacetylated Rictor internal deletions form mTORC2 with reduced kinase activity. In-cell-acetylation was performed with or without HA-Rictor-FLAG proteins with internal amino acid deletions as indicated, or WT and co-expression of mTORC2 components. Rictor-containing mTOR complexes were isolated and an immunokinase assay was performed as in Fig. 2B. B) Expression of Rictor Δ1041-1137 in HeLa cells results in reduced Akt S473 phosphorylation relative to Rictor WT. Hela cells were transfected with HA-Rictor proteins as indicated. Serum-starved cells were treated with or without IGF1 and extracts were analyzed by immunoblotting with the indicated antibodies. C) Diagram of Lysines within 1040-1140, identified as modules M1 (K1080, 1082), M2 (K1092, K1095), M3 (K1107, K1108), and M4 (K1116, K1119, K1125). "K" in bold are conserved in vertebrates. D) Acetylation of Rictor M2 and M4 is required for increased mTORC2 kinase activity. Immunokinase assays were performed as in A using Rictor mutants with K→R (KR) mutations of M1, M2, M3, M4, M1-4(inclusive), Rictor Δ1041-1137, Rictor WT and vector control.

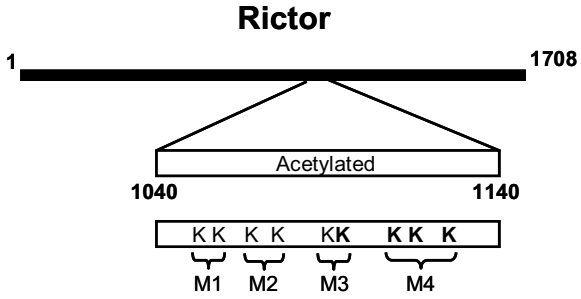
A.


HA/FLAG-Rictor
vc
Δ1041-1137
Δ1041-1188
Δ1141-1188
WT
Akt pS473
pan Akt
Ac-Rictor
HA-mTOR
HA-Rictor
HA-mSin 1.1
HA-LST8

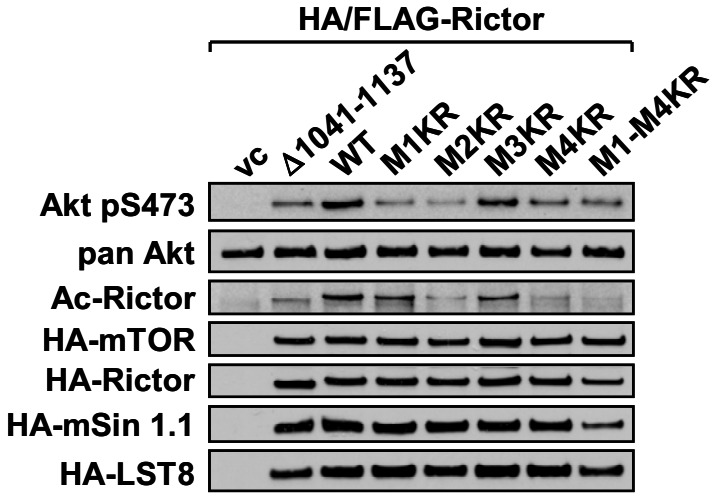
B.


HA/FLAG-Rictor
WT
Δ1041-1137
IGF1
- + - +
Akt pS473
pan Akt
HA

C.


Rictor
1
1708
Acetylated
1040
1140
K K K K KK K K K
M1 M2 M3 M4

D.


HA/FLAG-Rictor
vc
Δ1041-1137
WT
M1KR
M2KR
M3KR
M4KR
M1-M4KR
Akt pS473
pan Akt
Ac-Rictor
HA-mTOR
HA-Rictor
HA-mSin 1.1
HA-LST8

activity than either Rictor Δ1141-1188 or wild-type proteins. These data demonstrated that the acetylated region within Rictor 1041-1137 is required for acetylation-induced increases in mTORC2 kinase activity. To examine whether the Rictor Δ1041-1137 was defective in activating endogenous Akt phosphorylation, HeLa cells were transfected with Rictor expression constructs and cell extracts were analyzed for endogenous Akt S473 phosphorylation. Akt S473 phosphorylation was reduced in the cells expressing Rictor Δ1041-1137 relative to the cells expressing wild-type Rictor (Fig. 10B). This difference was not due to protein expression levels, since both constructs were detected at similar levels by Western analysis. To initiate experiments to identify critical lysine residues within Rictor 1041-1137, we divided the nine lysine residues into four modules (M1-4): M1 contains K1080 and K1082, M2 K1092 and K1095, M3 K1107 and K1108, and M4 K1116, K1119, and K1125 (Fig. 10A). Using a similar approach to the identification of critical acetylation sites in the p65 subunit of NF-κB (Chen *et al.*, 2002), we performed site-directed mutagenesis of the full length Rictor, where lysine (K) residues within each module were mutated to arginine (R) to "mimic" hypoacetylation. Rictor expression plasmids were generated that contained K→R single module mutations, where each K within distinct modules was converted to R, and the M1-M4, in which all 9 K residues were converted to R. Immunokinase assays were performed to analyze whether site-directed Rictor mutants were defective in governing mTORC2 activity, and whether this corresponded to a loss of Rictor acetylation. Of the single modules, Rictor K→R

M2 and M4 displayed a reduction in mTORC2 kinase activity and Rictor acetylation (Fig. 10C). Consistent with these two modules controlling Rictor function the Rictor K→R M1-M4 also showed a reduction in mTORC2 activity and acetylated Rictor. The Rictor K→R M1 showed a loss of mTORC2 activity, however this did not correlate with a loss of Rictor acetylation. In contrast, Rictor K→R M3 failed to show changes in either mTORC2 activity or Rictor acetylation, suggesting that the K residing within this region are most likely not important for Rictor function. The Rictor Δ1041-1137 deletion construct served as a positive control, and as expected, displayed reduced mTORC2 activity and a lack of Rictor acetylation. All site-directed Rictor mutants were equally expressed, suggesting that alteration of K→R did not affect the stability of Rictor or alter interaction with other members of the mTORC2 (Fig. 10C). Experiments shown in Fig. 10 highlight the importance of M2 and M4 within the acetylated region of Rictor 1041-1137, which are capable of dynamically regulating mTORC2 activity.

Figure 11: mSin1.1, mSin1.2 or mSin1.5 expression increases Rictor acetylation. In-cell-acetylation assay with co-expression of mTOR, Rictor and LST8 with myc-mSin1.1, myc-mSin1.2, myc-mSin1.4 or myc-mSin1.5. mTORC2 complexes were immunoprecipitated and analyzed by Western blotting for acetylated Rictor and total exogenously expressed protein.

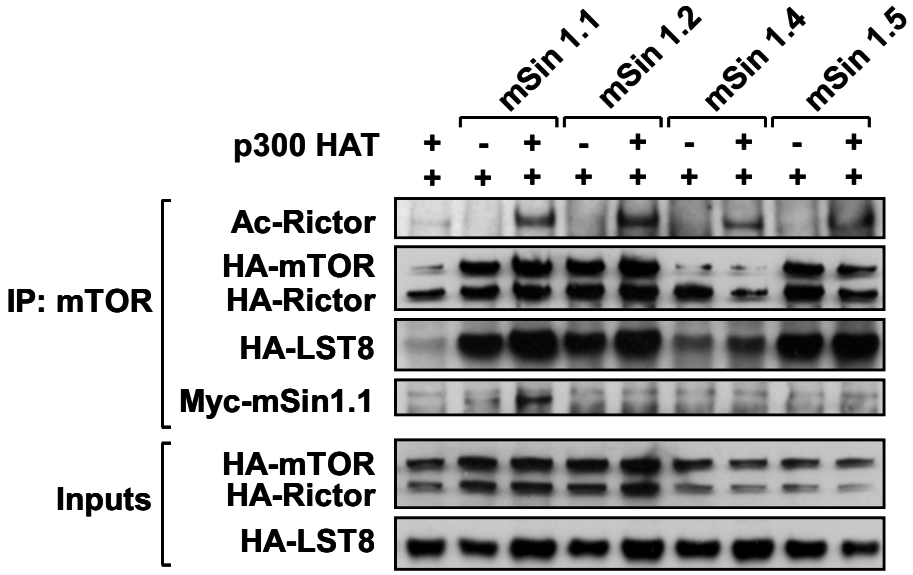
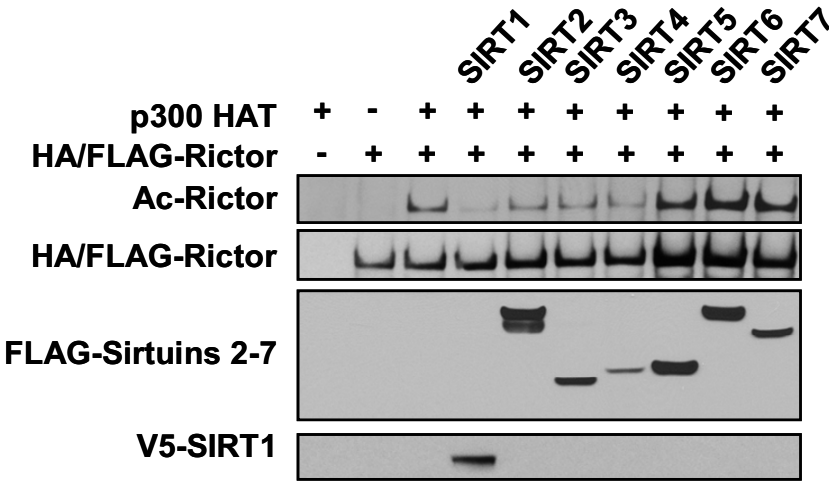

