Quantitative Phosphoproteomics Reveal that mTOR Regulates Cell Growth and Proliferation by Phosphorylating a Functionally Diverse Set of Substrates

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Aaron Mark Robitaille
Seattle, Washington, United States of America (USA)

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Prof. Dr. Michael N. Hall
Dr. Brian Hemmings
Dr. Paul Jenoe

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1.3: Summary

The atypical Ser/Thr kinase target of rapamycin (TOR) is a central controller of cell growth and proliferation. TOR forms two distinct multiprotein complexes, TORC1 and TORC2, which are structurally and functionally conserved from yeast to humans. Four major inputs control mammalian TOR (mTOR): growth factors, such as insulin; cellular energy levels, such as the AMP:ATP ratio; stress, such as hypoxia; and nutrients, such as amino acids. mTOR controls cell growth by the positive and negative regulation of several anabolic and catabolic processes, respectively, that collectively regulate cell size and proliferation. These cellular processes include autophagy, cytoskeleton rearrangement, glycolysis, lipogenesis, nutrient transport, ribosome biogenesis, and translation. Dysregulation of the mTOR signaling network has been associated with aging, and a multitude of diseases including cancer, cardiovascular disease, diabetes, inflammation, immune dysfunctions, and neurodegeneration. However, relatively few direct substrates of either one of the two mTOR complexes, mTORC1 and mTORC2, are known.

To determine downstream effectors of mammalian TOR (mTOR), we applied a functional, quantitative phosphoproteomics workflow to identify novel mTORC1 or mTORC2 regulated phosphorylations. Raptor and Rictor are essential components of mTORC1 and mTORC2, respectively. To distinguish phosphorylations regulated by mTORC1 or mTORC2, we specifically deleted Raptor or Rictor using an inducible gene knockout system in mouse embryonic fiberblasts (MEFs). We detected 4584 phosphorylation sites on 1398 proteins, and identified 335 novel mTOR effectors. Many of the novel effectors are implicated in cancer and metabolic diseases, but have no known links to mTOR. To distinguish direct mTOR substrates from indirect effectors, we combined peptide array in vitro kinase assays with phosphorylation motif analysis. This revealed that mTORC1 phosphorylates CAD in vivo and in vitro. CAD (carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase) is the initial and rate limiting enzyme in de novo pyrimidine synthesis. The macrolide rapamycin, which forms a complex with FKBP12, binds and acutely inhibits mTORC1 but
not mTORC2. Rapamycin treatment inhibited growth factor stimulated CAD phosphorylation and oligomerization, decreased \emph{de novo} pyrimidine synthesis, and delayed progression of S-phase where CAD activity is essential. Thus mTORC1 phosphorylates CAD and thereby stimulates \emph{de novo} pyrimidine synthesis to promote cell proliferation.

Separately, we characterize the autophosphorylation of mTOR on Ser2481. Insulin stimulates the phosphorylation of mTOR at Ser2481 specifically in mTORC2. Knockout of Rictor, but not Raptor, abolished mTOR autophosphorylation at Ser2481. Prolonged treatment with rapamycin, which indirectly inhibits mTORC2 complex formation, inhibited Ser2481 phosphorylation. Surprisingly, mTORC2 autophosphorylation at Ser2481 temporally occurs after the insulin-induced phosphorylation of Akt/PKB and the SGK1 substrate NDGR1. Mutation of Ser2481 to aspartic acid rendered mTOR unable to phosphorylate Akt/PKB \emph{in vitro}. However the function of mTOR-Ser2481 phosphorylation \emph{in vivo} remains elusive, as mutation of mTOR-Ser2481 did not alter Akt/PKB phosphorylation \emph{in vivo}.

In summary, mTORC1 and mTORC2 regulate the phosphorylation of a functionally diverse set of substrates to control several anabolic and catabolic processes that determine cell size and proliferation. As a central controller of cell growth and proliferation, mTOR plays a key role in regulating development, whereas dysregulation of mTOR signaling has been linked to aging and diseases such as cancer and metabolic disorders.
### 1.4: Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>4E-BP1</td>
<td>eukaryotic translation initiation factor 4E binding protein 1</td>
</tr>
<tr>
<td>AGC</td>
<td>cAMP-dependent protein kinase [PKA]/protein kinase G [PKG]/protein kinase C [PKC]</td>
</tr>
<tr>
<td>Akt/PKB</td>
<td>rac protein kinase alpha/ protein kinase B</td>
</tr>
<tr>
<td>CAD</td>
<td>carbamyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase</td>
</tr>
<tr>
<td>DEPTOR</td>
<td>DEP-domain-containing mTOR-interacting protein</td>
</tr>
<tr>
<td>DNA-PK</td>
<td>DNA-dependent protein kinase</td>
</tr>
<tr>
<td>IMAC</td>
<td>immobilized metal affinity chromatography</td>
</tr>
<tr>
<td>LC-MS/MS</td>
<td>liquid chromatography coupled to mass spectrometry</td>
</tr>
<tr>
<td>mLST8</td>
<td>mammalian lethal with SEC13 protein 8</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>mTOR</td>
<td>mammalian [or mechanistic] target of rapamycin</td>
</tr>
<tr>
<td>mTORC1</td>
<td>mTOR complex 1</td>
</tr>
<tr>
<td>mTORC2</td>
<td>mTOR complex 2</td>
</tr>
<tr>
<td>NDGR1</td>
<td>N-myc downstream regulated</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PIKK</td>
<td>phosphatidylinositol 3-kinase-related kinases</td>
</tr>
<tr>
<td>PRAS40</td>
<td>proline-rich Akt/protein kinase B [PKB] substrate 40 kDa</td>
</tr>
<tr>
<td>PRR5</td>
<td>proline-rich protein 5 [also called Protor]</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>Raptor</td>
<td>regulatory associated protein of mTOR complex 1</td>
</tr>
<tr>
<td>Rictor</td>
<td>rapamycin insensitive companion of mTOR</td>
</tr>
<tr>
<td>S6K</td>
<td>ribosomal protein S6 kinase</td>
</tr>
<tr>
<td>SGK</td>
<td>serum/glucocorticoid regulated kinase 1</td>
</tr>
<tr>
<td>SILAC</td>
<td>stable isotope labeling with amino acids in cell culture</td>
</tr>
<tr>
<td>Sin1</td>
<td>SAPK [stress-activated protein kinase]-interacting protein 1</td>
</tr>
<tr>
<td>SREBP-1</td>
<td>sterol regulatory element binding protein-1</td>
</tr>
<tr>
<td>TSC</td>
<td>tuberous sclerosis complex</td>
</tr>
<tr>
<td>ULK1</td>
<td>unc-51-like kinase 1</td>
</tr>
</tbody>
</table>
2.0: Introduction

To appropriately control cell growth and proliferation, cells respond to a variety of environmental cues to regulate the synthesis of essential building blocks such as proteins, lipids, sugars, nucleotides and other metabolites. The mammalian target of rapamycin (mTOR) is an atypical Ser/Thr kinase structurally and functionally conserved from yeast to human that positively regulates cell growth, proliferation, and survival, while inhibition of mTOR signaling extends lifespan (reviewed in (Laplante and Sabatini, 2009b; Wullschleger et al., 2006)). mTOR forms two multiprotein complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2).

Nutrients and growth factors activate mTORC1, whereas low cellular energy levels or hypoxic stress inhibit mTORC1. Upon activation, mTORC1 positively regulates protein translation, ribosome biogenesis, lipogenesis, and inhibits autophagy. mTORC2 is activated by growth factors via PI3K (phosphoinositide 3-kinase) dependent ribosome association (Zinzalla et al., 2011). Upon activation, mTORC2 regulates the phosphorylation of several AGC kinases including Akt/PKB, SGK, and canonical PKCs to control cell survival and cytoskeleton rearrangement (reviewed in (Jacinto and Lorberg, 2008)). Thus mTORC1 and mTORC2 link external growth signals to the internal growth capacity of the cell.

As central controllers of cell growth and proliferation, the mTORC1 and mTORC2 signaling networks are key regulatory nodes whose functions regulate development and aging, while dysregulation of mTOR signaling has been linked to cancer and metabolic disorders (reviewed in (Zoncu et al., 2010)). mTORC1 and mTORC2 signal via distinct substrates to collectively control spatial and temporal cell growth, which ultimately determines cell size and regulates proliferation (reviewed in (Robitaille and Hall, 2008; Yang and Guan, 2007)). However, relatively few direct substrates of either one of the two mTOR complexes are known.

Regulation and function of mTOR phosphorylation

mTOR is the founding member of the phosphatidylinositol 3-kinase-related kinases (PIKK) family (Manning et al., 2002), which includes Ataxia telangiectasia mutated (ATM); ATM and Rad3 related (ATR); DNA-dependent
protein kinase (DNA-PK); suppressor with morphological effect on genitalia 1 (SMG1); and transformation/transcription domain-associated protein (TRRAP). Despite the homology to lipid kinases, mTOR has not been demonstrated to have lipid kinase activity, and the significance of mTOR homology to lipid kinases remains unknown.

mTOR has a predicted molecular weight of 280 kDa, and an apparent molecular weight of 220 kDa. The amino (N) terminus of mTOR contains 20 tandem HEAT (Huntingtin, Elongation factor 3, PR65/A subunit of protein phosphatase 2A, TOR) repeats (Diagram 1). This region is followed by a FAT (FRAP (FKBP12-rapamycin-associated protein)/TOR, ATM, TRRAP) domain, an FK506-binding protein 12 (FKBP12)-rapamycin binding (FRB) domain, the kinase domain, and a carboxy (C)-terminal FATC domain. The HEAT repeats mediate protein-protein interactions (Perry and Kleckner, 2003), while the FAT and FATC domains that flank the catalytic site modulate mTOR activity via an unknown mechanism. The FAT and FATC domains are found as a pair in all PIKK family members.

The c-terminal region of mTOR situated between the catalytic domain and the FATC contains a repressor domain (RD) (Scott et al., 1998; Sekulic et al., 2000). Addition of an antibody (mTAB1) that recognizes the repressor domain (Brunn et al., 1997), or partial deletion of the repressor domain (amino acids 2430-2450) increases mTOR in vitro kinase activity (Edinger and Thompson, 2004). mTOR is phosphorylated at three sites within the repressor domain: Thr2446, Ser2448, and Ser2481. AMPK phosphorylates Thr-2446 (Cheng et al., 2004) whereas S6K phosphorylates Ser2448 (Chiang and Abraham, 2005; Holz and Blenis, 2005) and mTOR autophosphorylates Ser2481 (Peterson et al., 2000). However, mutation of Thr2446 or Ser2448 has no effect on mTOR kinase activity (Cheng et al., 2004; Chiang and Abraham, 2005; Holz and Blenis, 2005). Thus an unidentified residue within the repressor domain may play an important regulatory role.
Diagram 1: mTOR. A schematic of mTOR domain organization and phosphorylation sites are shown. Repressor domain (RD). Images were generated with Prosite’s MyDomains Image Creator.

**mTORC1 and mTORC2 are distinct complexes**

mTOR forms two distinct multiprotein complexes, mTORC1 and mTORC2, which are structurally and functionally conserved from yeast to human (Diagram 2).

mTORC1 is composed of mTOR, Raptor, and mLST8 (also called GβL). Whole-body knockout of mTOR is embryonic lethal in mice. Homozygous mTOR-/- mice die at embryonic (E) day E5.5 to E6 (Gangloff et al., 2004; Murakami et al., 2004). Similar to mTOR, Raptor-/- mice die at day E5.5 to E6.5, suggesting that both mTORC1 components are required for progressing past the same stage of embryonic development (Guertin et al., 2006). Additionally, mTORC1 associates with PRAS40 and DEPTOR, proteins that negatively regulate mTORC1 activity (Peterson et al., 2009; Thedieck et al., 2007; Vander Haar et al., 2007; Wang et al., 2007).

mTORC2 is composed of mTOR, Rictor, Sin1, mLST8, and PRR5/PRR5L (also called Protor 1/Protor 2) (reviewed in (Cybulski and Hall, 2009)). PRR5 is not required for the interaction between mTOR, Rictor, Sin1, and mLST8. Deletion of Rictor or Sin1 is embryonic lethal, as mice die after day E10-E10.5 (Guertin et al., 2006; Jacinto et al., 2006). Additionally, loss of Sin1 disrupts Rictor association with mTORC2. Surprisingly, although mLST8 is a component of both mTORC1 and mTORC2, mLST8 knockout mice die at day E10.5, phenotypically resembling Rictor-/- mice (Guertin et al., 2006). Furthermore, mLST8 is required for Akt/PKB hydrophobic motif (HM)
phosphorylation, but not S6K signaling. These data suggest that mLST8 has an essential role only in mTORC2.

Studies in yeast, flies and mammalian cells have shown that mTOR is found in oligomeric supercomplexes, that are likely to be mTORC1-mTORC1 and mTORC2-mTORC2 homodimers (Wang et al., 2006; Wullschleger et al., 2005; Zhang et al., 2006). Multimeric mTOR may have higher kinase activity than monomeric mTOR. It remains to be determined how oligomerization is regulated and how it may lead to increased kinase activity.

