Regulation of adipogenesis and adipose maintenance by the mammalian TOR complex 1

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Summary

mTOR, an atypical serine/threonine kinase, is a central component of a highly conserved signal transduction cascade that controls cell growth. It functions as part of two distinct multiprotein complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTORC1 contains mTOR, raptor, mLST8, and PRAS40, and is sensitive to the immunosuppressive and anti-cancer drug rapamycin. mTORC1 controls protein synthesis via phosphorylation of two well characterized effectors, the kinase S6K and the translational repressor 4E-BP1. mTORC2 contains mTOR, mLST8, rictor and mSin1, and is not directly inhibited by rapamycin, although long term rapamycin treatment can inhibit mTORC2 indirectly in certain cell types. It controls organization of the actin cytoskeleton. Both complexes are conserved in structure and function from yeast to human.

The mTOR signaling pathway is controlled by nutrients, cellular energy status, and growth factors such as insulin. Since mTOR is regulated by metabolic signals, we focused our research on the roles of the mTOR signaling pathway in metabolic tissues, in particular adipose tissue. My research project concentrated on studying how mTORC1 signaling affects adipocytes, in tissue culture and in mice.

Adipose tissue functions mainly as a long term fat storage depot. However, it is also an important endocrine organ, which secretes hormones, cytokines and complement factors. In this thesis, I first present evidence confirming that mTORC1 is required for the differentiation and maintenance of adipocytes *in vitro.* In tissue culture, inhibition of mTORC1 caused a decrease in the expression of adipose transcription factors, which led to a decreased expression of genes related to fat metabolism and storage. This resulted in de-differentiation of the cells, manifested as loss of intracellular triglycerides. I further focused my research on the key adipogenic transcription factor PPARγ, and tried to elucidate the molecular mechanism by which mTORC1 regulates its activity. The results suggested that rapamycin treatment acts to inhibit PPARγ downstream of its ligands.

To investigate a role of adipose mTORC1 in regulation of adipose and whole body metabolism, we generated mice with an adipose-specific knockout of *raptor* (*raptor*$^{ad-/-}$). Compared to control littermates, *raptor*$^{ad-/-}$ mice had substantially less adipose tissue, were protected against diet-induced obesity and hypercholesterolemia, and exhibited improved insulin sensitivity. Leanness was despite reduced physical activity and unaffected caloric intake, lipolysis, and absorption of lipids from the food. White adipose tissue of *raptor*$^{ad-/-}$ mice displayed enhanced expression of genes encoding mitochondrial uncoupling proteins characteristic of brown fat. Leanness of the *raptor*$^{ad-/-}$ mice was attributed to elevated energy expenditure due to mitochondrial uncoupling. These results suggest that adipose mTORC1 is a regulator of adipose metabolism and thereby controls whole body energy homeostasis.