mSin 1.1
mSin 1.2
mSin 1.4
mSin 1.5
p300 HAT
+ - + - + - + - +
+ + + + + + + + +
IP: mTOR
Ac-Rictor
HA-mTOR
HA-Rictor
HA-LST8
Myc-mSin1.1
Inputs
HA-mTOR
HA-Rictor
HA-LST8

Figure 12: Sirtuins 1-4 decrease Rictor acetylation. In-cell-acetylation assay with co-expression of V5-SIRT1 or FLAG-SIRT2-7. HA/FLAG-Rictor was immunoprecipitated and analyzed for acetylation by Western blotting. Total Rictor and Sirtuin proteins were detected by Western blotting.


SIRT1
SIRT2
SIRT3
SIRT4
SIRT5
SIRT6
SIRT7
p300 HAT + - + + + + + + + +
HA/FLAG-Rictor - + + + + + + + + +
Ac-Rictor
HA/FLAG-Rictor
FLAG-Sirtuins 2-7
V5-SIRT1

2.10: Discussion

Our studies have identified acetylation of the mTORC2 component, Rictor, as a mechanism of potentiating IGF1-dependent activation of mTORC2. Acetylation of Rictor increases mTORC2 phosphorylation of Akt Ser473 and is sensitive to deacetylases, including the Sirtuin NAD^+-dependent deacetylases. We have described two unique regions within the relatively uncharacterized Rictor protein that are critical for mTORC2 formation and for acetylation-dependent mTORC2 augmentation. The acetylated region in Rictor contains two lysine-rich acetylated modules, M2 and M4, consisting of K1092 and K1095, and K1116, K1119, and K1125, respectively, that positively regulate mTORC2 activity. Results presented here illustrate the importance of M2 and M4 acetylation in controlling mTORC2 kinase activity.

The required components for active mTORC2, namely mTOR, Rictor, Sin1 and LST8, are conserved in *S. cerevisaie* TORC2 as TOR2, AVO3, AVO1 and LST8 (Jacinto, 2008). Studies of the molecular organization of TORC2 have suggested a model in which Sin1/AVO1 and Rictor/AVO3 form the foundation of the complex, upon which mTOR/TOR2 and LST8 assemble (Wullschleger *et al.*, 2005; Jacinto *et al.,* 2006, Frias *et al.*, 2006; Guertin *et al.*, 2006). LST8 co-precipitates with the mTOR kinase domain and cryo-electron microscopy confirms the proximity of LST8 to the mTOR kinase domain in the mTORC1 complex (Wullschleger *et al.*, 2005; Kim *et al.,* 2003; Yip *et al.*, 2010). Direct binding between Sin1 and Rictor is inferred based on the interdependence of the two subunits for protein stability (Jacinto *et al.,* 2006; Frias *et al.*, 2006).

Additionally, Rictor knockdown prevents Sin1 from interacting with mTOR, while Sin1 knockdown prevents Rictor from interacting with mTOR (Jacinto *et al.,* 2006; Frias *et al.*, 2006). These observations have suggested that Rictor and Sin1 cooperate in mTOR binding. Moreover, in *S. cerevisiae*, the interaction between LST8 and AVO3 is disrupted by depletion of AVO1 (Wullschleger *et al.*, 2005). Collectively, experimental evidence supports the idea that Rictor and Sin1 require each other to associate with the mTORC2 complex. However, Rictor and Sin1 alone are not sufficient to complex with mTOR, since LST8-deficient cells fail to show Rictor-mTOR interaction (Guertin *et al.*, 2006). Collectively, these studies support the idea that Rictor, Sin1 and LST8 all cooperate to assemble Rictor-mSin1 with LST8-mTOR.

We have identified a novel, 64 residue region in Rictor (975-1039), that is required for Rictor to precipitate both mSin1.1 and LST8. The loss of mSin1.1 and LST8 binding to Rictor∆975-1039 is associated with a reduction, but not a complete loss, of mTOR-Rictor interaction. Our work supports a model by which protein-protein interactions between mSin1, LST8 and Rictor stabilize the association between mTOR and Rictor. We provide evidence that Rictor residues 975-1039 are critical for coordinating interactions with mSin1.1 and LST8 to cooperatively bind mTOR. However, since Rictor-971-1040 alone does not pull down mSin1.1 or LST8, we conclude that Rictor 975-1039 is necessary but not sufficient for mSin1.1/LST8 binding. This result was not a surprise since our data also show that both Rictor termini are required for interaction with mSin1.1 and LST8. Together, these data suggest that Rictor residues 975-1039

contain one of the critical sites in Rictor that form cooperative contacts with mSin1.1 and LST8.

Directly adjacent to the mTORC2 stabilization region in Rictor is the acetylated region (residues 1041-1137) which is required for acetylation-induced increases in mTORC2 activity. We approached the identification of functionally important Lys within Rictor by testing progressively smaller deletion constructs for acetylation by p300. This strategy narrowed the pool of potential sites from a total of 96 to 9 Lys located between residues 1041 and 1137. This region was confirmed to contain acetylated Lys, since GST-1040-1140 was robustly acetylated by p300 and because deletion of this region resulted in hypoacetylation. The functional importance of this acetylated region was further supported by the observed decrease in the mTORC2 *in vitro* kinase activity associated with RictorΔ1041-1137, relative to WT. In cells expressing Rictor proteins lacking the acetylated region, endogenous Akt S473 phosphorylation was reduced compared to that in cells expressing WT Rictor.

Since this region possesses no known structural features and acetyltransferases often modify proximal Lys within Lys-rich sequences (Thompson *et al.*, 2004), we subdivided the 9 Lys into 4 Lys-rich modules (M1 through M4). Mutational analysis of the lysine-rich modules demonstrated that Rictor K→R M2 and M4 were hypoacetylated and when reconstituted with other mTORC2 components showed reduced kinase activity, relative to WT Rictor. These observations highlight the contribution of acetylation of both M2 and M4 to the kinase activity of mTORC2. Thus, acetylation of multiple modules across this

region is required for acetylation-induced increases in mTORC2 kinase activity, suggesting that the acetylation of lysines within the M2 and M4 may act as an mTORC2 activity potentiation switch. Although the mechanism of mTORC2 activation by acetylation of Rictor is not known, a hypothetical comparison can be made to the activation of p300 by autoacetylation. p300 is activated by autoacetylation of 12 proximal sites, which relieves repression of an autoinhibitory loop (Thompson *et al.*, 2004). Since acetylation of lysine residues neutralizes the positive charge, acetylation across a region can significantly alter intra- or intermolecular interactions. Thus, Rictor acetylation may alter its interaction with other mTORC2 members. In support of this hypothesis, increased association of LST8 with Rictor-complexes is observed upon expression of p300, while a decrease in LST8 binding is seen with hypoacetylated Rictor mutants. Since LST8 is known to promote the kinase activity of mTOR, these results merit further study into the possible effect of Rictor acetylation on the association of LST8 with mTORC2 complexes (Guertin *et al.*, 2006).