Diagram 2: mTORC1 and mTORC2 are conserved regulators of cell growth and proliferation. mTOR forms two distinct, multiprotein complexes, mTORC1 and mTORC2, which are structurally and functionally conserved from yeast to human.

Pharmacological inhibition of mTORC1 and mTORC2
The macrolide rapamycin, which forms a complex with FKBP12, binds and allosterically inhibits mTORC1 but not mTORC2. In some cells, prolonged treatment with rapamycin (>24 hours) can inhibit mTORC2 complex assembly (Sarbassov et al., 2006). Recently, several groups have independently developed ATP-competitive inhibitors of mTOR, which include PP242, Torin1, and Ku-0063794 (reviewed in (Sparks and Guertin, 2010)). Pharmacological inhibition of mTOR signaling with ATP-competitive inhibitors lead to the discovery that mTORC1 has rapamycin-resistant functions. In mammals, rapamycin treatment incompletely mimics Raptor deletion (Feldman et al.,...
2009; Thoreen et al., 2009). mTOR ATP-competitive inhibitors target both mTORC1 and mTORC2, thus limiting the usefulness of these compounds as research tools. However, ATP-competitive inhibitors of mTOR may prove useful as future cancer therapeutics. PP242 was shown to inhibit the proliferation of cancer cells to a greater extent than rapamycin (Janes et al., 2010).
2.1: Upstream regulation of mTORC1

Four major inputs control mTORC1: growth factors, such as insulin; cellular energy levels, such as the AMP:ATP ratio; stress, such as hypoxia; and nutrients, such as amino acids (Diagram 3) (reviewed in (Robitaille and Hall, 2008)). mTORC1 integrates signaling from growth factors, cellular energy levels, and hypoxia through an upstream negative regulator called the tuberous sclerosis complex (TSC1-TSC2), a heterodimeric GTPase-activating protein (reviewed in (Huang and Manning, 2008)).

Growth factors activate mTORC1 by inhibiting TSC1-TSC2

Growth factors, such as insulin, inhibit TSC1-TSC2 through receptor tyrosine kinase (RTK) mediated activation of the phosphoinositol-3-kinase (PI3K) pathway. Insulin binds and activates the insulin receptor (IR), a receptor tyrosine kinase (RTK). The insulin receptor substrate (IRS), and subsequently active PI3K, are then recruited to the insulin receptor (reviewed in (Taniguchi et al., 2006)). The cellular level of phosphatidylinositol-3,4,5-trisphosphate (PI(3,4,5)P3) are maintained by the antagonistic actions of the lipid kinase PI3K and the lipid phosphatase PTEN. Activation of PI3K promotes Akt/PKB and PDK1 recruited to the plasma membrane (reviewed in (Fayard et al., 2010)). At the plasma membrane, Akt/PKB and PDK1 bind directly to PI(3,4,5)P3, where PDK1 phosphorylates and activates Akt/PKB (Alessi et al., 1997). Activated Akt/PKB then phosphorylates and functionally inactivates TSC2 (Gao et al., 2002; Inoki et al., 2002; Tee et al., 2003). The TSC1-TSC2 complex can also be inhibited by canonical MAPK signaling. Similar to Akt/PKB, active ERK or RSK can phosphorylate and inhibit TSC2 GAP activity (Ma et al., 2005). Inactivation of TSC1-TSC2 allows GTP-bound Rheb to activate mTORC1 (Garami et al., 2003; Inoki et al., 2003). In addition to inhibiting TSC1-TSC2, Akt/PKB phosphorylates PRAS40 at Ser247 and inactivates it, which leads to mTORC1 activation (Sancak et al., 2007; Vander Haar et al., 2007). mTORC1 can further phosphorylate PRAS40 at Ser183 and Ser221 (Fonseca et al., 2007; Oshiro et al., 2007). Growth factor activation of mTORC1 leads to the phosphorylation and subsequent degradation of DEPTOR, a negative regulate of mTORC1 activity (Peterson et al., 2009).
Low cellular energy and hypoxic stress inhibit mTORC1 via TSC1-TSC2

In contrast to growth factors that inhibit TSC1-TSC2, low cellular energy and stress activate TSC2 GAP activity. Low energy (high AMP:ATP ratio) activates AMP-activated protein kinase (AMPK) to phosphorylate TSC2 (Inoki et al., 2003). AMPK phosphorylation of TSC2 inhibits mTORC1 by increasing TSC2 GAP activity toward Rheb, a Ras-like GTPase. In addition to phosphorylating TSC2, AMPK also inhibits mTORC1 by directly phosphorylating Raptor (Gwinn et al., 2008). Mutations of the AMPK upstream activating kinase, LKB1, results in hyperactive mTORC1 signaling, thereby linking LKB1 to the TSC1-TSC2 mTORC1 pathway (Corradetti et al., 2004). Hypoxia inhibits mTORC1 signaling through the HIF1-mediated up regulation of two homologous proteins REDD1 and REDD2 (Regulated in Development and DNA damage response genes 1 and 2 ). REDD acts to activate TSC1-TSC2, independently of LKB1-AMPK, in order to inhibit mTORC1 (Brugarolas et al., 2004; Sofer et al., 2005). The stress and energy signaling pathways are likely to be further associated, as prolonged hypoxia leads to ATP depletion and activation of AMPK.

Amino acids activate mTORC1 downstream of TSC1-TSC2

In contrast to growth factor stimulation, amino acid activation of mTORC1 occurs independently of TSC1-TSC2 (Smith et al., 2005). Activation of mTORC1 by amino acids requires the Ras-like GTPases Rheb and Rag, both of which bind directly to mTORC1 (reviewed in (Duran and Hall, 2012)). Amino acids stimulate mTORC1 localization to a late endosomal/lysosomal compartment containing active Rheb. mTORC1 is recruited to the lysosomal compartment by the actions of lysosome associated protein complexes including Rag heterodimers, Ragulator, and vacuolar-ATPase (v-ATPase). Rag heterodimers (RagA or RagB binding to RagC or RagD) promote the translocation of mTORC1 to the lysosomal compartment (Sancak et al., 2008). Furthermore, Rag heterodimers are recruited to the lysosomal compartment via Ragulator, a scaffolding protein complex that is composed of MP1, p14, and p18 (Sancak et al., 2010). Additionally, v-ATPase, which interacts with Ragulator, is required for amino acid activation of mTORC1.
Thus amino acid stimulated v-ATPase-to-Ragulator-to-Rag-to-mTORC1 binding facilitates the activation of mTORC1 by lysosome associated, active Rheb. hVps34 has also been implicated in the amino acid activation of mTORC1 (Gulati et al., 2008; Nobukuni et al., 2005). However, in flies Vps34 functions downstream of TOR (Juhasz et al., 2008). How amino acids GTP-load, and thereby activate, the Rag GTPases to ultimately activate mTORC1 remains to be determined. Intriguingly, MAP4K3 acts upstream of Rag-mediated mTORC1 activation (Yan et al., 2010). Amino acids stimulate MAP4K3 activity to phosphorylate an unknown substrate upstream of the Rag/Ragulator complex. Where as under amino acid starved conditions, PP2A T61 epsilon inhibits MAP4K3 and thus subsequent mTORC1 activation. Upon activation, mTORC1 promotes protein translation, ribosome biogenesis, lipogenesis, and inhibits autophagy.

Diagram 3: mTORC1 signaling network. Amino acid and growth factor activation of mTORC1 promotes cell growth and proliferation.
2.2: Upstream regulation of mTORC2

In contrast to the detailed understanding of mTORC1 activation, the upstream regulators of mTORC2 have only recently been described (Diagram 4). Further work is necessary to refine how growth factor signaling activates mTORC2.

Growth factors activate mTORC2 via PI3K signaling

Growth factors signal to mTORC2 via receptor tyrosine kinase (RTK) activation of PI3K. Similar to the activation of mTORC1, upon insulin stimuli, the insulin receptor substrate (IRS) and subsequently active PI3K are recruited to the insulin receptor. The cellular level of phosphatidylinositol-3,4,5-trisphosphate (PI(3,4,5)P3) are maintained by the antagonistic actions of the lipid kinase PI3K and the lipid phosphatase PTEN. Activation of PI3K promotes the association of mTORC2 with intact 80S ribosomes. The interaction between mTORC2 and the ribosome is required for mTORC2 activation, independent of proteincin translation (Zinzalla et al., 2011). How (PI(3,4,5)P3) promotes mTORC2 association with the ribosome and subsequent activation remains a mystery, but PI(3,4,5)P3-containing liposomes do not stimulate mTORC2 in vitro kinase activity (Frias et al., 2006). Growth factor activation of mTORC2 leads to the phosphorylation and subsequent degradation of DEPTOR, a negative regulate of mTORC2 activity (Peterson et al., 2009).

A mTORC1 mediated negative feedback loop inhibits mTORC2

The TSC1-TSC2 complex may function upstream of mTORC2, but this activity is independent of TSC2 GAP activity (Huang et al., 2008). Loss of TSC1-TSC2 inhibits the phosphorylation of mTORC2 substrates, while simultaneously hyper-activating mTORC1. Hyper-active mTORC1 inhibits mTORC2 signaling through multiple mechanisms involving S6K and Grb10. Active S6K directly phosphorylates Rictor and IRS, which is part of a negative feedback loop that attenuates insulin signaling (Dibble et al., 2009; Harrington et al., 2004; Shah et al., 2004; Treins et al., 2009). mTORC1 phosphorylates and stabilizes Grb10, which also attenuates PI3K signaling to mTORC2 (Hsu et al., 2011; Yu et al., 2011). Additionally, loss of TSC1-TSC2 increases ER
stress, which then inhibits mTORC2 via GSK3β mediated phosphorylation of Rictor (Chen et al., 2011). It is unclear to what degree S6K-mediated Rictor phosphorylation, IRS inhibition, and increased ER stress individually contribute to mTORC2 inactivation.

**Alternative mechanisms of mTORC2 activation**

In dictyostelium, RasC is an upstream regulator of TORC2, independent of PI3K and canonical MAPK signaling. RasC physically binds to TORC2 and activates it through an unknown mechanism (Cai et al., 2010; Charest et al., 2010). Mammalian Ras can also bind to Sin1 *in vitro*, but the physiological significance of this interaction has not been demonstrated (Schroder et al., 2007). Additionally, mTORC2 and mTORC1 bind to phosphatidic acid (PA), suggesting that PA could facilitate membrane localization of mTOR (Fang et al., 2001). Taken together, this data suggests that growth factors activate mTORC2 though multiple mechanisms. Alternatively, mTORC2 may form distinct sub-complexes that differentially respond to upstream signaling from RTK or G-protein coupled receptor (GPCR) inputs. In support of the second hypothesis, mTOR and Rictor associate with different Sin1 isoforms (Sin1.1, Sin1.2, or Sin1.5) to form three distinct mTORC2 complexes. Interestingly, the *in vitro* kinase activity of mTORC2 containing the Sin1.5 isoform is insensitive to insulin stimulation (Frias et al., 2006). Upon growth factor activation, mTORC2 promotes cell survival and cytoskeleton rearrangement.
Diagram 4: mTORC2 signaling network. Growth factor activation of mTORC2 promotes cell survival and cytoskeleton rearrangement.
2.3: Downstream effectors of mTORC1

**mTORC1 positively regulates protein translation**

mTORC1 activates cap-dependent translation initiation and elongation by phosphorylating the eukaryotic initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1) and the p70 ribosomal S6 kinase (S6K) (Beretta et al., 1996; Gingras et al., 2001; Hara et al., 1997; Pearson et al., 1995). The phosphorylation of 4E-BP1 prevents its binding to eIF4E, enabling eIF4E to then associate with eIF4G to stimulate translation initiation (Holz and Blenis, 2005). mTORC1 and phosphoinositide-dependent kinase 1 (PDK1) phosphorylate and activate S6K. Activated S6K then promotes translation initiation by phosphorylating eIF4B, programmed cell death protein 4 (PDCD4) and eEF2 kinase (eEF2K) (Dorrello et al., 2006; Wang et al., 2001). Phosphorylation of eIF4B and PDCD4 activates translation initiation, whereas phosphorylation of eEF2K up regulates translation elongation. S6K also promotes the translation efficiency of spliced mRNAs via S6K-Aly/REF-like substrate (SKAR) (Ma et al., 2008). SKAR associates with mRNAs in a splicing-dependent manner, where it then recruits activated S6K and thereby preferentially enhances translation of spliced mRNAs. Additionally, S6K phosphorylates 40S ribosomal protein S6, but the significance of this phosphorylation is unknown. mTORC1 associates with its substrates 4E-BP1 and S6K through Raptor and a TOR signaling (TOS) motif in 4E-BP1 and S6K (Nojima et al., 2003; Schalm and Blenis, 2002). The TOS motif is a conserved five-amino-acid sequence that is necessary for the in vivo phosphorylation of these proteins by mTORC1. A similar substrate recognition motif has not been identified for mTORC2.