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Introduction

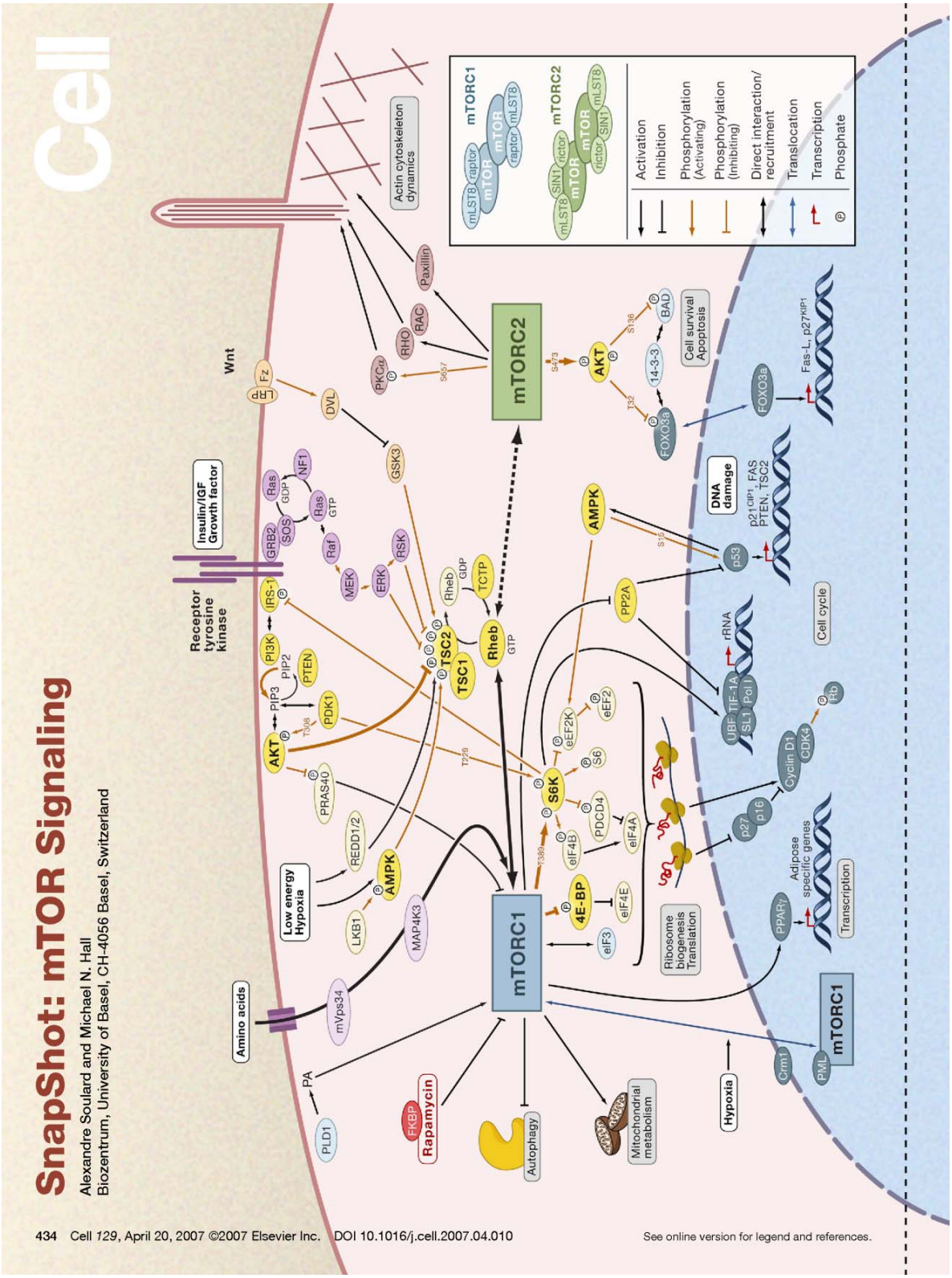
General overview

TOR (Target of Rapamycin) kinase is a highly conserved, central controller of cell growth (Corradetti and Guan, 2006; Guertin and Sabatini, 2007; Hay and Sonenberg, 2004; Wullschleger et al., 2006). It was originally identified in *S. cerevisiae* (Heitman et al., 1991), as the molecular target of the anti-fungal drug rapamycin. Later on, TOR genes were identified also in algae, slime mold, plants, worms, flies, and mammals (Wullschleger et al., 2006). The fundamental importance of TOR is underscored by genetic studies showing TOR to be essential for cell growth and development; disruption of the *TOR* gene is lethal in all examined species (Gangloff et al., 2004; Kunz et al., 1993; Long et al., 2002; Menand et al., 2002; Murakami et al., 2004; Oldham et al., 2000). In humans, dysfunctional mTOR signaling plays an important role in many if not most cancers, as well as in diseases such as tuberous sclerosis complex (TSC) and lymphangiomyelomatosis.