However, our experiments cannot rule out the possibility that Rictor acetylation alters its interaction with mTORC2 regulatory proteins, tuberous sclerosis complex proteins (TSC) -1/2 or Deptor. The TSC1/2 complex was recently found to be required for PI3K-activation of mTORC2 kinase activity and to specifically interact with Rictor-containing mTORC2 but not Raptor-containing mTORC1 (Huang *et al.*, 2008). Acetylation of Rictor may enhance its interaction with TSC1/2. Alternatively, acetylation also regulates protein function by

disrupting protein-protein interactions (Cohen *et al.*, 2004). Therefore, Rictor M1-M4 acetylation may reduce its interaction with the mTOR-inhibitor Deptor. Future work is needed to elucidate whether Rictor acetylation alters the association of Deptor or TSC1/2 with mTORC2.

The activation of mTORC2 by Rictor acetylation is a unique case in which acetylation of the adaptor alters the activity of the catalytic subunit. Modulation of catalytic activity of a multiprotein complex by modification of an adaptor protein has been shown for phosphorylation (Foster *et al.*, 2010), but has not previously been appreciated for acetylation. Large-scale proteomic analysis of acetylated proteins has identified a preponderance of acetylated proteins in enzymatic protein complexes, including acetylation of multiple lysines of adaptor proteins (Choudhary *et al.*, 2009). Our work suggests that the acetylation of these adaptor proteins may contribute to regulation of their catalytic binding partners.

The “multi-site protein modification” hypotheses have highlighted the impact of the interplay between protein modifications in regulating diverse protein functions (Sims and Reinberg, 2008; Yang, 2005; Yang and Seto, 2008b). The Rictor acetylated region contains an S6K1 phosphorylation site, T1135. This raises questions as to whether these modifications influence one another (Julien *et al.*, 2009; Dibble *et al.*, 2009; Treins *et al.*, 2009). However, we found that T1135 was not important for Rictor acetylation and previous work has shown that T1135 phosphorylation does not affect mTORC2 kinase activity (Julien *et al.*, 2009; Dibble *et al.*, 2009; Treins *et al.*, 2009). These findings suggest that T1135

phosphorylation is not involved in the enhancement of mTORC2 activity by Rictor acetylation.

Our data suggests that Rictor acetylation is either promoted or protected when it is assembled into the mTORC2 complex. Expression of the entire mTORC2 complex enhances Rictor acetylation relative to expression of Rictor alone, while deletion of regions resulting in a loss of mTORC2 formation reduces Rictor acetylation. In addition to mSin1.1, mSin1.2 and mSin1.5, but not mSin1.4, form mTORC2 complexes that promote Rictor acetylation (Fig. 11). Since all mSin1 variants, except mSin1.4, have previously been shown to assemble into mTORC2, this supports the finding that mTORC2 formation promotes Rictor acetylation (Frias *et al.*, 2006). Importantly, the activity of mSin1.5-containing mTORC2 is growth factor-independent, suggesting that acetylated Rictor regulates growth factor-dependent and independent mTORC2.

In addition to growth factors, mTORC1 is regulated by the availability of amino acids, cellular energy status and oxidative stress (reviewed by Sengupta *et al.*, 2010). Similarly, mTORC2 activity is activated by amino acids, resulting in phosphorylation of Akt S473 (Tato *et al.*, 2010). Work presented here provides a possible mechanism for coupling mTORC2 activity to cellular metabolism. We show that the Sirtuin inhibitor NAM increases Akt S473 phosphorylation and Rictor acetylation. Sirtuins have been shown to regulate numerous metabolic signaling proteins and enzymes, linking these functions to NAD^+ metabolism (Li *et al.*, 2008; Bao and Sack, 2010; Lan *et al.*, 2008; Longo, 2009; Zhong *et al.*, 2010). We have found that SIRT1, SIRT2, SIRT3 or SIRT4 are capable of

decreasing Rictor acetylation in cells and that SIRT1 deacetylates Rictor *in vitro* in an NAD^+-dependent manner (Fig. 12 and Fig. 5D). Additionally, Rictor acetylation and mTORC2 activity are sensitive to both NAM and TSA treatment and are therefore regulated by both Sirtuin and Class I/II/IV HDACs. This redundancy in the regulation of mTORC2 by HDACs is not surprising, since it is a critical cell signaling complex and because most acetylated targets are regulated by both families of HDACs (Glozak *et al.*, 2005; Zhang and Kraus, 2010).

Acetylation has recently been linked to cell metabolism not just by the Sirtuins, but also by the availability of the acetyl-group donor and metabolite signaling molecule, acetyl CoA. ACL produces cytosolic acetyl-CoA from the TCA cycle intermediate, citrate, and is required for growth factor induced increases in global histone acetylation (Wellen *et al.*, 2009). Expression of genes, including glucose transporter Glut4, is positively regulated by the availability of glucose in an ACL-dependent manner (Wellen *et al.*, 2009). Thus two nutrient-sensitive metabolite signaling molecules, NAD^+ and acetyl-CoA can modulate acetylation in response to growth factors, glucose availability, and metabolic processes. The acetylation of Rictor within M1-M4 is one clear example where nutrient-responsive enzymes dynamically regulate mTORC2 activity.

2.11: Materials and Methods

Cell culture, reagents, and plasmid constructs

HeLa and HEK293T cells were obtained from ATCC and maintained in DMEM (CellGro), 10% FBS (Invitrogen) and penicillin/streptomycin (Invitrogen). The plasmid encoding HA-Akt was described previously (Madrid *et al.*, 2000). Plasmids encoding myc-mSin1.1 (Addgene plasmid 12576), myc-mSin1.2 (Addgene plasmid 12577), myc-mSin1.4 (Addgene plasmid 12578), myc-mSin1.5 (Addgene plasmid 12579), HA-mSin1.1 (Addgene plasmid 12582) and HA-LST8 (Addgene plasmid 1865) were from DM Sabatini through Addgene. pcDNA3-HA/FLAG-Rictor, pcDNA3-HA-mTOR, and pcDNA3-HA-Raptor were a generous gift from JC Lawrence. Plasmids encoding V5-SIRT1 and FLAG-tagged SIRT2-7 were generous gifts from E Verdin. The antibodies used were: α-M2 FLAG-agarose (Sigma); α-HA.11 and α-HA.11-sepharose (Covance); and α-GST (Santa Cruz). All other antibodies were from Cell Signaling. Glutathione sepharose 4B were from GE Healthcare. Recombinant SIRT1 was from Enzo, recombinant human inactive Akt1 was from Millipore and recombinant IGF1 was from Invitrogen. NAM, splitomicin, TSA and Wortmannin were from Calbiochem. ATP was from Roche. Baculogold protease inhibitors were from BD Biosciences. All other chemicals were from Sigma.

Subcloning and site-directed mutagenesis

The plasmid encoding p300 HAT was generated by subcloning the region encoding residues 1066-1707 of human p300 into pcDNA3. The HA-tagged pcDNA3-Rictor 1-1188 construct was generated by restriction digest of pcDNA3-HA/FLAG-Rictor with Kpn I and Apa I, creation of blunt ends with Klenow and re-ligation of the large fragment containing vector backbone, HA-tag and encoding Rictor 1-1188. RictorΔ975-1188, RictorΔ1041-1188, and RictorΔ1141-1188 were generated by PCR of a fragment of Rictor with an engineered Kpn I site immediately downstream of the nucleotides encoding amino acids 974, 1040 and 1140, respectively. These PCR fragments were subcloned into pcDNA3-HA/FLAG-Rictor using endogenous Sac II and Kpn I sites. All other Rictor constructs were generated by subcloning a PCR fragment into pCMV-FLAG2, or for GST-constructs, pEBG, using pcDNA3-HA/FLAG-Rictor as the template. HA/FLAG-Rictor K→R mutants were generated by site-directed mutagenesis at the following sites: M1 (K1080, 1082), M2 (K1092, K1095), M3 (K1107, K1108), M4 (K1116, K1119, K1125) and M1-M4 (K1080, K1082, K1092, K1095, K1107, K1108, K1116, K1119, K1125).

Deacetylase inhibitor and IGF1 treatments

Prior to IGF1 treatment, cells were starved of serum to reduce basal growth factoring signaling and increase responsiveness to IGF1. Subconfluent Hela cells were starved of serum overnight in DMEM containing 5 mM glucose. For deacetylase inhibition, cells were treated with deacetylase inhibitor doses

optimized by a post-doc in the lab, namely 500 nM TSA, 500 μM NAM, 10 μM Splitomicin or DMSO (vehicle control). Cells were treated with deacetylase inhibitors for 4 hours prior to IGF1 addition, to maximize responsiveness while minimizing increases in gene expression. Deacetylase inhibitor treatments were the same for 293T cells, except that cells were starved of serum overnight in DMEM containing 25 mM glucose overnight prior to inhibitor and IGF1 treatment. All IGF1 treatments used 10 ng/mL IGF1 for 15 minutes for acute, sub-maximal stimulation.