**mTORC1 promotes ribosome biogenesis**

mTORC1 promotes the synthesis of ribosomes and transfer RNAs (tRNAs). Rapamycin blocks the biosynthesis of ribosomes by inhibiting transcription of RNA polymerase I (Pol I)-dependent rRNA genes, Pol II-dependent ribosomal protein genes (RP genes), and Pol III-dependent tRNA genes (reviewed in (Martin and Hall, 2005)). mTOR controls Pol I via the essential transcription initiation factor TIF-1A (Transcriptional Intermediary Factor 1A). Rapamycin treatment leads to TIF-1A inactivation, and thus
impairs formation of the transcription initiation complex. Furthermore, TIF1A translocates from the nucleus to the cytoplasm upon rapamycin-mediated mTORC1 inactivation (Mayer et al., 2004). In yeast, the forkhead-like transcription factor FHL1 functions as a TOR-dependent regulator of Pol II-dependent RP gene expression (Martin et al., 2004). mTORC1 regulates of Pol III-mediated gene expression by directly phosphorylated and inhibiting MAF1, a Pol III transcriptional repressor (Michels et al., 2010). mTORC1 associates with TFIIIC, is recruited to Pol III-transcribed genes, and relieves MAF1 mediated repression, thus allowing Pol III transcription to occur (Kantidakis et al., 2010). mTOR also controls ribosome biogenesis by promoting translation of 5’ oligopyrimidine (TOP) mRNAs (Schwab et al., 1999). 5’ TOP mRNAs, defined by a 5’ oligopyrimidine tract, encode components of the translation apparatus, including ribosomal proteins and elongation factors.

**mTORC1 positively regulates lipogenesis**

mTORC1 positively regulates de novo lipogenesis by activating sterol regulatory element binding protein-1 (SREBP-1) (reviewed in (Laplante and Sabatini, 2009a)). SREBP-1 is an basic helix loop helix transcription factor that regulate lipogenesis by controlling the expression of genes required for cholesterol, fatty acid, and triglycerides synthesis. mTORC1 positively regulates the activity of SREBP-1 by phosphorylating and inhibiting Lipin1 (Peterson et al., 2011). Lipin1 is phosphatidic acid phosphatase that negatively regulates SREBP-1 activity. Growth factor stimulated, mTORC1 mediated phosphorylation of Lipin1 inhibits Lipin1 nuclear localization. Lipin1 that has been phosphorylated by mTORC1 cannot enter the nucleus and inhibit the transcriptional activity of SREBP-1. It remains to be determined how the phosphatidic acid phosphatase activity of Lipin1 inhibits SREBP-1.

**mTORC1 inhibits autophagy**

mTORC1 negatively regulates macroautophagy, a starvation-induced catabolic process where bulk cytoplasm is enclosed in a double membrane structure and delivered to the vacuole for degradation. Rapamycin induces autophagy in yeast and human tissue culture, demonstrating the conserved
role of mTORC1 as an inhibitor of autophagy (Kamada et al., 2000; Paglin et al., 2005). mTORC1 directly inhibits autophagy by phosphorylating and repressing unc-51-like kinase 1 (ULK1), component of a conserved protein complex composed of ULK1, autophagy-related gene 13 (ATG13) and focal adhesion kinase family-interacting protein of 200 kDa (FIP200) (Ganley et al., 2009; Hosokawa et al., 2009; Jung et al., 2009). mTORC1 also directly phosphorylates and represses DAP1 (Koren et al., 2010). Unlike the ULK1/ATG13/FIP200 complex, DAP1 is a negative regulator of autophagy. Thus via positive and negative regulation, mTORC1 activity tightly controls the absolute level of cellular autophagy. Additionally, dysregulation of mTORC1 may contribute to cancer cell survival, as tumor cells may temporarily activate autophagy to overcome nutrient deprivation under poor growth conditions.
2.4: Downstream functions of mTORC2

Unlike mTORC1, which can be specifically inhibited by rapamycin, mTORC2 is insensitive to acute rapamycin treatment. Thus, many of the downstream effectors and physiological functions of mTORC2 remain unknown.

**mTORC2 controls AGC kinase activation and protein stability**

mTORC1 and mTORC2 phosphorylate and thereby activate several members of the AGC kinase family (reviewed in (Jacinto and Lorberg, 2008)). The AGC kinase activated by mTORC1 includes S6K, while mTORC2 regulates Akt/PKB, SGK, and PKCα (Chung et al., 1992; Facchinetti et al., 2008; Garcia-Martinez and Alessi, 2008; Sarbassov et al., 2005). mTOR phosphorylates the hydrophobic motif (HM) in the AGC kinases to stimulate kinase activity. In S6K and SGK, phosphorylation of the HM creates a docking site for PDK1 and subsequent phosphorylation of the AGC kinase activation loop (reviewed in (Pearce et al., 2009)).

In response to growth factors, mTORC2 phosphorylates Akt/PKB at the hydrophobic motif (Akt/PKB-Ser473) (Sarbassov et al., 2005). Where as, in response to DNA damage, DNA-PK regulates Akt/PKB HM phosphorylation (Bozulic et al., 2008). PDK1 phosphorylates Akt/PKB at the activation loop (Akt/PKB-Thr308) (Alessi et al., 1997). Phosphorylation of both the activation loop and hydrophobic motif is required for the full activation of Akt/PKB. Ablation of Sin1 or Rictor results in loss of Akt/PKB HM phosphorylation, yet the phosphorylation of several Akt/PKB targets is unaffected (Guertin et al., 2006; Jacinto et al., 2006). This suggests that either the phosphorylation of Akt/PKB-Ser473 alters substrate specificity, or that in the absence of active Akt/PKB, other AGC kinases such as SGK, S6K or RSK can phosphorylate a subset of Akt/PKB substrates. Notably, mTORC2 is required for Akt/PKB mediated phosphorylation of Foxo1/3a.

Additionally, mTORC2 regulates phosphorylation of the turn motif (TM) in Akt/PKB and canonical PKCs to promote AGC kinase protein stability (Facchinetti et al., 2008; Ikenoue et al., 2008). In yeast, TOR phosphorylates and activates the AGC kinases Gad8, Ypk2, and Sch9 (Kamada et al., 2005; Matsuo et al., 2003; Urban et al., 2007). Future studies may reveal additional AGC kinases that are regulated by mTOR.
**mTORC2 promotes cell survival**

mTORC2 deficient cells are sensitive to stress induced apoptosis (Jacinto et al., 2006). mTORC2 promotes cell survival through the activation of Akt/PKB and serum and glucocorticoid-inducible kinase (SGK), two AGC kinases that have both distinct and overlapping substrates. Akt/PKB negatively regulates the pro-apoptotic protein BAD, while SGK regulates the phosphorylation of NDRG1, and both kinases negatively regulate FOXO (Brunet et al., 1999; Datta et al., 1997; Murray et al., 2004). Interestingly, mTORC2 activity is required for prostrate tumorigenesis in PTEN null tumors, and not for normal prostate function (Guertin et al., 2009). This suggests that inhibiting mTORC2-mediated cell survival would be attractive cancer therapeutic target with few side affects to healthy tissue.

**mTORC2 regulates cytoskeleton remodeling**

mTORC2 regulates actin cytoskeleton remodeling in yeast, and is important for chemotaxis and cell migration in dictyostelium and mammals respectively (Charest et al., 2010; Jacinto et al., 2004; Kamada et al., 2005). Furthermore, loss of mTORC2 perturbs cell morphology and inhibits dendritic tiling (Koike-Kumagai et al., 2009). mTORC2 dependent cytoskeleton remodeling is likely mediated by the mTORC2 effectors PKCα, Paxillin, and Rac1 (reviewed in (Cybulski and Hall, 2009)). The molecular mechanism by which mTORC2 regulates these effectors to control cytoskeleton remodeling has not been well characterized.
Identification of novel mTORC1 and mTORC2 substrates

The mTORC1 and mTORC2 signaling networks have emerged as central controllers of cell growth that are important for development, aging, and diseases such as cancer and diabetes. In mice, tissue specific knockouts of mTOR or mTORC1/mTORC2 components have demonstrated that mTOR plays a key role in regulating muscle atrophy, glucose metabolism, and lipid metabolism (reviewed in (Polak and Hall, 2009)). To better understand how cell growth and proliferation is regulated, we sought to determine which novel substrates mTORC1 and mTORC2 phosphorylate. Relatively few direct substrates of either mTORC1 or mTORC2 are known. Identifying novel mTOR substrates may reveal new therapeutic options to target mTOR dysregulation in cancer and metabolic disorders.

Here we developed a functional proteomics workflow to quantitatively identify novel mTORC1 or mTORC2 regulated phosphorylations, and distinguish between direct mTOR substrates and indirect effectors. In total, we identified 335 novel mTORC1 or mTORC2 substrates or indirect effectors. We identified CAD as a potential mTORC1 substrate. CAD, (carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase), is the initial and rate limiting enzyme of the de novo pyrimidine synthesis pathway, a conserved and essential metabolic pathway important for cell growth and proliferation (Tatibana and Shigesada, 1972). CAD activity is essential in highly proliferating cells, particularly in S-phase. (Karle and Cysyk, 1984; Swyryd et al., 1974). We validated that CAD is a direct substrate of mTORC1, and determined that mTORC1 positively regulates growth factor stimulated de novo pyrimidine synthesis to promote S-phase progression. Thus our quantitative phosphoproteomic workflow revealed a novel mTORC1 substrate that positively regulates cell proliferation. Our research provides new insights into how signaling networks regulate the synthesis of essential metabolic building blocks such as proteins, lipids, and nucleotides.

mTOR autophosphorylation is specific to mTORC2

Separately, we set out to investigate the functional consequence of mTOR-Ser2481 phosphorylation, which occurs within a repressor domain. We show that mTOR autophosphorylation at Ser2481 is specific to mTORC2,
requires an intact mTORC2 complex, and is stimulated by growth factors. Unexpectedly, mTORC2 autophosphorylation at Ser2481 temporally occurred after the phosphorylation of mTORC2 substrates. Furthermore, mTOR autophosphorylation at Ser2481 negatively regulated mTORC2 in vitro activity towards Akt/PKB. However the function of mTOR-Ser2481 phosphorylation in vivo remains elusive, as mutation of mTOR-Ser2481 did not alter Akt/PKB phosphorylation in vivo.
3.0: Results

3.1: Quantitative phosphoproteomics reveal that mTORC1 phosphorylates CAD and positively regulates de novo pyrimidine synthesis

Quantitative phosphoproteomics reveals 335 novel mTORC1 and mTORC2 effectors

Relatively few direct substrates of either mTORC1 or mTORC2 are known. Here we developed a functional proteomics workflow to quantitatively identify novel mTORC1 or mTORC2 regulated phosphorylations, and distinguish between direct mTOR substrates and indirect effectors. We integrated stable isotope labeling by amino acids in cell culture (SILAC) (Ong et al., 2002), preparative SDS-PAGE fractionation (Li et al., 2007), phosphopeptide enrichment (Bodenmiller et al., 2007), high accuracy mass spectrometry (Olsen et al., 2005), and bioinformatic programs (Cox and Mann, 2008) to quantify and identify mTORC1 or mTORC2 regulated phosphorylation changes in vivo. Mass spectrometry (MS) has been used successfully to analyze the global, in vivo phosphorylation targets of kinases and phosphatases (Bodenmiller et al., 2010; Pan et al., 2009). To identify the specific mTORC1 and mTORC2 regulated phosphoproteomes, we deleted Raptor or Rictor using an inducible gene knockout system in mouse embryonic fibroblasts (MEFs) (Cybulski et al., 2012; Patursky-Polischuk et al., 2009). Raptor and Rictor are essential components of mTORC1 and mTORC2, respectively (Figure 1A).

We then performed a series of quantitative phosphoproteomic experiments to identify novel proteins whose phosphorylation changed in an mTORC1 or mTORC2 dependent manner (Figure 1B). Using a SILAC triple label strategy, we analyzed four biological replicate experiments, rotating each SILAC label per biological replicate. Each biological replicate was measured as two technical replicates yielding a total of 220 nano-LC-MS/MS runs. Phosphopeptide quantification was reproducible across technical and biological replicates, with a R² value of 0.9884 and 0.6131 respectively (Figure 1C and Figure 1D). Using a 1% false discovery rate (FDR), we detected 4584 unique phosphorylation sites on 1398 proteins.
For a phosphorylation to be identified as being regulated by mTORC1 or mTORC2, we chose a stringent cut-off of >1.5 standard deviations (SD) from the log2 transformed mean ratio (iRapKO/Control, iRicKO/Control, or iRicKO/iRapKO) (Figure 1E). Furthermore, the phosphorylation must have been detected in 2 out of 4 biological replicates in the Raptor knockout or Rictor knockout experiments. In total, the phosphorylations of 335 proteins were significantly changed, identifying these as mTOR substrates or indirect effectors (Table 1). We detected and correctly identified 18 previously validated mTORC1 or mTORC2 regulated phosphorylations on 9 mTOR effectors including Raptor, Rictor, GSK3β, NDRG1, PRAS40, and S6. We confirmed by western blot that the in vivo phosphorylation of GSK3β-pS9, NDRG1-pT330, and S6-pS235/236 match our observed SILAC ratios, further validating our phosphoproteomic analysis (Figure 2B).