TOR is found in two functionally and structurally distinct multiprotein complexes termed TOR complex 1 (TORC1) and TORC2 (Jacinto et al., 2004; Loewith et al., 2002; Sarbassov et al., 2004). In mammals, mTORC1 consists of mTOR, raptor, PRAS40, and LST8, and is sensitive to rapamycin (Fonseca et al., 2007; Haar et al., 2007; Hara et al., 2002; Kim et al., 2002; Kim et al., 2003; Loewith et al., 2002; Oshiro et al., 2007; Sancak et al., 2007; Thedieck et al., 2007; Wang et al., 2007b). mTORC2 contains mTOR, rictor, SIN1, and LST8 (Frias et al., 2006; Jacinto et al., 2006; Jacinto et al., 2004; Sarbassov et al., 2004; Yang et al., 2006a), and is not directly inhibited by rapamycin, although long term rapamycin treatment can inhibit mTORC2 indirectly in certain cell types (Sarbassov et al., 2006). Knockout of *mTOR*, *raptor*, *SIN1* or *rictor* in mice is embryonic lethal, indicating that both mTORC1 and mTORC2 are essential (Gangloff et al., 2004; Guertin et al., 2006; Jacinto et al., 2006; Murakami et al., 2004; Shiota et al., 2006; Yang et al., 2006a).

mTORC1 is activated by nutrients (amino acids), anabolic growth factors (e.g., insulin and insulin-like growth factor), and cellular energy (ATP). The growth factor signal and energy status are transmitted to mTORC1 via the essential tuberous sclerosis complex proteins TSC1 and TSC2 (Crino et al., 2006; Kwiatkowski, 2003). The TSC heterodimer (TSC1-TSC2) is a GTPase activating protein (GAP) that inhibits the essential small GTPase Rheb (Garami et al., 2003; Zhang et al., 2003b). Rheb-GTP binds and activates mTORC1 (Avruch et al., 2006). Akt phosphorylates and inactivates TSC2 in response to growth factors (Shaw and Cantley, 2006), whereas AMP kinase (AMPK) phosphorylates and activates TSC2 in response to low energy (high AMP) (Dennis et al., 2001; Inoki et al., 2003b). Nutrients impinge on mTORC1 at the level of Rheb or mTORC1 by a poorly understood mechanism involving the type III PI3K hVps34 (Avruch et al., 2006; Nobukuni et al., 2005). The upstream regulators of the more recently identified mTORC2 are not known, but mTORC2 appears to respond at least to growth factors, possibly via TSC1-TSC2 (Yang et al., 2006b). The mammalian TOR signaling network is depicted in illustration 1 (Soulard and Hall, 2007).

mTORC1 and mTORC2 separately control many cellular processes that collectively determine cell growth and development. mTORC1 controls transcription, protein synthesis, ribosome biogenesis, nutrient transport, and autophagy, among other processes. mTORC1 controls protein synthesis via phosphorylation of S6 kinase (S6K) and eIF-4E binding protein (4E-BP), two key regulators of translation initiation (Brunn et al., 1997; Burnett et al., 1998; Hay and Sonenberg, 2004). mTORC2 controls organization of the actin cytoskeleton via small Rho-type GTPases and Protein Kinase C (Jacinto et al., 2004; Sarbassov et al., 2004; Schmidt et al., 1997), and thereby determines the shape and possibly motility of the cell. In addition, mTORC2 phosphorylates Ser473 within the hydrophobic motif of Akt and thereby activates Akt toward substrates such as the Forkhead transcription factor FOXO and the apoptosis regulator BAD (Jacinto et al., 2006; Sarbassov et al., 2005; Shiota et al., 2006; Yang et al., 2006a).



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See online version for legend and references.

Illustration 1. The mammalian TOR signaling pathway *(Soulard and Hall, 2007).*

Although mTORC1 is present in essentially all tissues, the findings that it is controlled by metabolic signals and is implicated in metabolic disorders suggest that it plays a particularly important role in metabolic tissues. Thus, we have focused on studying the role of the mTORC1 signaling pathway in metabolic tissues, in particular adipose tissue. My research project concentrated on studying how mTORC1 signaling affects adipocytes, in tissue culture and in mice.

Adipose tissue functions mainly as a long term fat storage depot. However, it is also an important endocrine organ, which secretes hormones, cytokines and complement factors such as leptin, adiponectin, TNFα and many others (Gimeno and Klaman, 2005; Shi and Burn, 2004), that regulate energy homeostasis, lipid metabolism, appetite, fertility, immune and stress responses. Excess or deficiency of adipose tissue can lead to severe metabolic diseases such as type 2 diabetes, cardiovascular disorders and cancer.