Transfections

Plasmids were transiently transfected into HEK 293T and HeLa using Polyfect reagent (Qiagen) according to the manufacturer's instructions. For HeLa cells transfected with HA/FLAG-Rictor constructs, cells were washed in PBS and fresh DMEM with 10%FBS was added 4 hrs post-transfection. The media was replaced again after 24 hrs. Cells were incubated an additional 72 hrs prior to lysis and analysis of lysates by Western blotting.

Cell lysis, Immunoprecipitation and Western blot

For immunoprecipitation, cells were lysed in 0.3% CHAPS, 50 mM Hepes and 200 mM NaCl, pH 7.5, with 5 mM NAM, 500 nM TSA, 1 mM DTT, 1X phosphatase and 1X Baculogold protease inhibitors. Cell lysates were pre-cleared with mouse IgG and protein-G agarose for 30 min (Santa Cruz) at 4°C with tumbling. Pre-cleared lysates were mixed with primary antibodies and

protein-G agarose or resin-conjugated primary antibodies and rotated end-over-end at 4°C for 2 hrs. Immune-complexes were washed 3 times in lysis buffer and either analyzed by Western blotting or subjected to *in vitro* enzymatic assays described below. Western blotting was performed using the NuPAGE system (Invitrogen). Briefly, proteins were separated on 4-12% Bis-Tris polyacrylamide gels and transferred onto nitrocellulose membranes (Whatman). Primary antibodies were used at 1:1000 dilution, except for α-HA, which was used at 1:5000, in blocking solution. HRP-conjugated secondary antibodies (Promega) were used at 1:5000 in blocking solution. Signals were detected by chemiluminescence using ECL plus reagent (Amersham) exposed to film (Kodak).

In-cell-acetylation assays, in vitro deacetylation assay and immunokinase assays

In-cell-acetylation assays were performed as described (Chen *et al.*, 2002). HEK 293T cells were transfected with expression plasmids encoding HA-tagged mTOR, Rictor, Raptor, mSin1.1, LST8, or Akt, with or without p300 HAT. Cells were lysed after 24 hrs and target proteins were immunoprecipitated with α-HA antibodies (mTOR, Rictor, Raptor, mSin1.1) or α-HA-sepharose (Akt, LST8) and analyzed by immunoblotting with α-pan acetyl-Lys antibody (Ac-Lys). In-cell-acetylation assays with GST construction were performed as described, except that glutathione-sepharose was used for GST protein precipitation. For in-cell-acetylation assays with the mTORC2 complex, HEK 293T cells were co-

transfected with expression plasmids encoding HA/FLAG-Rictor, HA-mTOR, HA-LST8 and HA-mSin1.1 with or without p300 HAT. 24 hours later, cell lysates were subject to immunoprecipitation with α-FLAG agarose. *In vitro* deacetylation assays were performed by incubating immunoprecipitated HA/FLAG-Rictor protein in deacetylase buffer (Biomol) containing 500 μM NAD^+ and 5 U recombinant SIRT1 enzyme, for 2 hr at 30°C. For kinase assays, 293T cells were transfected with mTORC2 as described and the medium was replaced after 4 hrs. 24 hrs later, cells were washed twice and incubated in serum-free DMEM overnight. Cells were treated with IGF1 prior to lysis and immunoprecipitation. Following standard washes, immunoprecipitants were washed once in kinase assay buffer containing 25 mM Hepes, pH 7.5, 100 mM potassium acetate, 0.01% BSA, 1 mM DTT and 1X phosphatase inhibitor cocktail 1 (Sigma). Immunoprecipitants were mixed with 0.5 μg recombinant Akt1 and 1 mM ATP in a total volume of 15 μl kinase assay buffer and incubated at 37°C for 30 min. For mTOR inhibition, 2 μM Wortmannin was added to the kinase assay buffer. The reactions were stopped by addition of 2X LDS-PAGE sample buffer (Invitrogen) containing 100 mM dithiothreitol and boiling for 10 minutes. Samples containing 10 ng Akt1 were analyzed by immunoblotting as described above.

2.12: References

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Chapter 3: General Discussion and Future Directions

3.1: General Discussion

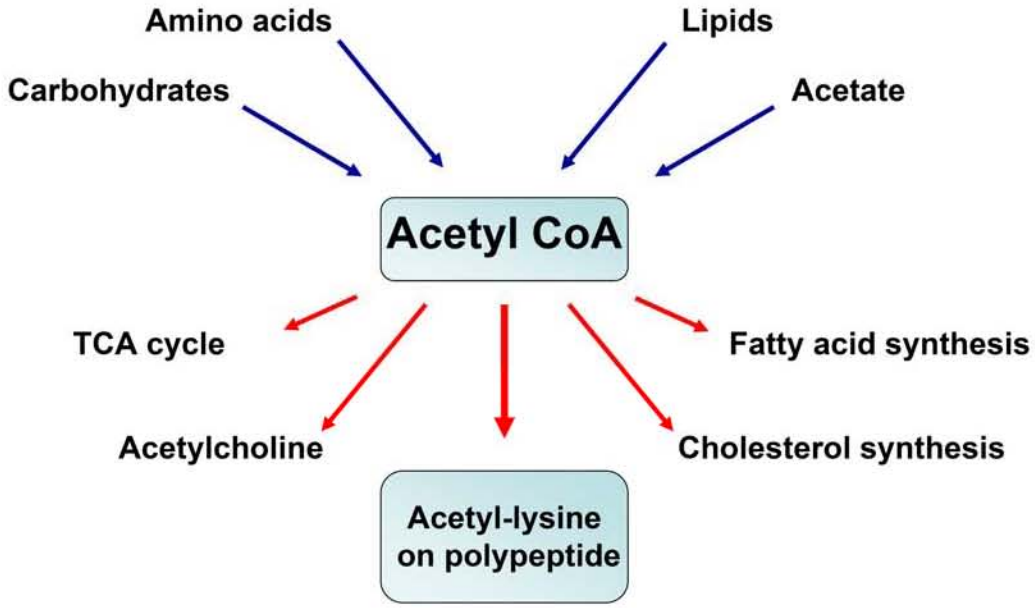
Insulin and IGF1 are homologous endocrine signaling proteins that activate PI3K upon binding to their physiological receptors. Canonical insulin signaling is limited to specific tissues that express insulin receptor. Since the insulin receptor is important for regulation of glucose homeostasis, it is not surprising that it is expressed in skeletal muscle, liver, kidney, and fat. In contrast, the IGF1 receptor is expressed in most cell types, resulting in IGF1 signaling to peripheral tissues. Activation of PI3K in response to insulin or IGF1 stimulates phosphorylation of Akt by PDK1 at T308 and by mTORC2 at S473, as shown in Figure 2. Phosphorylation at both of these sites is required for increased activation of Akt, which phosphorylates substrates to promote cell growth, proliferation, survival and metabolism (Bellacosa *et al.*, 1998; Alessi *et al.*, 1996).

mTORC2 is a nutrient-responsive kinase complex activated by growth factors, insulin and amino acids (Figure 3). mTORC2-mediated Akt phosphorylation at S473 is particularly important for regulation of cell survival and metabolism via FOXO transcription factors and glucose uptake and glycogen synthesis in insulin-responsive tissues. Thus, mTORC2 is a key regulator of cellular and physiological metabolism in response to the availability of nutrients and growth factors.