To identify a direct substrate of mTOR, we then focused on the phosphorylations that were down regulated by mTORC1 or mTORC2 inhibition, respectively. Many of the novel effectors are implicated in cancer and metabolic diseases, but have no known links to mTOR (Figure 1F). Thus mTORC1 and mTORC2 control cell growth and proliferation by regulating a diverse set of direct substrates and indirect effectors. Additionally, we compared our mTOR regulated phosphoproteome to previously published studies (Hsu et al., 2011; Yu et al., 2011). These studies and our own incompletely identified the known mTOR effectors, suggesting additional novel mTORC1 or mTORC2 substrates remain to be revealed (Figure 1G). Thus all three datasets are likely sampling different parts of the global mTOR regulated phosphoproteome.
Figure 1

A

1μM 4-OHT:

Raptor
S6K-pT389
S6K
Actin

1μM 4-OHT:

Rictor
Akt/PKB-pS473
Akt/PKB
Actin

* non-specific band

B

Metabolic labeling
Protein fraction and digestion
Phosphopeptide enrichment
Quantification and identification
Direct mTOR phosphorylation

SLAC labeled MEFs
SDS-PAGE and tryptic digestion
TQ2 and IMAC
nano LC-MS/MS (LTQ-Orbitrap) and MaxQuant
Peptide array in vitro kinase assay

Blue = Control RKO
Red = iRapKO R8K4
Orange = iRicKO R10K8

C

Technical Replicate 2

Log Ratio

R² = 0.9884

Log Ratio

D

Biological Replicate 1

Log Ratio

R² = 0.6131

E

phosphopeptide count

Log (iRapKO or iRicKO / Control)
Global phosphopeptide distribution

F

KEGG pathway
RNA transport
Tight junction
Endometrial cancer
mTOR signaling pathway
Ribosome biogenesis in eukaryotes
Colorectal cancer
MAPK signaling pathway
ErbB signaling pathway
Prostate cancer
Neurotrophin signaling pathway
Focal adhesion
Insulin signaling pathway
DNA replication
Gloma
Cell cycle
Adherens junction
Asthma/myeloid leukemia
Pathways in cancer
RNA degradation

G

mTOR effectors:
direct substrates of mTOR, S6K, SGK, and Akt/PKB

Robitaille et al. 2012
Yonghao et al. 2011
Hsu et al. 2011
mTOR effectors
285
43
36
9
3
171
26
6
4
**Figure 1:** **Functional phosphoproteomics reveals 335 novel mTORC1 or mTORC2 regulated phosphoproteins.**

**A.** Knockout of Raptor or Rictor inhibits mTORC1 or mTORC2 signaling, respectively. We generated floxed Raptor or Rictor mouse embryonic fibroblasts with an inducible 4-OHT inducible Cre. Three days after 4-OHT treatment, Raptor or Rictor protein levels are decreased by 90%. This decrease corresponds to a decrease in mTORC1 or mTORC2 signaling. * denotes non-specific band.

**B.** Quantitative phosphoproteomic workflow used to identify mTORC1 or mTORC2 regulated phosphorylations. We detected 4584 phosphorylation sites on 1398 proteins and identified 335 novel mTOR effectors.

**C.** Phosphopeptide quantification is robust across technical replicates, $R^2=0.9884$. **D.** Phosphopeptide quantification is reproducible between biological replicates, $R^2=0.6131$.

**E.** Global distribute of all 4584 phosphopeptides. Regulated phosphorylations are +/- >1.5 SD of the log$_2$ transformed ratio mean.

**F.** KEGG pathways that are statistically enriched in the mTOR regulated phosphoproteins.

**G.** Comparison of mTOR regulated phosphoproteins identified in three different phosphoproteomic studies.

**Identification of mTOR consensus motif**

To determine if mTOR controls the phosphorylation of specific motifs in vivo, we analyzed the primary amino acid sequence surrounding the down regulated phosphorylations with Motif-X (Schwartz and Gygi, 2005). Phosphorylation motif analysis revealed that mTORC1 and mTORC2 regulates the phosphorylation of pS-P and pT-P sites (potential direct mTOR phosphorylation sites) and R-x-x-pS sites (consensus site for AGC kinases, including mTOR effectors proteins S6K, SGK, Akt/PKB, and canonical PKCs) (Figure 2A). We confirmed the in silico results by examining the phosphorylation of R-x-x-pS/T sites known to be regulated by AGC kinases Akt/PKB, SGK, and S6K (Figure 2B). These results suggest that mTOR regulates the novel effectors directly or via AGC kinase activation.

To distinguish between direct mTOR substrates and indirect effectors, we performed a peptide array mTOR in vitro kinase assay. mTOR robustly phosphorylated peptides corresponding to validated mTOR substrates Akt/PKB and 4E-BP1 (Figure 2C). In total 33% of peptides identified in the mTORC1 and mTORC2 regulated phosphoproteomes were directly phosphorylated by mTOR in vitro (Figure 2D and Table 1). Thus the proteins these phosphorylation sites correspond to are identified as potential direct mTOR substrates.
Phosphorylation motif analysis of the peptides which mTOR can phosphorylate in vitro revealed that mTOR phosphorylated two distinct types of phosphorylation motifs: pS/T-P sites and hydrophobic motif (HM) F-x-x-F-pS-Y sites (Figure 2E). We confirmed that mTORC1 and mTORC2 regulate HM phosphorylations in vivo using phospho-specific motif antibodies against bulky hydrophobic (Φ) sites (Figure 2F). Many of the putative phosphorylations detected by the motif antibodies were similar, and therefore mTOR independent. However, we detected thirteen phosphorylations that were regulated by mTORC1 or mTORC2.

The remaining sites that mTOR phosphorylated in vitro did not contain a well defined amino acid motif. In vivo and in vitro, mTORC1 can also phosphorylate serine followed by glutamine residues (pSer-Gln) such as those in MAF1 (Michels et al., 2010). This is consistent with our data that mTOR directly phosphorylated a wide range of motifs. Thus mTOR is a promiscuous kinase, having a preference rather than an absolute requirement for serine or threonine followed by a proline (Ser/Thr-Pro), and serine or threonine flanked by bulky hydrophobic residues (Φ-Ser/Thr-Φ).
Figure 2

A

Enriched in iRapKO and iRicKO
down regulated phosphorylations

5.13

in vivo

4.08

fold
enriched

3.35

B

1μM 4-OHT:

<table>
<thead>
<tr>
<th>Protein</th>
<th>Control</th>
<th>iRapKO</th>
<th>iRicKO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raptor</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rictor</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S6-pS235/236</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GSK3β-pS9</td>
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<td></td>
</tr>
<tr>
<td>GSK3β</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>NDRG1-pS330</td>
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<tr>
<td>NDRG1</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

C

<table>
<thead>
<tr>
<th>Peptide Type</th>
<th>wt peptide</th>
<th>S/T-A peptide</th>
<th>wt peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Akt/PKB-S473</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4EBP1-T46</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Green = direct
mTOR phosphorylation
Red = nonspecific signal

D

mTOR         mock         overlay

E

in vitro

Relative abundance

F

1μM 4-OHT:

<table>
<thead>
<tr>
<th>Protein Type</th>
<th>DE</th>
<th>RHK</th>
</tr>
</thead>
<tbody>
<tr>
<td>small/nonpolar</td>
<td>AVFPMILW</td>
<td></td>
</tr>
<tr>
<td>basic</td>
<td>FFSY</td>
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</tr>
<tr>
<td>acidic</td>
<td>DE</td>
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</tr>
<tr>
<td>polar</td>
<td>STYCNGQ</td>
<td></td>
</tr>
</tbody>
</table>

kDa

F-pS/T-Y

F-x-x-F-pS/T-Y

→ = mTOR regulated
Figure 2: 33% of the phosphopeptides identified by mass spectrometry can be directly phosphorylated by mTOR. A. Analysis of the amino acid sequence surrounding phosphorylations down regulated by deletion of Raptor or Rictor. Motif-x was used to determine phosphorylation motifs that were significantly enriched above the mouse proteome. B. Phosphorylation at Akt/PKB, SGK, or S6K regulated R-x-x-S/T sites are decreased by Raptor or Rictor knockout. Four days after 4-OHT treatment, cells were harvested and mTOR signaling pathway was analyzed by western blot. Protein lysates are the same as those in Figure 3F, and Supplemental Figure 7. C. Peptide array mTOR in vitro kinase positive controls are shown in insert. D. In vitro phosphorylation of novel mTOR regulated peptides by recombinant mTOR. Peptides identified in the mTORC1 and mTORC2 regulated phosphoproteomes were synthesized on cellulose membranes using a MultiPep robotic spotter. Membranes were then subjected to an in vitro kinase assay using gamma-\(^{32}\)P ATP and recombinant mTOR. Each peptide was synthesized in duplicate with a matching peptide where Ala was substituted for Ser/Thr. Image was false colored for visual clarity E. mTOR primarily phosphorylates pS/T-P sites (37.5%) and F-x-x-F-pS-Y sites (25.0%). In vitro kinase motif analysis was performed with WebLogo. F. mTORC1 and mTORC2 regulate at least 13 distinct hydrophobic (Φ) site phosphorylations in vivo. iRapKO and iRicKO MEFs were treated as in Figure 2B. Protein lysates are the same as those in Figure 2B, and Figure 3F.

Identification of CAD as a novel mTORC1 substrate

To identify a direct substrate of mTORC1 or mTORC2, we focused further on the novel effectors whose peptide could be phosphorylated by mTOR in vitro. One putative substrate of particular interest to us was CAD (carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase). CAD is the initial and rate limiting enzyme of the de novo pyrimidine synthesis pathway, a conserved and essential metabolic pathway important for cell growth and proliferation (Tatibana and Shigesada, 1972). In our phosphoproteome data, CAD-Ser1859 phosphorylation was specifically down regulated by Raptor knockout (Table 1), and mTOR robustly phosphorylated the same peptide in vitro (Figure 3A). Additionally, we identified that CAD contains a putative TOS motif of FELVI (aa 1389 – 1393) (Figure 3B and 3C). CAD enzymatic activities and protein are conserved from worms to human, while in yeast CAD function is conserved by the action of two genes, URA2/URA4. In mammals, similar to the activation of mTORC1, de novo pyrimidine synthesis is positively regulated by growth factors (Graves...
et al., 2000; Smith and Buchanan, 1979). Thus CAD was an ideal candidate to be an mTORC1 substrate.

To validate that mTORC1 regulation of CAD phosphorylation was not specific to MEFs, we examined if rapamycin could inhibit CAD phosphorylation in HeLa cells. Growth factors robustly stimulated the phosphorylation of CAD-pS1859 in a rapamycin sensitive manner (Figure 3D), without altering CAD expression (Figure 3E). Therefore we generated a phospho-specific antibody against CAD-Ser1859. We validated that the antibody specially recognized CAD-Ser1859 phosphorylation (Figure 3F). In agreement with our mass spectrometry results, western blot experiments confirmed that CAD-Ser1859 phosphorylation was stimulated by growth factors in a rapamycin sensitive manner (Figure 3E). Similarly, CAD-Ser1859 phosphorylation was specifically inhibited upon Raptor, but not Rictor, knockout (Figure 3G). We then confirmed that mTOR could phosphorylate CAD in vitro (Figure 3H). In agreement with our results in vivo, mass spectrometry identified that mTOR phosphorylated CAD in vitro at Ser1859 (data not shown). Thus CAD is a novel mTORC1 substrate. Finally, to determine if mTORC1 mediated phosphorylation of CAD was physiologically relevant, we examined the phosphorylation of CAD in liver specific TSC1 knockout mice (L-TSC1 KO). Deletion of tuberous sclerosis protein 1 (TSC1) leads to hyper activation of mTORC1 (Shah et al., 2004). In the liver, CAD-Ser1859 phosphorylation was increased in TSC1 knockout mice. Furthermore, the increase in CAD Ser1859 phosphorylation was specifically inhibited by rapamycin treatment (Figure 3I). Thus mTORC1 directly regulates the rapamycin sensitive phosphorylation of CAD in vivo and in vitro.
Figure 3: **CAD is a novel mTORC1 substrate**

### A. Peptide array mTOR in vitro kinase spots for CAD-Ser1859.

### B. CAD contains a putative TOS motif of FELVI (aa 1389–1393). TOS motif conservation in human mTORC1 substrates.

### C. Diagram of CAD protein, mTORC1 regulated phosphorylation site, and TOS motif. The first three metabolites of the de novo pyrimidine synthesis pathway are synthesized by the CPS (carbamoyl-phosphate synthetase), ATC (aspartate transcarbamylase) and DHO (dihydroorotase) domains of CAD, respectively.

### D. Rapamycin inhibits growth factor stimulated phosphorylation of CAD-pS1859. Values are expressed as mean +/- SD. ** indicates a statistical difference of **p<0.01. n=3. HeLa cells were grown in SILAC media for 7 days and then treated with rapamycin for 1h. CAD was immunoprecipitated, trypsinized, and analyzed by nano-LC-MS/MS.