Several studies in tissue culture have demonstrated that rapamycin treatment strongly inhibits differentiation and maintenance of adipocytes (Bell et al., 2000; Cho et al., 2004; El-Chaar et al., 2004; Gagnon et al., 2001; Kim and Chen, 2004; Yeh et al., 1995a). In addition, rapamycin is being used as an immunosuppressive medication, with the major adverse reaction of hyperlipidemia (Morrisett et al., 2002) , which might also suggest a function for mTORC1 in adipose tissue.

In this thesis, I will present experiments that show the importance of mTORC1 for the development and maintenance of adipose tissue, and examine the molecular basis for mTORC1 requirement for adipogenesis and adipose maintenance.

The next parts of the introduction will include a deaper overview of the published literature concerning the most relevant aspects of my research work: mTORC1 structure, regulation and signaling, adipose tissue function, and the links between them.

The TOR protein

TOR is a large protein (~280 kDa), that was first identified in 1991 in *S. cerevisiae* as the target of the anti-fungal drug rapamycin (Heitman et al., 1991). It was subsequently isolated and sequenced (Cafferkey et al., 1993; Helliwell et al., 1994; Kunz et al., 1993), and found to have homologs in many other species, including the fungi *S. pombe* (Weisman and Choder, 2001), *C. neoformans* (Cruz et al., 1999) and *C. albicans* (Cruz et al., 2001), and the higher eukaryotes *A. thaliana* (Menand et al., 2002), *C. reinhardtii* (Crespo et al., 2005), *Dictyostelium* (Lee et al., 2005), *D. melanogaster* (Oldham et al., 2000; Zhang et al., 2000), *C. elegans* (Long et al., 2002), and mammals. The mammalian TOR is called mTOR (also named FRAP, RAFT1) (Brown et al., 1994; Sabatini et al., 1994; Sabers et al., 1995). TOR homologs were found in every examined eukaryotic organism to date, indicating that it might be conserved in all eukaryotes. Budding yeast contain two TOR proteins, TOR1 and TOR2, that are 67% identical in their amino acids sequence (Helliwell et al., 1994). Other fungi also contain two TOR proteins, but higher eukaryotes contain only one TOR protein.

The C-terminus of the TOR proteins bares homology to the catalytic subunits of PI3Ks from bovine and yeast (Cafferkey et al., 1993; Kunz et al., 1993). Based on this homology, TOR was classified and is the founding member of the PI kinase-related kinases (PIKKs) family, together with the yeast TEL1 and MEC1, fly MEI41, worm SMG-1, and human DNA-PK, ATM, ATR, and FRP1 (Abraham, 2001; Keith and Schreiber, 1995). Despite the homology to lipid kinases, PIKK kinases are in fact Ser/Thr kinases.

The N-terminus of TOR contains two large blocks of tandem HEAT motifs, that were named so because they appear, among others, in the proteins Huntingtin, elongation factor 3, A subunit of protein phosphatase 2A, and TOR1 (Andrade and Bork, 1995). These motifs consist of 37-43 amino acids that occur in blocks of 3-22 tandem repeats, not well conserved in sequence but baring a common structure architecture of hydrophobic and hydrophilic residues that form antiparallel α-helices (Kobe et al., 1999). They are thought to mediate protein-protein interactions. Next to the HEAT repeats is

the FAT domain, another domain common to all PIKKs. This domain is always coupled with a FATC domain, that is found at the very C-terminus of TOR. These two domains are thought to interact with each other to mediate protein-protein interactions. Subsequent to the FAT domain is the FKBP-rapamycin binding domain (FRB), the binding site for the FKBP-rapamycin complex. Finally there is the kinase domain, which is the active site of TOR. To summarize, the outline of the TOR domains order from N- to C-terminus is HEAT-HEAT-FAT-FRB-kinase-FATC.