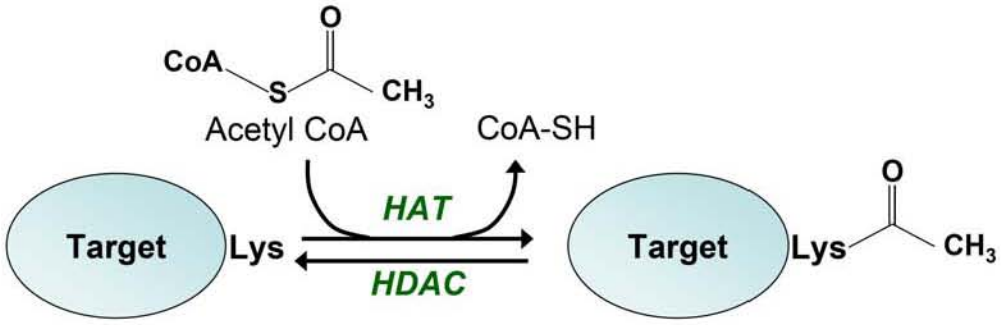
Like phosphorylation, acetylation is a reversible regulatory post-translational modification. Acetylation is linked to cell metabolism by the metabolite signaling molecule acetyl CoA, which is used by HATs as the donor of

Figure 13: Acetyl CoA is a metabolite signaling molecule. A) Acetyl CoA is produced (blue arrows) from the breakdown of nutrients and utilized (red arrows) in various processes including the TCA cycle, the synthesis of macromolecules, and in the acetylation of lysines within polypeptides. B) Histone acetyltransferases (HATs) use acetyl CoA to catalyze the addition of an acetyl group to lysine residues within polypeptides. Lysine acetylation is reversed by the action of histone deacetylases (HDACs), which remove the acetyl moiety, regenerating the unmodified protein target.

A.


Amino acids
Lipids
Carbohydrates
Acetate
Acetyl CoA
TCA cycle
Fatty acid synthesis
Acetylcholine
Cholesterol synthesis
Acetyl-lysine
on polypeptide

B.


CoA
S
O
CH3
Acetyl CoA
CoA-SH
HAT
HDAC
Target
Lys
Target
Lys
O
CH3

the acetyl moiety. Figure 13 shows the roles of acetyl CoA in both cell metabolism and protein acetylation. Acetyl CoA is an important metabolite produced by the breakdown of carbohydrates, lipids and amino acids. It also can be synthesized from acetate, particularly when nutrient sources are scarce (Shimazu *et al.*, 2010). Acetyl CoA is used in the TCA cycle and in the synthesis of fatty acids, cholesterol and the neurotransmitter acetylcholine. Thus, acetyl CoA is poised to connect the availability of nutrients for its synthesis to the activity of HATs. In support of the idea this idea, the enzyme that converts citrate to acetyl CoA in the cytoplasm and nucleus, ATP-citrate lyase, is required for growth-factor induced increases in global histone acetylation (Wellen *et al.*, 2009).

The regulation of acetylated targets within the cell is also linked to cell metabolism through the activity of Sirtuins, which utilize oxidized nicotinic adenine dinucleotide (NAD^+) as a cosubstrate to deacetylate protein targets (Bao and Sack, 2010; North and Verdin, 2004). Sirtuins are activated in response to nutrient depletion due to a rise in the intracellular levels of NAD^+ (Lin *et al.*, 2000; Anderson *et al.*, 2002; Dioum *et al.*, 2009). Thus, acetylation is linked to cell metabolism through the availability of two metabolite signaling molecules, acetyl-CoA and NAD^+. When nutrients are abundant, the availability of acetyl CoA may promote acetylation, whereas depletion of nutrients may promote NAD^+-dependent deacetylation by Sirtuins.

Since acetylation is linked to cell metabolism and mTORC2 is a nutrient sensor, we hypothesized that mTORC2 is regulated by dynamic acetylation. Our

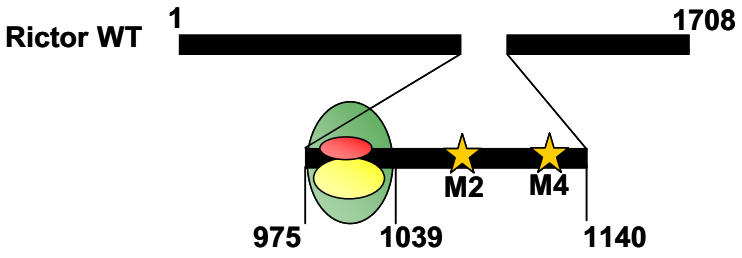
original hypothesis, shown in Figure 4, predicted that acetylation of mTORC2 under conditions of nutrient abundance, promotes mTORC2-mediated phosphorylation of Akt in response to IGF1. In contrast, we predicted that depletion of cellular nutrients promotes Sirtuin-mediated deacetylation of mTORC2 components resulting in reduced IGF1-stimulated mTORC2 activity.

Results presented in Chapter 2 identify the mTORC2 component Rictor as a direct target of acetylation. Dynamic acetylation of Rictor by p300 modulates mTORC2 kinase activity, suggesting that the metabolite signaling molecule acetyl CoA may promote Akt S473 phosphorylation. Rictor is deacetylated by SIRT1 *in vitro* in an NAD^+-dependent manner, suggesting that Rictor can be directly deacetylated by the NAD^+-dependent deacetylases. Surprisingly, acetylation of Rictor is sensitive to inhibitors of both Sirtuin and Class I/II/IV deacetylases, suggesting that Rictor is deacetylated by multiple deacetylase families. This finding is importance since an increase in Rictor acetylation, following the inhibition of deacetylases, enhanced Akt phosphorylation at S473. Together, these findings suggest that members of both deacetylase families target Rictor, resulting in reduced mTORC2 activity upon IGF1 stimulation. These conclusions are presented in Figure 14B. These results are not surprising given the importance of regulating mTORC2 activity in response to multiple nutrient and stress signals.

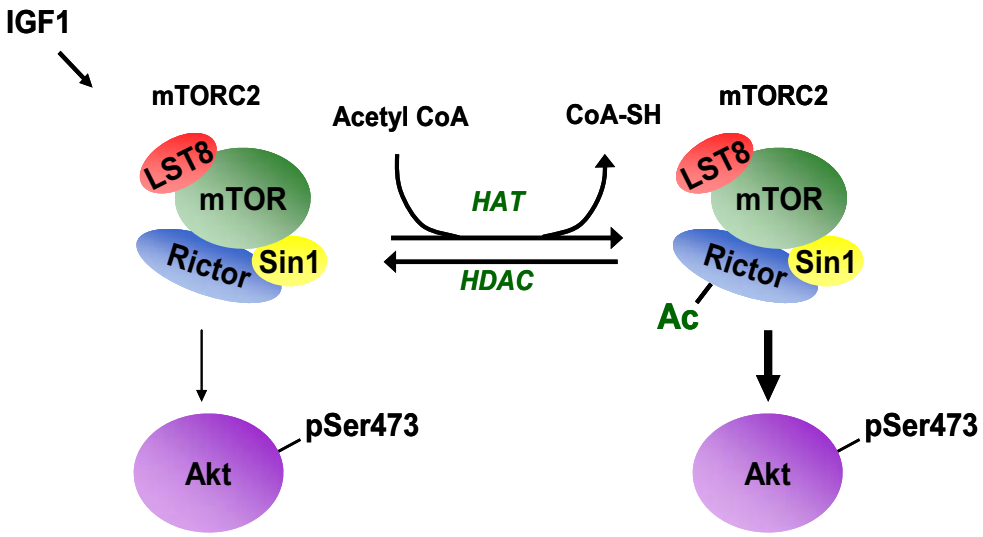
During my work two adjacent functional regions were identified within the relatively uncharacterized Rictor protein (Figure 14A). The region between amino acids 975 and 1039 was shown to be required for Rictor association with

Figure 14: Multi-site acetylation of Rictor links the availability of acetyl CoA to IGF1-stimulated mTORC2 activation. A) Identification of two adjacent functional regions within Rictor. Rictor residues 975-1039 contain a region required for mTORC2 integrity (yellow, green and red shaded ovals). The acetylation region contained between amino acids 1040 and 1140 contains multiple acetylated Lys within M2 and M4 (yellow stars) that contribute to increased mTORC2 activity. B) The availability of the metabolite signaling molecule acetyl CoA is predicted to increase acetylation of Rictor resulting in increased mTORC2-mediated Akt phosphorylation at S473 in response to IGF1. In contrast, deacetylation of Rictor by HDACs reduces IGF1 stimulated mTORC2 activity, resulting in decreased Akt S473 phosphorylation.

A.


Rictor WT
1
1708
M2
M4
975
1039
1140

B.


IGF1
mTORC2
LST8
mTOR
Rictor
Sin1
Acetyl CoA
CoA-SH
HAT
HDAC
mTORC2
LST8
mTOR
Rictor
Sin1
Ac
Akt
pSer473
Akt
pSer473

mSin1.1 and LST8 and is thus essential for the integrity of the entire mTORC2 complex. Adjacent to this region, between amino acids 1040 and 1140, resides the acetylated region containing the Lys-rich modules M1-M4. Work presented here indicates that acetylation of multiple Lys within M2 and M4 promotes mTORC2 kinase activity.