### E. 1 hour rapamycin treatment in HeLa cells does not alter CAD expression, while CAD-Ser1859 phosphorylation is inhibited. HeLa cells were serum starved for 16 hours, prior to restimulation with 10% dialyzed serum +/- 100nM rapamycin.

### F. G9c cells, which lack endogenous CAD, were transfected with either wild type or mutant CAD in the presence of growth factors. The mTORC1 dependent phosphorylation of CAD is only detected in wild type CAD.

### G. Deletion of Raptor inhibits CAD-Ser1859 phosphorylation. iRapKO and iRicKO MEFs were treated as in Figure 2B. Protein lysates are the same as those in Figure 2B, and Supplemental Figure 7.

### H. mTOR phosphorylates CAD in vitro in a PP242 sensitive manner. CAD was immunoprecipitated and incubated with mTOR for 30min at 37C. S6K is an mTORC1 in vitro kinase.
assay positive control. I. CAD-Ser1859 phosphorylation is regulated by mTORC1 in the mouse liver. Ctrl: TSC1-fl/fl mice, KO: TSC1-fl/fl; Albumin-Cre mice. 12 week old littermates were starved overnight and treated with rapamycin or sham (0.9% NaCl) for six hours prior to sacrifice.

**mTORC1 positively regulates de novo pyrimidine synthesis**

We then determined what biological relevance mTORC1 mediated phosphorylation of CAD had on de novo pyrimidine synthesis. In de novo pyrimidine synthesis, CAD synthesizes the pyrimidine ring from glutamine, bicarbonate, and aspartic acid. The pyrimidine ring is then attached to ribose to form a pyrimidine nucleotide. To measure the rate of de novo pyrimidine synthesis, we metabolically labeled newly synthesized pyrimidines with N15-amide labeled glutamine (Figure 4A). We then performed a quantitative metabolic flux analysis using targeted uHPLC-MS/MS to measure the rate of N15 incorporation into UDP and UTP, thus allowing us to determine the relative rate of de novo pyrimidine synthesis. N15-amide labeled glutamine was taken up by the cells independent of growth factors or rapamycin treatment (Figure 4B). N15 incorporation was not detected in an intermediate of the pentose phosphate pathway (6-phospho-gluconate (6-PG)) demonstrating the specificity of the N15 labeling to newly synthesized pyrimidines (Figure 4B).

Under starved conditions, we observed a low level of de novo pyrimidine synthesis, whereas growth factors stimulated an increased in the synthesis of UDP and UTP. Furthermore, rapamycin treatment completely inhibited the growth factor stimulated de novo pyrimidine synthesis, thereby decreasing the absolute de novo synthesis of UDP and UTP by 62.4% and 57.8%, respectively (Figure 4B). The observed low to high metabolic flux through de novo pyrimidine synthesis is consistent with a CAD enzymatic transition from a low activity to a high activity conformation (Cohen et al., 1985).

Additionally, rapamycin inhibited the growth factor stimulated increase in DHOA, the final CAD synthesized metabolite, as well as OA and UTP intercellular concentrations (Figure 4C). Conversely, rapamycin did not inhibit the growth factor stimulated increase in purine synthesis, as measured by GDP and GTP concentrations (Figure 4D). Thus, mTORC1 positively
regulates CAD activity to control growth factor stimulated *de novo* pyrimidine synthesis.
Figure 4: mTORC1 positively regulates growth factor stimulated de novo pyrimidine synthesis. A. Diagram depicting de novo pyrimidine synthesis pathway. B. Rapamycin treatment inhibits growth factor stimulated de novo pyrimidine synthesis of UDP and UTP. HeLa cells were serum starved for 16 hours, prior to restimulation with 10% dialyzed serum +/- 100nM rapamycin. At time point 0, pyrimidines were metabolically labeled by feeding HeLa cells 4mM N15-amide labeled glutamine. Metabolites were then measured using a targeted approach with uHPLC-MS/MS. C. Rapamycin inhibited the growth factor stimulated increase in DHOA, OA, and UTP cellular concentrations. D. Rapamycin does not inhibit the growth factor stimulated increase in GDP or GTP levels. Total cellular concentrations of metabolites...
were calculated by comparing the amount of metabolites in the cells to a N15 labeled internal standard and exogenous metabolite standard curves. Values are expressed as mean +/- SD. * indicates a statistical difference of *p<0.05, **p<0.01, ***p<0.001. n=3-6.

**mTORC1 regulates CAD oligomerization**

To identify a potential mechanism of how mTORC1 regulates CAD activity, we examined the cellular distribution of endogenous CAD. Growth factors stimulated the formation of punctate structures containing CAD. Whereas rapamycin treatment inhibited the formation of CAD punctate structures, closely resembling serum starvation (Figure 5A and 5B). Punctate structures were only observed in the presence of antibody specific to CAD (data not shown).

*In vitro* CAD can oligomerize into a hexamer (Lee et al., 1985). Furthermore, CAD orthologs assemble into large oligomers (Zhang et al., 2009). Thus the CAD punctate structures may be CAD oligomers. Using immunoelectron microscopy, we observed that growth factors stimulated the formation of CAD punctate structures in the cytoplasm (Figure 5C). In agreement with the immunofluorescence results, punctate structures were only observed in the presence of antibody specific to CAD (Figure 5C).

Additionally, we tested whether CAD could colocalize with proteins that form punctate structures in immunofluorescence microscopy. CAD did not colocalize with EEA1 (early endosomes), LAMP-2 (lysosomes), Catalase (peroxisomes), and Complex IV subunit I (mitochondria) supporting our hypothesis that the CAD punctate structures are CAD oligomers (Figure 5D). We then enriched for CAD oligomers using glycerol gradients (Figure 5E and 5F). Growth factors stimulated the formation of CAD oligomers in a rapamycin sensitive manner (Figure 5E and Figure 5G). Furthermore, mutation of CAD-Ser1859 to Ala inhibited the growth factor stimulated formation of CAD oligomers (Figure 5F and Figure 5H). Thus mTORC1-mediated phosphorylation of CAD-Ser1859 promotes growth factor dependent CAD oligomerization.
Figure 5: **Rapamycin regulates CAD oligomerization.**

A. Rapamycin inhibits the growth factor stimulated translocation of CAD to punctate structures. HeLa cells were synchronized in early s-phase using a double thymidine block. Following synchronization, cell were serum starved for 16h hours in D-MEM containing with 0.1% D-FCS + 2.5mM thymidine, followed by 30min AA starvation in 1xPBS. Cells were then released in DMEM (starved) or DMEM + 10% D-FCS +2xAA (stimulated) or DMEM + 10% D-FCS + 2xAA + 100nM rapamycin (rapamycin). Endogenous CAD was then visualized by immunocytochemistry after 1h rapamycin treatment.

B. Quantification of Figure 5A. Black bars represent the mean. *** indicates a statistical difference of p<0.001. n=116 cell counted per condition.

C. CAD forms oligomeric structures. HeLa cells were serum starved for starved 16 hours, prior to restimulation with 10% dialyzed serum for 1hour. Endogenous CAD was then visualized by immunoelectron microscopy. Three representative aggregate structures are shown. Punctate structures were only observed in the presence
of antibody specific to CAD. D. CAD punctate structures did not colocalize with EEA1 (early endosomes), LAMP-2 (lysosomes), Catalase (peroxisomes), and Complex IV subunit I (mitochondria). HeLa cells were serum starved for starved 16 hours, prior to restimulation with 10% dialyzed serum. Endogenous CAD and the corresponding membrane marker were then visualized by immunocytochemistry after 1h restimulation. E. HeLa cells were serum starved for 16h hours in D-MEM containing with 0.1% D-FCS, followed by 30min AA starvation in 1xPBS. Cells were restimulated with DMEM (starved) or DMEM + 10% D-FCS + 2x AA (stimulated) or DMEM + 10% D-FCS + 2x AA + 100nM rapamycin (rapamycin) for 1 hour. CAD oligomers were then isolated on 10-35% sucrose gradients. F. Mutation of CAD-Ser1859 to Ala inhibited the growth factor stimulated formation of CAD oligomers. G9c cells were transfected with either wild type or mutant CAD. CAD oligomers were isolated on 10-35% sucrose gradients. Gradient fractions are run across multiple SDS-PAGE gels. G. The peak fractions from CAD oligomers isolated in Figure 5E are shown. H. The peak fractions from CAD oligomers isolated in Figure 5F are shown.

**mTORC1 regulates cell cycle progression**

In the absence of exogenous pyrimidines, CAD activity is essential for cell proliferation (Patterson and Carnright, 1977), and particularly important for proper S-phase progression (Mitchell and Hoogenraad, 1975). Therefore, we examined if the decrease in de novo pyrimidine synthesis induced by rapamycin treatment had a physiological effect on cell cycle progression. Cells were synchronized in early S-phase using a double thymidine block to circumvent the G1/S arrest that prolonged rapamycin treatment induces (Heitman et al., 1991; Terada et al., 1993). Similar to serum starvation, rapamycin treatment delayed S-phase progression by 32% (Figure 6A). As the cells entered G2/M, the delay in cell cycle progression increased to 52%, consistent with an additional requirement for mTORC1 in G2/M (Ramirez-Valle et al., 2010). Furthermore, the rapamycin-induced delay in cell cycle progression was rescued by the addition of exogenous pyrimidines (Figure 6A).

In cells lacking endogenous CAD activity, cell proliferation is dependent on the supply of exogenous pyrimidines (Figure 6B). In the absence of exogenous pyrimidines, expression of wild type CAD rescued the cell proliferation defect, while expression of mutant CAD (Ser1859Ala) that cannot be phosphorylated by mTORC1 only partially restored cell proliferation (Figure...
This data supports our model where mTORC1 phosphorylation of CAD promotes cell proliferation by regulating CAD oligomerization, and thus a CAD enzymatic transition from a low activity state to a high activity oligomerized conformation. Thus mTORC1 controls cell proliferation in part by positively regulating \textit{de novo} pyrimidine synthesis.

To examine the potential clinical relevance of our results, we further tested the ability of rapamycin to inhibit cell proliferation. Everolimus, a synthetic rapamycin derivative, is used currently as a cancer therapeutic (Motzer et al., 2008). However, rapamycin does not strongly inhibit proliferation in all cell types (Figure 6C), (Neshat et al., 2001). Therefore we reasoned that inhibition of mTORC1 combined with a chemotherapeutic nucleoside (6-azauridine) would synergistically deplete pyrimidine pools, and thus be a potent inhibitor of proliferation in cell types that are resistant to rapamycin treatment. In HeLa cells, we observed that the addition of rapamycin and 6-azauridine inhibited cell proliferation to a greater extent than rapamycin or 6-azauridine alone (Figure 6D). Proliferation was inhibited by 42.8\% at low nanomolar concentrations of each drug (10nM rapamycin + 64nM 6-azauridine). Thus the combination of rapamycin and 6-azauridine together may increase the therapeutic window for cancer therapy, particularly in tumors that do not respond to rapamycin treatment. In summary (Figure 6E), we have shown that mTORC1 regulates cell proliferation, in part, by regulating CAD phosphorylation and oligomerization to promote \textit{de novo} pyrimidine synthesis.
**Figure 6: Rapamycin inhibits s-phase progression.**

**A.** Exogenous pyrimidines rescue the rapamycin-induced delay in cell cycle progression. HeLa cells were synchronized in early S-phase using a double thymidine block. Cells were then released in DMEM (starved) or DMEM + 10% D-FCS + 2x AA (stimulated) or DMEM + 10% D-FCS + 2x AA + 100nM rapamycin (rapamycin), +/- 30µM uridine. DNA content was analyzed by flow cytometry.

**B.** Expression of wild type CAD, but not mutant CAD, restored normal cell proliferation. G9c cells, which lack endogenous CAD activity, were transfected with either wild type or mutant CAD. Cells were visualized with crystal violet five days after transfection.

**C.** Rapamycin strongly inhibits proliferation in MEFs, but not HeLa cells. MEFs or HeLa cells were grown for three days in DMEM + 0.1% D-FCS (starved), DMEM + 10% D-FCS (stimulated), or DMEM + 10% D-FCS + 100nM rapamycin (rapamycin). Cells were then visualized with crystal violet.

**D.** Rapamycin and 6-azauridine inhibited cell proliferation to a greater extent than rapamycin or 6-azauridine alone. HeLa cells were grown for 42 hours +/- rapamycin and 6-azauridine. Cell proliferation was then measured with an AlamarBlue assay. The black bar represents cell proliferation in the presence of 10nM rapamycin. Values are expressed as mean +/- SD. * indicates a statistical difference between (rapamycin + 6-azauridine) vs (rapamycin) or (6-azauridine) alone. *p<0.05, **p<0.01. n=3

**E.**
mTORC1 promotes cell proliferation by regulating CAD phosphorylation, oligermization, and activity to positively regulate de novo pyrimidine synthesis.
3.2: mTOR autophosphorylation at Ser2481 regulates mTORC2 in vitro kinase activity

Growth factors stimulate mTOR autophosphorylation at Ser2481 specifically in mTORC2

To investigate the functional consequence of mTOR phosphorylation, we examined how growth factors affect mTOR-Ser2481 phosphorylation. mTOR phosphorylation at Ser2481 is stimulated by insulin or serum, and inhibited by wortmannin (Figure 7A). Thus mTOR phosphorylation at Ser2481 is growth factor responsive. This data is inconclusive to determine if Phosphoinositide 3-kinase (PI3K) is upstream of mTORC2 as wortmannin can directly inhibit mTOR. Acute (1 hour) rapamycin treatment inhibits mTORC1 but not mTORC2. As mTOR-Ser2481 phosphorylation is not sensitive to acute rapamycin treatment (Figure 7A) (Peterson et al., 2000), we hypothesized that this phosphorylation site might be specific to mTORC2. To test this, we isolated mTORC1 or mTORC2 complexes by immuno precipitating Raptor or Rictor, which are specific components of mTORC1 and mTORC2, respectively. We observed that mTOR phosphorylated at Ser2481 was found specifically in mTORC2, whereas mTOR phosphorylated at Ser2448 was observed in mTORC1 and mTORC2 (Figure 7B). We then confirmed that mTOR phosphorylation at Ser2481 requires mTOR kinase activity in vivo (Figure 7C) and in vitro (data not shown). Thus mTOR autophosphorylation at Ser2481 is observed specifically within mTORC2. Additionally, we observed that mTOR phosphorylated multiple interacting partners, including Raptor, Rictor, and Sin1, in vitro (Figure 7D).

Next we examined if mTOR autophosphorylation at Ser2481 required specific components of mTORC1 (Raptor) or mTORC2 (Rictor and Sin1). Using inducible Rictor or Raptor knockouts MEFS (Cybulski et al., 2012; Patarsky-Polischuk et al., 2009), we observed that knockout of Rictor blocked mTOR autophosphorylation of Ser2481, where Ser2481 phosphorylation was unaffected by Raptor knockout (Figure 7E). Phosphorylation of Ser2481 was also blocked in constitutive Sin1-/- mouse embryonic fiberblasts (MEFs) (Figure 7F). Finally, prolonged rapamycin treatment (24 hours), which indirectly inhibits mTORC2 complex formation (Sarbassov et al., 2006), inhibited mTOR-Ser2481 phosphorylation (Figure 7G). Thus growth factors
stimulate mTORC2 autophosphorylation at Ser2481, and mTOR-Ser2481 autophosphorylation is dependent on mTORC2 complex integrity.
Figure 7: Growth factors stimulate mTOR autophosphorylation at Ser2481 specifically in mTORC2. A. HEK293 cells were serum starved overnight in 0.1% FBS, then restimulated with 100nM insulin or 10% FBS for 15min. Cells were pretreated with 100nM rapamycin for 1h or 100nM wortmannin for 30 min as indicated. B. HEK293 cells were stimulated with 100nM insulin for 15min prior to lysis. mTORC1 or mTORC2 immunocomplexes were purified via Raptor or Rictor antibodies. Equal amounts of mTOR were loaded. C. HA-mTOR or HA-mTOR kinase dead (KD) were expressed in HEK293 cells. 48 hours post-transfection, cells were stimulated with 100nM insulin for 15min. HA-mTOR and HA-mTOR KD were then immunoprecipitated with HA antibodies coupled to protein-A sepharose. D. HEK293 or HeLa cells were serum starved overnight in 0.1% FBS, then restimulated with 100nM insulin. Endogenous mTORC1 or mTORC2 was immunoprecipitated with Raptor or Rictor specific antibodies treated as described for the in vitro kinase assay. 32P-ATP-labeled bands were excised and digested by trypsin, and the corresponding proteins identified by mass spectrometry. E. Knockout of Rictor or Raptor was induced with 1µM 4-OHT 72 hours prior to cell lysis. Cells were then serum starved overnight in 0.1% FBS, and stimulated with 100nM for 15min. F. Cell extracts from either constitutive Sin1 -/- MEFs or control MEFs. G. iRicKO MEFs were treated with 100nM rapamycin for the indicated time.
**mTORC2 autophosphorylation at Ser2481 temporally occurs after the insulin-induced phosphorylation of Akt/PKB**

Next we investigated the temporal regulation of the mTOR signaling network. Surprisingly, mTORC2 autophosphorylation at Ser2481 temporally occurred after the insulin-induced Akt/PKB and NDRG1 phosphorylation (Figure 8A). Maximal phosphorylation of Akt/PKB and NDGR1 occurred between 2min - 5min after insulin stimulation, whereas maximal mTORC2 autophosphorylation at Ser2481 occurred 15min – 30min after insulin stimulation. In contrast to the phosphorylation of mTOR-Ser2481, maximum mTOR phosphorylation at Ser2448 occurred between 30min - 45min and corresponded temporally to the maximum phosphorylation of S6K-Thr389. Thus maximum phosphorylation of mTOR-Ser2481 occurs after the phosphorylation of mTORC2 substrates \textit{in vivo}. This data suggests mTOR-Ser2481 phosphorylation may inhibit mTORC2 activity.

To examine the functional role of mTORC2 autophosphorylation at Ser2481, we mutated mTOR Ser2481 to either alanine (Ala) or aspartic acid (Asp). Mutation of Ser2481 to either alanine or aspartic acid did not disrupt mTORC1 or mTORC2 complex stability (Figure 8B). Thus mTOR-Ser2481 is not required for mTORC1 or mTORC2 complex integrity. To determine if mTOR-Ser2481 regulates mTOR kinase activity, we then preformed \textit{in vitro} kinase assays using Akt/PKB as a substrate. Wild type mTOR and mTOR-Ser2481Ala phosphorylated Akt/PKB-Ser473, while kinase dead mTOR and mTOR-Ser2481Asp could not phosphorylate Akt/PKB \textit{in vitro} (Figure 8C). As mTOR-Ser2481Asp was unable to phosphorylate Akt/PKB \textit{in vitro}, we hypothesized that mutation of mTOR-Ser2481 to alanine may render mTOR constitutively active. However, mTOR-Ser2481Ala immunoprecipitated from insulin stimulated cells phosphorylated Akt/PKB \textit{in vitro} to a greater extent than in serum-starved conditions (Figure 8D). Thus mutation of Ser2481 to alanine is not sufficient to render mTOR constitutively active. This data is in agreement with recent findings that growth factor stimulated mTORC2 activation requires a direct interaction with intact ribosomes (Zinzalla et al., 2011).

We then investigated the \textit{in vivo} function of mTOR-Ser2481 phosphorylation. Expression of kinase dead (KD) mTOR, mTOR-Ser2481Ala,
or mTOR-Ser2481Asp did not significantly affect the in vivo phosphorylation of Akt/PKB Ser473 (data not shown). As no dominate negative affect was observed with kinase dead mTOR, we concluded the levels of endogenous mTOR were sufficient to regulate Akt/PKB phosphorylation. Thus the functional role of mTORC2-Ser2481 autophosphorylation in vivo remains elusive.

We then further characterized the regulation of mTOR phosphorylation at Ser2448. We observed that mTOR-Ser2448 was phosphorylated in mTORC1 and mTORC2 (Figure 7B), and that mTOR-Ser2448 phosphorylation was reduced by Raptor, but not Rictor, knockout (Figure 7E). In agreement with our results, mTOR-Ser2448 phosphorylation was previously shown to be mediated by S6K1 (Chiang and Abraham, 2005; Holz and Blenis, 2005). Therefore, we investigated if there was possible crosstalk between mTORC1 and mTORC2 via S6K1 phosphorylation of Ser2448 in mTORC2. HEK293 cells were treated with rapamycin for 1 hour before specifically immuoprecipitating mTORC1 or mTORC2. We observed that acute rapamycin treatment did not block Ser2448 phosphorylation in mTORC2, whereas mTOR disassociated from Raptor upon rapamycin treatment (Figure 8E). In agreement with these observations, rapamycin treatment partially decreased mTOR-Ser2448 phosphorylation in vivo when S6K1 phosphorylation was completely inhibited (Figure 8E). Thus mTORC2 phosphorylation at Ser2448 is Rictor-independent and rapamycin insensitive. The functional of mTOR-Ser2448 phosphorylation in both mTORC1 and mTORC2 is unknown.

In summary (Figure 8F), we observed that growth factor stimulated mTOR autophosphorylation at Ser2481 was specific to mTORC2, occurred after the phosphorylation of mTORC2 substrates, and regulated mTORC2 in vitro activity towards Akt/PKB. Conversely, mTOR-Ser2448 phosphorylation was observed in both mTORC1 and mTORC2. mTOR-Ser2448 phosphorylation in mTORC1 was regulated by both rapamycin treatment and Raptor knockout, whereas mTOR-Ser2448 phosphorylation in mTORC2 was insensitive to rapamycin treatment or Rictor knockout. Future work is required to reveal the in vivo function of both mTOR-Ser2448 and mTOR-Ser2481 phosphorylation.
Figure 8: **Insulin-stimulated mTORC2 autophosphorylation temporally occurs after the phosphorylation of Akt/PKB.** A. HeLa cells were serum starved overnight in 0.1% FBS, then restimulated with 100nM insulin for the indicated time point. B. HA-mTOR, HA-mTOR kinase dead (KD), HA-mTOR
Ser-2481-Ala, or HA-mTOR Ser-2481-Asp were expressed in HEK293 cells. 48 hours post-transfection, cells were lysed and HA-mTOR constructs were immunoprecipitated with HA antibodies coupled to protein-A sepharose. C. Immunoprecipitations from Figure 9B were split into two equal tubes: one tube was used to assess total protein, and one for mTOR in vitro kinase assay. D. HA-mTOR Ser-2481-Ala or HA-mTOR Ser-2481-Asp were expressed in HEK293 cells. 48 hours post-transfection, cells were either serum starved overnight in 0.1% FBS or serum starved overnight in 0.1% FBS and then restimulated with 100nM insulin. In vitro kinases assays were preformed as described in Figure 9C. E. HEK293 cells were pretreated with 100nM rapamycin for 1h, then stimulated with 100nM insulin for 15min prior to lysis. mTORC1 or mTORC2 immunocomplexes were purified via Raptor or Rictor antibodies. F. A schematic of mTORC1 and mTORC2 phosphorylation sites and the kinases responsible for their phosphorylation are shown. Repressor domain (RD). Images were generated with Prosite’s MyDomains Image Creator.
4.0: Discussion

4.1: Identification of the mTOR regulated phosphoproteome

To identify novel mTORC1 or mTORC2 substrates, we used a quantitative and functional phosphoproteomic approach. We quantified the phosphorylation of 1398 unique proteins, which represents 52% coverage of the known mTORC1 and mTORC2 signaling networks. First, we performed a large-scale analysis of the mTORC1 and mTORC2 regulated phosphoproteomes by inducible knockout of essential mTORC1 or mTORC2 components, followed by phosphopeptide enrichment and high accuracy mass spectrometry. Then we analyzed which phosphopeptides identified by mass spectrometry could be phosphorylated by mTOR in vitro. This workflow allowed us to obtain a global view of the mTORC1 and mTORC2 regulated phosphoproteomes. A major challenge encountered by many phosphoproteomic datasets is the inability to distinguish between direct and indirect effectors. Our peptide array in vitro kinase assay provided the functional data necessary to identify potential direct mTOR substrates from indirect effectors. Using the inducible gene knockout of other kinases or phosphatases, followed by peptide array in vitro kinase or phosphase assay, this workflow could be more broadly applied to study the downstream functions of additional kinases and phosphatases.

In yeast, rapamycin sensitive phosphoproteomes have identified several novel TORC1 effectors (Huber et al., 2009; Soulard et al., 2010). However in mammals, rapamycin treatment incompletely mimics Raptor deletion, as mTORC1 has rapamycin-resistant functions (Feldman et al., 2009; Thoreen et al., 2009). Additionally, prolonged rapamycin treatment inhibits mTORC1 and mTORC2 (Sarbassov et al., 2006). Thus rapamycin will only identify a subset of mTORC1 substrates, where our approach to delete Raptor avoids the above stated limitations. Recently, ATP-competitive inhibitors of mTOR have revealed portions of the larger mTOR regulated phosphoproteome (Hsu et al., 2011; Yu et al., 2011). However ATP-competitive inhibitors of mTOR target both mTORC1 and mTORC2 (reviewed in Sparks and Guertin, 2010). Thus these studies cannot distinguish mTORC1 substrates from mTORC2 substrates. Inducible deletion of Raptor
or Rictor allowed us to overcome the above stated challenge and reveal the specific mTORC1 or mTORC2 regulated phosphoproteome. Additionally, these studies and our own incompletely identified the known mTOR effectors, suggesting additional novel mTORC1 or mTORC2 substrates remain to be revealed. Thus all three datasets are likely sampling different parts of the global mTOR regulated phosphoproteome. Currently, mTOR has been implicated to directly or indirectly regulate the phosphorylation of 803 distinct proteins. This corresponds to 3.9% of all mammalian genes. Given the broad role of mTOR in regulating cell physiology, many mTOR targets are expected and many may remain to be characterized.