Our studies have focused on the mTORC2-mediated activation of Akt1 in response to IGF1 because these molecules are expressed ubiquitously, and are not tissue-specifically restricted. However, based on our current work we predict that acetylation of Rictor most likely contributes to mTORC2-mediated activation of Akt2 in response to insulin as well. Akt2 is the predominant isoform involved in insulin-stimulated glucose uptake and regulation of glucose homeostasis (Cho *et al.*, 2001; Garofalo *et al*., 2003). We predict that the availability of acetyl CoA may provide an important link between glucose metabolism and mTORC2-mediated Akt2 activation in insulin-responsive tissues. In contrast, we predict that nutrient depletion may promote Sirtuin-mediated deacetylation of Rictor, thus inhibiting insulin-stimulated mTORC2-mediated Akt2 phosphorylation. In summary, dynamic regulation of Rictor acetylation in response to acetyl CoA and NAD^+ metabolism may provide important regulatory controls in insulin-regulated physiology.

The precise mechanism of insulin/IGF1-stimulated mTORC2 activation is not known, however, it has been postulated that mSin1.1 or mSin1.2 may be responsible for PI3K-dependent membrane recruitment via their respective pleckstrin homology domains. In other words, PI3K-dependent generation of

PIP_3 most likely is responsible for recruiting the mSin1.1/1.2-containing mTORC2 to the membrane where it subsequently phosphorylates Akt S473. Furthermore, there may also exist an additional positive feedback mechanism that links Akt S473 phosphorylation with increased p300 activity. Our lab has shown that Akt phosphorylates the acetyltransferase p300 at S1834, resulting in increased p300 catalytic activity (Huang and Chen, 2005; Liu *et al.*, 2006). S1834 is conserved in the p300 paralog CBP, suggesting that CBP may also be stimulated by Akt. Increased p300 catalytic activity upon phosphorylation at S1834 by Akt results in increased histone acetylation and gene expression (Huang and Chen, 2005; Liu *et al.*, 2006). Thus, this may establish a positive feedback loop in which activation of Akt potentiates p300-mediated acetylation of Rictor, resulting in increased mTORC2-mediated Akt activation. A positive feedback loop involving increased p300/CBP-mediated acetylation of Rictor represents a promising direction in the study of mTORC2 activation by growth factors and insulin.

3.2: Rictor acetylation promotes IGF1-stimulated Akt phosphorylation

Experimental evidence indicates that Rictor acetylation signals for increased activation of mTORC2. Induction of Rictor acetylation increases mTORC2 kinase activity, while mTORC2 reconstituted with hypoacetylated Rictor mutants has reduced kinase activity compared to WT Rictor-complexes (Fig. 6B, Fig. 10A and Fig. 10D). The physiological relevance of Rictor acetylation has been demonstrated by a reduction in cellular levels of IGF1-stimulated Akt phosphorylation upon expression of a hypoacetylated Rictor mutant in HeLa cells

(Fig. 10B). However, the caveat of these experiments is that they were performed in the presence of endogenous WT Rictor, which may mask the defect observed with RictorΔ1041-1137 expression. For this reason, future investigation of the contribution of Rictor acetylation to IGF1-stimulated mTORC2 phosphorylation of Akt at S473 will be performed in a Rictor-null background.

In order to accomplish this, future experiments will compare IGF1-stimulated Akt phosphorylation at S473 in Rictor$^{-/-}$ mouse embryonic fibroblasts (MEFs) reconstituted with exogenous Rictor WT or Rictor site-directed mutant K→R M1-M4. We have obtained Rictor$^{-/-}$ MEFs and WT MEFs, kindly provided by the laboratory of the late JC Lawrence, Jr with permission from the Magnuson laboratory (Shiota *et al.*, 2006). Rictor$^{-/-}$ MEFs stably expressing Rictor transgenes will be generated by infection with recombinant retroviruses encoding Rictor proteins. For virus generation, we will use a replication-deficient vesicular stomatitis virus G (VSV-G) retrovirus system. For Rictor expression we will use pBABE-puro, obtained from Dr. Bob Weinberg, which has an E2F promoter upstream of the multiple cloning site and encodes a puromycin resistance gene that enables selection of stably-transformed cells (Morgenstern and Land, 1990).

We will follow a protocol modified from the Wang lab (Yang *et al.*, 2003). DNAs encoding HA/FLAG-Rictor WT or HA/FLAG-Rictor K→R M1-M4 will be cloned into pBABE-puro. pBABE-puro Rictor WT, Rictor K→R M1-M4 and empty vector will each be used to generate recombinant VSV-G. Briefly, pBABE-puro constructs will be transfected into 293T cells together with pVSV-G and pGAG-

POL. After 2 days, virus will be collected from the cell media and stored at −80°C. Recombinant viruses will be used to infect Rictor$^{-/-}$ MEFs.

Pools of puromycin-resistant Rictor$^{-/-}$ MEFs infected with WT and M1-M4 Rictor will be assayed for Rictor expression by Western blotting with α-FLAG antibodies. If equivalent levels of Rictor are detected in stable pools, we will proceed to the Akt assay. If Rictor levels differ between the populations, we will expand populations, select for subclones, create clonal pools and assess the Rictor expression in each of these populations. To assay Akt phosphorylation, cells starved of serum overnight in Dulbecco's modified eagle medium (DMEM) containing 25 mM glucose will be stimulated with 10 ng/mL IGF1 for 15 minutes, washed and lysed in ice-cold lysis buffer. Cell lysates will be analyzed for phosphorylated Akt by Western blotting using α-phospho Ser473 Akt and α-phospho Thr308 Akt antibodies. Detection of total Akt levels will serve as a loading control.

Based on work described in this thesis, we predict that the ability of mTORC2 to phosphorylate Akt at S473 in response to IGF1 will be reduced in Rictor-K→R M1-M4 expressing MEFs relative to Rictor-WT MEFs. Further analysis of the phosphorylation of Akt substrates, such as Foxo1, Foxo3a, Bad, and GSK3β, in the Rictor-K→R M1-M4 and Rictor-WT MEFs will confirm the importance of Rictor acetylation in regulating Akt activity.

3.3: Determination of the temporal sequence of mTORC2 activation

My research has shown that Rictor acetylation increases mTORC2 activity in the presence of IGF1. However, it is not known whether IGF1 stimulates Rictor acetylation prior or subsequent to mTORC2 activation. To address this question, it will be important to detect endogenous acetylation of Rictor. This will also accomplish the important goal of verifying the modification of Rictor by acetylation under physiological conditions. To detect endogenous acetylated Rictor, future plans are to develop a site-specific acetyl-Rictor antibody. However, in order to develop a site-specific acetyl-Rictor an antibody, we need to identify specific acetylated Lys within M1-M4. There are two approaches that can be carried out in parallel to address this aim. One is the use of mass spectrometry (MS) to identify acetylated Lys in Rictor. The second method is to use site-directed mutagenesis of individual sites and evaluate the acetylation status and mTORC2 activity associated with single-site Rictor mutants.

For identification of acetylated Lys within Rictor by MS, we will use stable-isotope labeling by amino acid in cell culture (SILAC), which utilizes a “heavy” and “light” stable isotope of lysine to enhance detection of acetylated peptides (Ong *et al.*, 2002; Choudhary *et al.*, 2009). HEK 293T cells will be grown in medium containing either the “heavy” or “light” isotopes of lysine and transfected with GST-Rictor 1040-1140, with or without p300 HAT. To examine whether we can detect endogenous acetylation, cells transfected with GST-Rictor 1040-1140 will be either left alone or stimulated with IGF1 for 15 minutes. Based on Figures 8B and 9D, GST-Rictor 1040-1140 contains the Lys in M2 and M4 that are critical

to acetylation-mediated mTORC2 augmentation. Additionally, this Rictor construct can be expressed and purified with glutathione-sepharase under partially denaturing conditions to minimize co-precipitating contaminants and to preserve the post-translational modifications. Since M1-M4 are Lys-rich and trypsin cleaves proteins at Lys and Arg, we will use partial tryptic digest to generate peptides with Lys preserved. Purified peptides can be subjected to MS and analyzed for acetylated Lys.