Quantitatively defining the mTORC1 and mTORC2 regulated phosphoproteomes revealed that mTORC1 phosphorylates CAD to positively regulate de novo pyrimidine synthesis. Thus we identified a novel biological function of mTORC1, and identified 335 novel substrates or indirect effectors of mTORC1 or mTORC2, central controllers of cell growth.

For appropriate cell growth to occur, cells need to coordinate the production of essential building block such as proteins, lipids, sugars, nucleotides and other metabolites. mTORC1 and mTORC2, as central controllers of cell growth, have well established roles in regulating protein synthesis, translation, and an emerging role in lipid and glucose homeostasis (reviewed in (Laplante and Sabatini, 2009a; Polak and Hall, 2009). Here we have revealed a novel finding that mTORC1 directly phosphorylates CAD to positively regulate growth factor stimulated de novo pyrimidine synthesis, and thereby cell proliferation. There are several key findings in the literature which hinted that CAD may function downstream of mTORC1. In flies, mutations within the CAD gene, rudimentary, have smaller cell size (Fausto-Sterling and Hsieh, 1976). Furthermore, de novo pyrimidine synthesis is known to be positively regulated by growth factors (Smith and Buchanan, 1979).

Pyrimidines, in addition to being essential components of DNA and RNA, are used as an energy source to drive specific cellular functions. UTP is the source of energy for activating glucose and galactose, while CTP is an energy source in lipid metabolism. Thus mTORC1 likely regulates de novo pyrimidine synthesis to both promote cell proliferation, and as part of a larger role in controlling lipid and glucose homeostasis in vivo. Furthermore, in
contrast to purines, which are synthesized in the mitochondria, pyrimidines are synthesized in cytoplasm providing a spatial localization where regulation of de novo pyrimidine synthesis would be accessible to direct mTOR phosphorylation. We showed that mTORC1 regulates CAD phosphorylation, oligomerization, and function. mTORC1 mediated phosphorylation of CAD promotes CAD oligomerization, and increased de novo pyrimidine synthesis in vivo. This data suggests that mTORC1 mediated CAD oligomerization may facilitate steric channeling of CAD synthesized metabolites, and thus explain the increase the rate of de novo pyrimidine synthesis.

De novo pyrimidine synthesis requires PRPP (5-phosphoribosyl-1-pyrophosphate), which is produced via the pentose phosphate pathway. Thus it is likely that regulation of the de novo pyrimidine synthesis pathway and the pentose phosphate pathway are coordinated. Interestingly, TSC1 -/- MEFs, which have hyperactive mTORC1 signaling, display an increase flux through the oxidative branch of the pentose phosphate that is partially dependent on SREBP-1 (Duvel et al., 2010). Our data demonstrates that mTORC1 acutely (1 hour) regulates the production of pyrimidines by directly phosphorylating and activating CAD, the initial and rate limiting step of de novo pyrimidine synthesis. Separately and in a prolonged manner (12-20 hours), mTORC1 regulates the production of PRPP via SREBP-1 dependent transcriptional activity. Thus it is likely that mTORC1 controls de novo pyrimidine synthesis through a combination of direct and indirect effectors to properly regulate cell proliferation.

4.2: Regulation of mTOR phosphorylation

Separately, we investigated the regulation of mTOR phosphorylation at two sites, mTOR-Ser2481 and mTOR-Ser2448. Here we report that mTOR autophosphorylation at Ser2481 is specific to mTORC2, requires an intact complex, and is stimulated by growth factors. Growth factor activation of mTORC2 leads to the rapid phosphorylation of Akt/PKB and SGK1 substrate NDGR1. If mTOR-Ser2481 phosphorylation was a marker of active mTORC2, we hypothesize that mTORC2 autophosphorylation would precede the phosphorylation of mTORC2 substrates. However, in vivo mTORC2 activity towards Akt/PKB-Ser473 phosphorylation preceded mTORC2
autophosphorylation at Ser2481. This data suggests mTOR-Ser2481 phosphorylation may contribute to inhibition of mTOR activity towards Akt/PKB. Furthermore, mTOR-Ser2481 is within a repressor domain of mTOR. The repressor domain is 95% conserved in vertebrates, yet poorly conserved in invertebrates. This data suggests the repressor domain may be required for vertebrate specific functions of mTOR. Interesting, other regulatory sites in the mTOR signaling network are also solely conserved in vertebrates, such as ERK (Ma et al., 2005) and IKKβ (Lee et al., 2007) phosphorylation of TSC2. Addition of an antibody (mTAB1) that recognizes the repressor domain (RD) (Brunn et al., 1997), or partial deletion of the repressor domain (amino acids 2430-2450) increases mTOR in vitro kinase activity (Edinger and Thompson, 2004). We identified that mTORC2 autophosphorylation at Ser2481 inhibits mTORC2 in vitro kinase activity. One possible mechanism could be that mTOR-Ser2481 phosphorylation blocks substrate presentation. Alternatively, mutation of mTOR-Ser2481 to aspartic acid may not properly mimic the functional role of phosphorylation at this site. Furthermore, as the expression of mTOR-Ser2481 mutated to aspartic acid did not alter mTORC2 signaling in vivo, there are likely additional regulatory mechanisms that inactivate growth factor regulated mTORC2 activity. Many phosphorylations sites have recently been discovered in mTOR and mTOR interaction proteins (Gwinn et al., 2008; Hayashi et al., 2007; Wang et al., 2009b). mTOR can phosphorylate Raptor, Rictor, and Sin1 at multiple sites in vivo (Acosta-Jaquez et al., 2009; Foster et al., 2009; Wang et al., 2009a). Thus multiple phosphorylations may each contribute a small portion to the regulation of mTOR activity. It is likely that certain phosphorylations will positively regulate mTOR activity, while others may contribute to a feedback inhibition mTOR activity. Therefore it will be important to examine these phosphorylations under specific physiological conditions to determine their function role.

We also characterized the phosphorylation of mTOR at Ser2448. mTOR-Ser2448 phosphorylation was first proposed to be an Akt/PKB site (Sekulic et al., 2000). Subsequent work demonstrated phosphorylation of mTOR-Ser2448 was mediated by S6K (Chiang and Abraham, 2005; Holz and Blenis, 2005). Our data suggest S6K phosphorylates mTORC1, but an
unidentified Raptor and Rictor independent-kinase phosphorylates Ser2448 in mTORC2 (Fig 2). A recent study showed that cytoplasmic mTOR-Ser2448 phosphorylation correlated with tumor progression and poor survival in human gastric cancer. Interestingly, nuclear mTOR-Ser2448 phosphorylation was associated with better overall survival (Murayama et al., 2009). Thus mTOR-Ser2448 phosphorylation may prove useful as a clinical biomarker. Similarly, while this work was in progress, another group published the observation that mTOR-Ser2481 phosphorylation was specific to mTORC2, and can be utilized as a biomarker for mTORC2 sensitively to rapamycin (Copp et al., 2009). Although the functional significance of mTOR-Ser2448 and mTOR-Ser2481 phosphorylation remains to be clarified, our results suggests that further investigation of mTOR phosphorylation may identify important functional roles and/or prove useful as clinical biomarkers.

4.3: Closing remarks
The mTORC1 and mTORC2 signaling networks have emerged as central controllers of cell growth and proliferation that are important for development, aging, and diseases such as cancer and diabetes. Future work to identify direct substrates of mTOR may reveal new cellular processes that mTORC1 and mTORC2 regulate to ultimately determine cell size and positively regulate proliferation.
5.0: Experimental Procedures

Antibodies: CAD antibody for western/IP (#A301-374A), and CAD antibody for IF/ImmunoEM (#IHC-00280) from Bethyl. Anti-Akt/PKB and Anti-mTOR (for endogenous IP) from Santa Cruz Biotechnology. Alexa Fluor 488 anti-rabbit and Alexa Fluor 594 anti-mouse secondary antibodies from Invitrogen. HRP-coupled anti-mouse and anti-rabbit secondary antibodies from Pierce; HRP-coupled anti-IgG, light chain specific, anti-rabbit secondary antibody from Jackson Immunoresearch; HRP-coupled anti-rabbit IgM from Abcam; Ten-nanometer gold particles coupled to goat anti-rabbit IgG from BBIInternational; Anti-Rictor and anti-Raptor antibodies were produced as previously described (Jacinto et al., 2004). All other commercial antibodies from Cell Signaling Technology.

Antibody production: CAD-Ser1859 phospho-specific, rabbit polyclonal antibodies were raised against a KLH-coupled peptide (RIHRApSDPGLPAE), which corresponds to residues 1854–1866 of human CAD. Antibodies were generated and affinity purified by Eurogentec.

Bioinformatics: In vivo kinase motif analysis was performed with Motif-X (Schwartz and Gygi, 2005). In vitro kinase motif analysis was performed with WebLogo (Crooks et al., 2004). We searched for human, fish, fly, worm, and yeast orthologs of our novel mouse mTOR effectors using g-profiler (Reimand et al., 2007). KEGG pathway analysis was done using the Benjamini-Hochberg FDR in g-profiler. Using the previously identified TOS motifs found in PRAS40, S6K, and 4E-BP, (Lee et al., 2008) we developed a consensus TOS motif corresponding to: F-[DEV]-[MLI]-[DEV]-[LIE]. We then searched for putative TOS motifs using ScanProsite (Gattiker et al., 2002). Sequence alignments were done with ClustalW2 (Larkin et al., 2007). Venn diagrams were created with VENNY (Oliveros, 2007). HeLa cell volume was calculated from BioNumbers (Milo et al., 2010). Previously identified growth factor regulated phosphorylations were searched for in PhosphositePlus (Hornbeck et al., 2004).
**Cell culture:** Cells were maintained in D-MEM containing 10% dialyzed fetal bovine serum (D-FCS). For overnight serum deprivation, cells were grown for 16 hours in D-MEM containing with 0.1% D-FCS. iRapKO and iRicKO are inducible Raptor and Rictor, respectively, knockout MEF cell lines. Generation of the iRapKO and iRicKO cell lines was previously described (Cybulski et al., 2012; Patusky-Polischuk et al., 2009). Briefly, immortalized MEFs contain floxed Raptor or Rictor alleles, and 4-hydroxytamoxifen (4-OHT) inducible Cre recombinase. iRapKO (Raptor knockout) cells or iRicKO (Rictor knockout) cells were compared to control cells treated with 1 μM 4-OHT. 72 hours after 4-OHT treatment, the deletion of Raptor and inhibition of mTORC1 signaling or deletion of Rictor and inhibition of mTORC2 signaling was confirmed by western blot. For SILAC experiments, MEFs or HeLa cells were culture for 7 passages in SILAC media + 10% D-FCS. Cells were then subjected to 4-OHT treatment (MEFs) or cell harvest (HeLa). HeLa cells were transfected with Lipofectamine 2000 following the manufacture’s instructions. Wild-type and mutant CAD was transfected into G9c cells with FuGene, following the manufacture’s instructions. G9c cells lack functional CAD activity, and require the addition of 30μM uridine to the media to maintain cell growth and proliferation (Qiu and Davidson, 2000). Cell proliferation was measured using Crystal Violet staining or an AlamarBlue assay as previously described (Colombi et al., 2010).

**Chemicals:** SILAC media and D-FCS from DundeeCellProducts; Glutamine and Pen/Strep from Invitrogen. N15-amide glutamine from Cambridge Isotope Labs. Lipofectamine 2000, and recombinant mTOR from Invitrogen. PALA was a kind gift from the NCI/DTP Open Chemical Repository. Protein A-agarose from Zymed. Soluble peptides were synthesized by GenScript. CAD siRNA and Non-targeting siRNA SMARTpools from Dhharmacon. Additional cell culture reagents including D-MEM, insulin, and rapamycin, wortmannin, and PP242 from Sigma.

**Flow cytometry:** HeLa cells were arrested in early S-phase using a double thymidine block. 40% confluent cells were subsequently grown in complete
media + 2.5mM thymidine for 18h, released in complete media for 9h, and arrested in S-phase with a second 16 hour incubation in complete media + 2.5mM thymidine. Cells were then released, ± 30μM uridine, in DMEM (starved) or DMEM + 10% D-FCS + 2x AA (stimulated) or DMEM + 10% D-FCS + 2x AA + 100nM rapamycin (rapamycin). Cells were trypsinized and washed 2x in 1xPBS + 0.1% BSA. Cells were fixed in 75% ice-cold EtOH for 1h at 4C. After washing 2x 1xPBS, cells were permeabilized with 0.25% Triton-100 in 1x PBS for 5min at 4C. Cells were washed, and then treated with 30ug/mL RNase A for 15min at 37C. After washing, cells were resuspended at 1x10^6 cells/mL in PI staining buffer (1xPBS + 3.8mM NaCitrate + 50 ug/mL propidium iodide + 10ug/mL RNase A) and incubated in the dark for 2h at 4C. The PI staining was then measured with a Beckman Coulter Cell Lab Quanta SC Flow cytometer.