This strategy is predicted to provide important confirmation of acetylation within M1-M4 of Rictor. Additionally, detection of acetylated Lys in the absence of p300 HAT will comprise the first evidence of direct acetylation of Rictor by an endogenous HAT in response to IGF1. Moreover, acetylated Lys detected within M2 and M4 from samples generated in the absence or presence of p300 HAT will be used to identify residues critical for mTORC2 activity.

The second strategy is to identify critical acetylated Lys in Rictor using single-site directed mutagenesis. We have shown that Rictor M2 and M4 contain acetylated Lys that contribute to mTORC2 activity (Fig. 10D). Single Rictor K→R mutants of K1092, K1095, K1116, K1119 and K1125 will be evaluated for acetylation status and mTORC2 activity using an in-cell-acetylation assay and immunokinase assay, respectively (Fig. 10D).

Once critical sites have been identified, we will generate site-specific α-acetyl-Rictor antibodies. The Mayo lab has prior experience with generating site specific α-acetyl-specific antibodies that will assist in the selection of immunogenic peptides and generation of α-Rictor acetyl-lysine specific

antibodies (Hoberg *et al.*, 2006; Ramsey, Yeung and Mayo, unpublished data). Acetyl-Rictor antibodies, once tested for specificity, can be used in a number of applications. First, they will be used to detect endogenous acetylation of Rictor in response to IGF1, an important step in verifying the physiological relevance of Rictor acetylation. Second, experiments investigating the acetylation status of Rictor in response to different physiological conditions will be possible. For example, one will be able to determine whether Rictor is acetylated in response to numerous growth factors. These important questions can be addressed by manipulation of the cellular growth media and performing Western blots with the acetyl-Rictor antibody. The predicted results of these experiments are that Rictor acetylation is stimulated by the presence of glucose and growth factors, including IGF1. These results will initiate the investigation into the order of Rictor acetylation and mTORC2 activation, since mTORC2 activity is known to increase in response to growth factors (Sarbassov *et al.*, 2005; Frias *et al.*, 2006). Finally, the development of a specific α-acetyl-Rictor antibody will allow one to interrogate the order of Rictor acetylation and mTORC2-dependent phosphorylation of Akt in response to IGF1 stimulation.

3.4: O-GlcNAc modification sites predict critical acetylated Lys

Another potential strategy for identifying critical acetylated Lys is to make a prediction based on primary sequence information and the post-translational modification O-GlcNAc. David Allison and colleagues in the Mayo lab have identified that O-GlcNAc modification of the RelA/p65 subunit of NF-κB promotes

acetylation of K310, a key event in the transcriptional activation of NF-κB (Allison *et al.*, in preparation). In our lab, increased GlcNAcylation via expression of OGT has also been found to increase the acetylation status of SMRT and MEK (Popko and Mayo, unpublished data; Yeung and Mayo, unpublished data). Additionally, previous work from the Groner lab has demonstrated preferential association of CBP with GlcNAcylated Stat5 (Gewinner *et al.*, 2004), suggesting that GlcNAcylation may promote HAT recruitment to proteins. The results of the work of Allison and colleagues support a hypothesis that GlcNAcylation of p65 at T305 recruits p300 to K310, stimulating acetylation at this site. We have performed a multiple sequence alignment of p65 K310 with two biologically significant acetyl-lysine sites, K382 in p53 and K27 in histone H3 (Zhao *et al.*, 2006; Lau and Cheung, 2011). Interestingly, the alignment of the amino acid sequences surrounding known p300-mediated acetyl-lysine also shows alignment of either a Ser or Thr with the GlcNAcylation site of p65, T305 (Figure 15). Since both Ser and Thr can be modified with O-GlcNAc, our analysis of this alignment predicts a consensus sequence for dual post-translational modification involving O-GlcNAcylation and acetylation T/SXXXXK, where T/S is O-GlcNAc modified, X is any amino acid and K is a critical acetylation site. With this consensus O-GlcNAcylation and acetylation site prediction in mind, we next performed a multiple sequence alignment of p65 K310 with each of the Lys in Rictor M2 and M4 (Figure 16). This alignment shows that the sequences surrounding Rictor K1095 and K1116, but not the other three Lys, fit the T/SXXXXK predicted consensus site.

Figure 15: Multiple sequence alignment with p65 T305 and K310 predict consensus acetylation site T/SXXXXK. Multiple sequence alignment of segments of human RelA/p65, human p53 and human histone H3 with critical acetylated Lys indicated at position 0: K310 in p65, K382 in p53 and K27 in H3. p65 is GlcNAcylated at T305, indicated at -5.

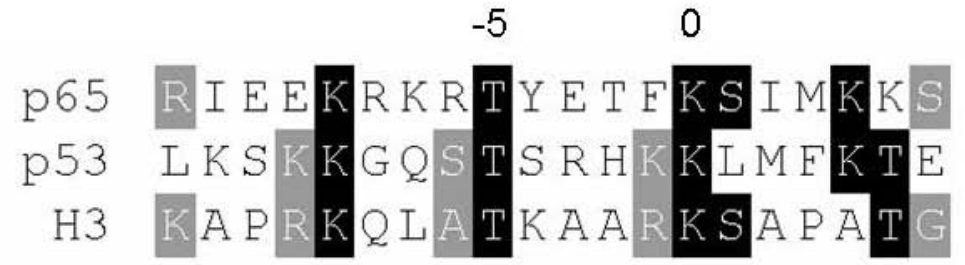
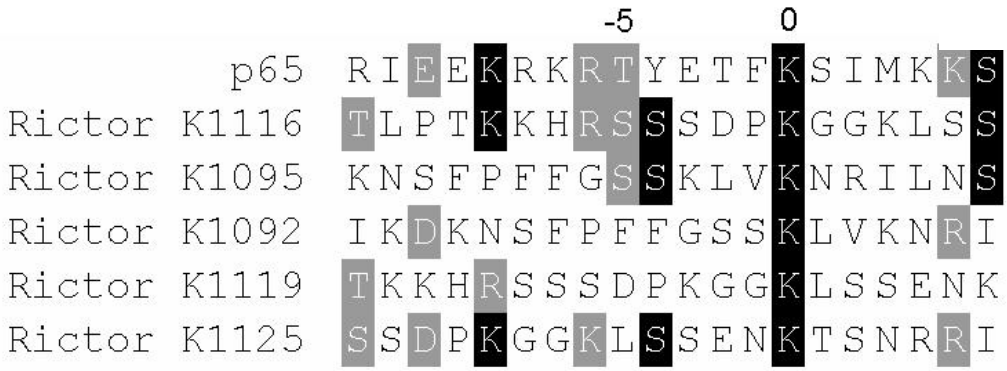

-5
0
p65 RIEEKRKRTYETFKSIMKKS
p53 LKSKKGQSTSRHKKLMFKTE
H3 KAPRKQLATKAARKSAPATG

Figure 16: Rictor K1116 and Rictor K1095 align with p65 consensus acetylation site prediction. Multiple sequence alignment of murine Rictor sequences with M2 and M4 Lys at position 0 indicated at left (i.e. Rictor K1116). Alignment with p65 K310 at position 0 shows T/S at position -5 in sequence around Rictor K1116 and Rictor K1095.

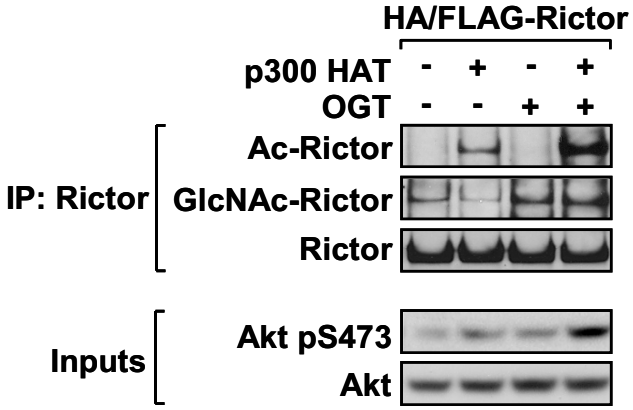

-5
0
p65 RIEEKRKRTYETFKSIMKKS
Rictor K1116 TLPTKKHRSSSDPKGGKLSS
Rictor K1095 KNSFPFFGSSKLVKNRILNS
Rictor K1092 IKDKNSFPFFGSSKLVKNRI
Rictor K1119 TKKHRSSSDPKGGKLSSENK
Rictor K1125 SSDPKGGKLSSENKTSNRRI

There are two hypotheses generated by this analysis. The first is that Rictor K1095 and K1116 are biologically significant acetylation sites in the regulation of mTORC2 kinase activity. The second is that Rictor is O-GlcNAcylated at S1090 and S1111, which promotes Rictor acetylation at K1095 and K1116, respectively, resulting in increased mTORC2 kinase activity. This hypothesis predicts that GlcNAcylation of Rictor positively regulates mTORC2-mediated Akt phosphorylation at S473. Since Akt regulates processes leading to UDP-GlcNAc synthesis, including glucose uptake, glycolytic flux, acetyl CoA synthesis and ATP synthesis, GlcNAcylation of Akt may constitute a positive feedback loop modulated by the availability of UDP-GlcNAc precursors (Kane *et al.*, 2002; Gottlob *et al.*, 2001; Nogueira *et al.*, 2008; Hahn-Windgassen *et al.*, 2005; Berwick *et al.*, 2002; Deprez *et al.*, 1997).