**Fluorescence Microscopy:** HeLa cells were grown on cover slips to 60% confluence. Cells were serum starved for 16h hours in D-MEM containing with 0.1% D-FCS, followed by 30min AA starvation in 1xPBS. Cells were restimulated with 10% D-FCS and double the concentration of AA s in D-MEM for 1h. Cells were then washed 3x 1xPBS, and fixed with 4% paraformaldehyde in 1xPBS for 15min at 24C. After washing 2x 1xPBS, cells were quenched with 50mM ammonium chloride in 1xPBS for 10min at 24C. Cells were washed 2x 1xPBS and permeabilized with 0.1% Triton-100 in 1xPBS for 5min. After washing 2x 1xPBS, cells were blocked in 2% BSA in 1xPBS for 1h at 24C. Cells were washed 2x 1xPBS and incubated with primary antibody in 1xPBS + 0.3% BSA (1:200 dilution) for 2h at 24C. After washing 3x 1xPBS, cells were incubated with secondary antibodies in 1xPBS + 0.3% BSA (1:200 dilution) and keep in the dark for 1h at 24C. Cells were washed 3x 1xPBS, stained 3min with DAPI (1:1000 dilution) in 1xPBS, before mounting on microscope slides. Images were taken with a 40x objective on a Zeiss AxioImager Z1 microscope and an AxioCam MRm camera (150-300ms exposure time). Inducible knockout of Raptor was performed as previously described, and MEFs were images as described above.
**Glycerol Gradient Ultracentrifugation:** Cells (1x10^7) were suspended in 250µL of lysis buffer containing 100mM Tris-HCL pH 8.0, 100mM KCl, 25 mM MgCl2, 1 mM DTT, 7.5% (vol/vol) DMSO, and 2.5% (vol/vol) glycerol. CAD oligomers were then isolated on 10–35% (vol/vol) glycerol gradients as previously described (Lee et al., 1985).

**Immunoelectron microscopy:** HeLa cells were serum starved for 16h hours in D-MEM containing with 0.1% D-FCS, followed by 30min AA starvation in 1xPBS. Cells were restimulated with 10% D-FCS and double the concentration of AAs in D-MEM for 1h. Cells were then fixed and treated for immunoelectron microscopy as previously described (Stettler et al., 2009). Ten-nanometer gold particles coupled to goat anti-rabbit IgG were used to detect rabbit anti-CAD antibodies.

**Immunoprecipitations:** Cells were rinsed twice with ice-cold PBS before lysis. Lysis buffer contained 40mM HEPES pH 7.4, 150mM NaCl, 0.3% CHAPS supplemented with protease and phosphatase inhibitors (1x Roche complete protease inhibitor cocktail, 1 µg/ml Aprotinin, 1 µg/ml Pepstatin, 10 mM NaF, 10 mM β-glycerophosphate, 0.5mM ortho-vanadate). Cells were harvested and incubated on ice for 5 minutes. Cells suspensions were then gently sonicated for 5 seconds on ice. Cell debris was removed by centrifugation for 10 minutes at 10000g. The protein concentration was determined with a Bio-Rad assay. For immunoprecipitations (IP), 5 µg of antibody was incubated with 1mL of cleared cell lysate from a 10 cm plate, and rotated for 2 hours (HA IP) or overnight (mTOR IP) at 4°C. Normal rabbit IgG was used for mock IPs. 50 µl of a 50% slurry of protein A-agarose was added and then additionally incubated for one hour. Beads were collected by centrifugation, and immunoprecipitations were washed three times in lysis buffer before being resuspended in 2x SDS-PAGE sample buffer.

**Metabolic Measurements:** Prior to metabolic labeling, HeLa cells were serum starved for 16h hours in D-MEM containing with 0.1% D-FCS, followed by 30min AA starvation in 1xPBS. Cells were restimulated with 10% D-FCS and 2x AA (minus glutamine) + 4mM N15-amide labeled glutamine. Cells were
washed 2x with ice-cold 1xPBS. Cells were then quenched by flash freezing the plates in liquid nitrogen. Metabolites were extracted with 75% EtOH in 10mM ammonium acetate, pH 7.5 for 5min at 80C. Cell debris was removed via centrifugation at 3000g for 5min at 4C. Samples were then dried in a speed-vac at stored at -80C until measurement. Metabolites were then measured using a targeted approach with uHPLC-MS/MS as previously described (Buescher et al., 2010). Total cellular concentrations of metabolites were calculated using a HeLa cell volume of 2425 µM$^3$, and then comparing the relative amount of metabolites in cells to a heavy isotope labeled internal standard and exogenous metabolite standard curves.

**mTOR endogenous in vitro kinase assay:** *In vitro* kinase protocol was modified from (Sarbassov et al., 2005). Briefly, immunoprecipitations of mTOR or HA-mTOR were prepared as previously described. After three washes in lysis buffer, immunoprecipitations were split into two equal tubes: one tube was used to assess mTOR phosphorylation at Ser-2481, and one for *in vitro* kinase assay. Immunoprecipitations for *in vitro* kinase assay were resuspended in kinase reaction buffer (25 mM Hepes pH 7.4, 50mM NaCl, 1 mM MgCl2, and 1mM ATP) at 37C for 30min plus inactive 1µg Akt/PKB1 (Upstate Biotechnology) as a substrate.

**mTOR recombinant in vitro kinase assay:** Cells were rinsed three times with ice-cold 1xPBS before lysis. Cells were then lysed in RIPA buffer (50mM Tris, pH 7.4, 150mM NaCl, 1mM EDTA, 1.0% NP-40, and 0.1% Deoxycholate with protease and phosphatase inhibitors: 1x Roche complete protease inhibitor cocktail, 1µg/ml Aprotinin, 1µg/ml Pepstatin, 10mM NaF, 10mM β-glycerophosphate, 0.5mM ortho-vanadate). Cells were harvested by scrapping and incubated on ice for 20 minutes. Cell debris was removed by centrifugation for 10 minutes at 1000g. The protein concentration was determined with a BCA assay. 5µg of antibody was incubated with 1mg of cleared cell lysate and rotated for 3 hours at 4°C. Normal rabbit IgG was used for mock IPs. 40µl of a 50% slurry of protein A-agarose was added and then incubated for one additional hour. Beads were collected by centrifugation, and immunoprecipitations were washed four times in lysis buffer before being
resuspended in kinase reaction buffer (25mM HEPES pH 7.4, 50mM NaCl, 0.01% Tween, 1mM MnCl₂, 1mM DTT, and 1mM ATP + 10uCi ³²P-ATP) at 37C for 30min with 100ng mTOR (Invitrogen) +/- 200nM PP242.

**Peptide array spotting:** An array of 600 peptides was synthesized on cellulose paper using an intavis AG MultiPep robotic spotter following the manufacture’s instructions. 12-mer peptides (6aa-pS/T-5aa) of each pSer/Thr site identified as mTOR regulated in 2/3 SILAC labels were spotted in duplicate. Additionally, Ala was substituted for each pSer/Thr identified via MS and spotted in duplicate (6aa-A-5aa).

**Peptide array in vitro kinase assay:** The printed peptide array was moistened in 95% EtOH, and blocked overnight at 24C in peptide kinase buffer (25mM HEPES pH 7.2, 50mM NaCL, 0.01% Tween-20, 10mM MnCl₂, 1mM EDTA) supplemented with 0.2 mg/mL BSA. The array was then additionally blocked with a cold-kinase assay (peptide kinase buffer + 1.0mg/mL BSA + 1mM ATP and incubated at 37C for 30min). The mTOR kinase reaction was carried out with peptide kinase buffer + 0.1mg/mL BSA + 500uM ATP + 500uCi ³²P-ATP + 100ng mTOR (Invitrogen) for 3h at 37C. After extensive washing (3x for 10min in [50mM Tris, pH 6.8, 8M urea, 1% SDS, 0.5% B-ME], 2x ddH2O, 3x for 10min in [50mM Tris, pH 6.8, 1M NaCL], 2x ddH2O, 3x for 10min in [5% phosphoric acid, pH 1.5], 2x ddH2O, 2min in 95% EtOH) the array was air dried and incorporation of ³²P into the peptides was measured with a PhosphorScreen (Molecular Dynamics) and a STORM scanner (Amersham/GE).

**Peptide in vitro kinase assay:** Soluble peptides were incubated in peptide kinase buffer (25mM HEPES pH 7.2, 50mM NaCL, 0.01% Tween-20, 10mM MnCl₂, 1mM EDTA + 0.1mg/mL BSA + 500uM ATP + 500uCi ³²P-ATP + 100ng mTOR (Invitrogen) +/- 500nM PP242) for 30min at 37C. Peptides were then spotted and air dried on Whatman P81 paper. After washing 3x [0.5% phosphoric acid, pH 1.5] and 1x 100% acetone, the incorporation of ³²P into the peptides was measured with a liquid scintillation counter.
Phosphoproteomics: Protein extraction, SDS-PAGE protein fractionation, in-gel digestion, peptide desalting, phosphopeptide enrichment, nano-LC-MS/MS analysis, database search, and quantitation were previously described (Soulard et al., 2010) with the following changes: 2mg of each SILAC labeled protein extract was mixed together prior to SDS-PAGE fractionation. Identification of phosphopeptides was done with MaxQuant 1.13.13 against the mouse or human Swiss-Prot/TrEMB database downloaded from Uniprot on June 30th, 2009. The databases contained forward and reverse mouse or human sequences as well as common contaminants.

Plasmids: HA-mTOR, and HA-mTOR kinase dead (KD) were described previously (Dennis et al., 2001). Human CAD cloned into pCMV-SPORT6 was obtained from Open Biosystems (accession# BC065510). CAD-Ser-1859-Ala, HA-mTOR Ser-2481-Ala, and HA-mTOR Ser-2481-Asp were produced via site-directed mutagenesis and verified by DNA sequencing.

Quantification and statistics: Quantification of immunoblot signals was performed with ImageQuant v7.0 using a STORM scanner and ECL Plus (from Amersham) or 32P-ATP via PhosphorScreens (Molecular Dynamics). Phosphospecific signals were normalized to the total amount of the corresponding protein. Statistical significance was determined using a Student’s t-Test. Data is presented as the mean ± standard deviation.

TiO2 phosphopeptide enrichment: 0.40mg TiO2 spheres were used per 1.00mg total peptides. TiO2 beads were primed with 100% acentronitrile (AcCN). TiO2 beads and total peptides were then combined in binding buffer (80% AcCN, 5% TFA, 1M Glycolic Acid), and incubated for 30min. TiO2 beads were then washed 1x binding buffer, and 2x with washing buffer (80% AcCN, 1% TFA). Phosphopeptides were eluted with 30% AcCN, 300mM NH4OH.
6.0: Acknowledgements

I gratefully acknowledge the Werner-Siemens Foundation for fellowship funding, Nadine Cybulski for generating the iRapKO and iRicKO cell lines, Lee Graves for providing the G9c cells, Estella Jacinto for providing the Sin1-/- MEFs, Anna Santamaria for advice on peptide array spotting protocols, and Erich Nigg for use of the MultiPep robotic spotter. Also, I thank Michael Hall and Paul Jenoe for supervising the research, Brian Hemmings for being a member of my PhD committee, Anne Spang for chairing the PhD defense, and the entire Hall lab for great conversations.

Michael Hall proposed the project, and Aaron M. Robitaille designed the experimental setup. Aaron M. Robitaille and Luca Fava performed the peptide spotting. Stefan Christen carried out the metabolic measurements. Mitsugu Shimobayashi created CAD point mutation. Marion Cornu generated L-TSC1 KO mice. Cristina Prescianotto-Baschong performed immunoelectron microscopy. Additional experiments and data analysis by Aaron M. Robitaille. Paul Jenoe and Suzette Moes maintained the nano-LC-MS/MS Orbitrap. Michael Hall, Paul Jenoe, and Uwe Sauer supervised the research.
8.0: References


protein kinase A toward some but not all substrates. Mol Biol Cell 21, 3475-3486.


Wang, L., Lawrence, J.C., Sturgill, T.W., and Harris, T.E. (2009b). Mammalian target of rapamycin complex 1 (mTORC1) activity is associated with phosphorylation of raptor by mTOR. J Biol Chem.


Table 1: mTOR regulated phosphorylations identified by mass spectrometry.

An excel version of the mTOR regulated phosphorylations is available online at: edoc.unibas.ch
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RS(ph)PS(ph)PAAMSER
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mTOR regulated phosphorylations

Raptor down
iRapKO/Control

Peptide array +
SILAC 1 / Biological 1

Raptor down

Peptide array +
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**Table Note:**
- H/L Ratio: Ratio of heavy to light label intensity.
- SILAC: Stable Isotope Labeling by Amino Acid Exchange.
- Peptide array: Indicate the specific peptide sequence involved in the phosphorylation event.
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**Note:** The table above represents peptide phosphorylation data for the given uniprot entries. The columns indicate the uniprot ID, modified sequence, MS1 and MS2 ratios, NM ratio, experiment, and SILAC ratios for different biological conditions.
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DTAATFQSVDGS(ph)PQAEQSPLESTSK
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Raptor up

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SILAC 2 / Biological 3
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13. *iRicKO/IRapKO* | 0.4919 | 0.2055 | 0.0516 |
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