3.5: O-GlcNAc modification of Rictor increases Rictor acetylation and Akt pS473

The alignment of Rictor Lys within the acetylated modules M2 and M4 with the consensus prediction for dual O-GlcNAcylated and acetylated sequences led to the hypothesis that Rictor acetylation is promoted by direct GlcNAcylation. In testing this hypothesis, we first sought to determine whether expression of OGT potentiated Rictor acetylation. We performed in-cell-acetylation assays in which OGT was co-expressed with Rictor. As shown previously in Figure 5B, p300 effectively acetylated Rictor. Importantly, a substantial increase in Rictor acetylation was observed in cells co-expressing OGT (Figure 17). O-GlcNAc

Figure 17: Increased Rictor GlcNAcylation is associated with increased Rictor acetylation and Akt phosphorylation at S473. In-cell-acetylation and GlcNAcylation of Rictor was performed by co-expression of HA/FLAG-Rictor with or without p300 HAT and OGT. Rictor proteins were immunoprecipitated and subjected to Western blotting for post-translational modifications using α-*pan* Acetyl-Lys or α-*pan* O-GlcNAc antibodies or for total Rictor using α-FLAG antibodies. Cell lysates (inputs) were analyzed by Western blotting with the indicated antibodies.


HA/FLAG-Rictor
p300 HAT - + - +
OGT - - + +
IP: Rictor
Ac-Rictor
GlcNAc-Rictor
Rictor
Inputs
Akt pS473
Akt

modification of immunoprecipitated Rictor was detected using a *pan* O-GlcNAc-specific antibody and was increased in the presence of exogenous OGT. Total Rictor protein levels were unchanged by the expression of OGT. Additionally, we detected elevated endogenous Akt pS473 activity in cells co-expressing both p300 and OGT, while Akt protein levels remained the same in the presence of p300 and OGT expression. Altogether, the results of these experiments suggest that acetylation and O-GlcNAcylation of Rictor is biologically important. Additional experiments are needed to determine if O-GlcNAcylation of Rictor contributes to mTORC2 activation.

These results support the hypothesis that GlcNAcylation of Rictor recruits acetyltransferase to acetylate Rictor at Lys that activate mTORC2 activity. Based on the dual modification consensus site prediction, we hypothesize that Rictor is O-GlcNAcylated at S1090 and S1111 to promote Rictor acetylation at K1095 and K1116, respectively, resulting in an increase in mTORC2 kinase activity. Future directions in the Mayo lab are to determine whether these specific Rictor Ser and Lys are post-translationally modified and to evaluate their contribution to IGF1-stimulated mTORC2 activity and Akt-mediated biological functions.

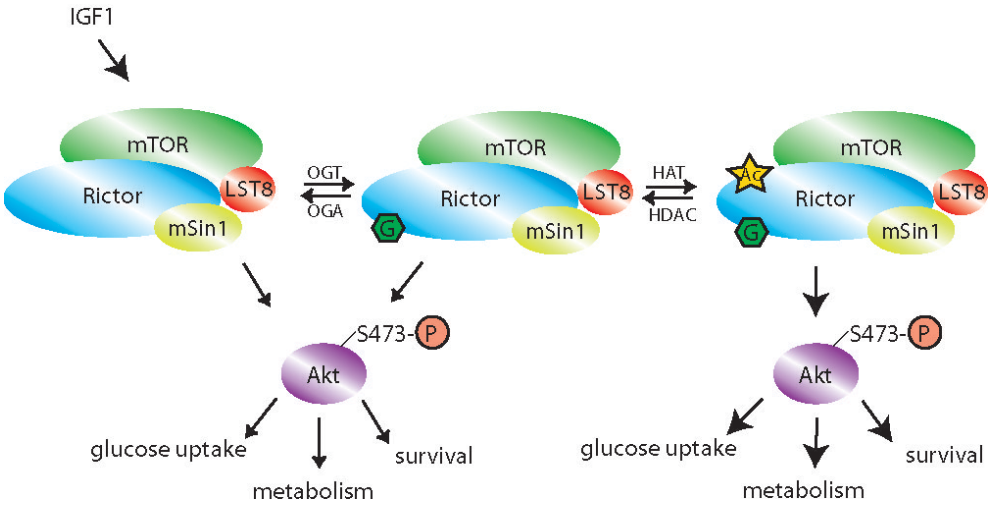
3.6: Sequential O-GlcNAcylation and Acetylation of Rictor Positively Regulate mTORC2 and Akt in the fed state

Preliminary data suggests that GlcNAcylation of Rictor may promote its acetylation and mTORC2 activity. Together with the work of Allison *et al.*,

demonstrating that O-GlcNAc modification specifically increases acetylation of RelA/p65, this led us to construct a model predicting the activation of mTORC2 by sequential GlcNAcylation and acetylation of Rictor. This model predicts that in the absence of GlcNAcylation, Rictor is unacetylated and IGF1 treatment stimulates mTORC2-mediated basal phosphorylation of Akt at S473. GlcNAcylation of Rictor recruits p300 to acetylate Rictor, stimulating mTORC2 kinase activity and increasing IGF1-stimulated Akt phosphorylation at S473. Reversal of Rictor acetylation and O-GlcNAcylation through the activities of HDACs and OGA, respectively, is predicted to decrease IGF1-stimulated mTORC2 activity, resulting in basal Akt phosphorylation at S473. An illustration of this model is shown in Figure 15.

Although GlcNAcylation of protein targets in the insulin/IGF1 pathway has previously been associated with the development of insulin-resistance and decreased Akt phosphorylation at S473 (Hanover *et al.*, 2010; Teo *et al.*, 2010), several lines of evidence point to activation of OGT upon insulin stimulation. In adipocytes, OGT was found to be tyrosine phosphorylated and catalytically activated upon insulin treatment (Whelan *et al.*, 2008). The OGT enzyme contains a unique PIP_3 binding motif, which mediates OGT recruitment to the plasma membrane within 90 seconds of serum stimulation (Yang *et al.*, 2008). OGT membrane translocation is dependent on PI3K, suggesting that OGT is localized to sites of Akt activation. Increased HBP flux has previously been shown to modulate the O-GlcNAc levels of insulin/IGF1-pathway proteins including IRS, PDK1 and Akt (Park *et al.*, 2005; Gandy *et al.*, 2006; Kang *et al.*,

Figure 18: Sequential GlcNAcylation and Acetylation of Rictor Increases IGF1-stimulated mTORC2 activity to Activate Akt. In the absence of GlcNAcylation, Rictor is unacetylated and IGF1 treatment stimulates mTORC2-mediated basal phosphorylation of Akt S473. GlcNAcylation of Rictor recruits p300 to acetylate Rictor, stimulating increased mTORC2 kinase activity and increasing IGF1-stimulated Akt phosphorylation at S473. The activities of OGT and HAT are reversed by OGA and HDACs, respectively.


IGF1
mTOR
Rictor
LST8
mSin1
OGT
OGA
G
HAT
HDAC
Ac
S473-P
Akt
glucose uptake
metabolism
survival

2008; Whelan *et al.*, 2010). This suggests that once localized at the membrane, OGT activity is regulated by UDP-GlcNAc levels. Since UDP-GlcNAc synthesis involves major metabolic pathways, OGT may signal nutrient availability to activate Akt-mediated metabolic and survival signaling via GlcNAcylation of Rictor. Upon depletion of cellular energy, Sirtuin-mediated deacetylation of Rictor may inhibit mTORC2-mediated Akt phosphorylation. Future work in the study of Rictor acetylation and O-GlcNAcylation will determine the physiological conditions and specific enzymes that dynamically regulate Rictor post-translational modification and mTORC2 activity.

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