TORC1

Composition and localization

TORC1 was first described in *S. cerevisiae*, where it contains either TOR1 or TOR2, in addition to LST8, KOG1, and TCO89 (Loewith et al., 2002; Reinke et al., 2004). Deletion of any of TORC1 components except TCO89 disrupts the complex, and is lethal for the yeast (Heitman et al., 1991; Loewith et al., 2002). The mammalian counterparts of mTORC1 are mTOR, mLST8, PRAS40 and raptor (homolog of KOG1) (Fonseca et al., 2007; Haar et al., 2007; Hara et al., 2002; Kim et al., 2002; Kim et al., 2003; Loewith et al., 2002; Oshiro et al., 2007; Sancak et al., 2007; Thedieck et al., 2007; Wang et al., 2007b). Tissue RNA expression profiling revealed ubiquitous expression of the mTORC1 members. Especially high levels were detected in skeletal muscle, and to a lesser extent in kidney, heart, brain, liver, and placenta (Kim et al., 2002; Loewith et al., 2002). The subcellular localization of mTORC1 is under debate, as several groups have shown very different localizations including the cytoplasm, nucleus, mitochondria and ER/golgi (Desai et al., 2002; Drenan et al., 2004; Kim and Chen, 2000; Paglin et al., 2005; Park et al., 2002; Sabatini et al., 1999; Schieke et al., 2006; Zhang et al., 2002).

KOG1 is highly conserved, with homologs found in every sequenced eukaryotic genome (Kim et al., 2002). raptor (regulatory associated protein of mTOR), the mammalian homolog, is a 150 kDa protein that contains in its N-terminus a novel domain

named RNC (raptor N-terminal conserved) that is conserved among raptor homologs. This domain is composed of three blocks with at least 67%–79% sequence similarity, predicted to form α-helices (Kim et al., 2002). raptor also contains four HEAT repeats and seven WD-40 repeats (Shinozaki-Yabana et al., 2000). The interaction between raptor and mTOR involves the HEAT repeats of mTOR, and multiple sites in raptor (Kim et al., 2002). raptor directly interacts with substrates of mTORC1, and functions as a scaffold protein to tether the substrates and mTOR together (Nojima et al., 2003). The interaction between mTOR and raptor is disrupted by rapamycin treatment (Kim et al., 2002; Oshiro et al., 2004).

LST8 is an essential protein of unknown function. The mammalian homolog mLST8 (previously also called GβL) is a 36 kDa protein that consists almost entirely of seven WD-40 repeats (Kim et al., 2003). It binds the C-terminus of mTOR and positively regulates its activity (Kim et al., 2003).

Upstream regulators

mTORC1 integrates signals from several inputs, namely growth factors, nutrients, energy and stress. An overview on the entire mTOR upstream regulatory network can be found in illustration 1 (Soulard and Hall, 2007).

The best described signaling pathway to activate mTORC1 is via the growth factor insulin. Insulin binds to its receptor, a tyrosine kinase, which in response phosphorylates and activates the insulin receptor substrate (IRS). IRS recruits then PI3K, that phosphorylates and converts phosphatidylinositol-4,5-phosphate (PIP2) in the membrane into phosphatidylinositol-3,4,5-phosphate (PIP3). PIP3 then recruits both PDK1 and Akt to the membrane, and Akt is phosphorylated and activated by PDK1. Akt then activates mTORC1 via two pathways. The first is by an inhibitory phosphorylation of the TSC complex (Inoki et al., 2002; Manning et al., 2002; Potter et al., 2002), that when active acts as a GAP for the small GTPase rheb (Garami et al., 2003; Tee et al., 2003; Zhang et al., 2003b). Rheb can directly bind and activate mTOR (Inoki et al., 2003a; Long et al., 2005a).

The second way via which Akt activates mTOR is by an inhibitory phosphorylation of PRAS40, which functions to inhibit the activity of mTORC1 (Fonseca et al., 2007; Haar et al., 2007; Oshiro et al., 2007; Sancak et al., 2007; Thedieck et al., 2007; Wang et al., 2007b).

The Wnt signaling pathway is another growth regulator that positively controls mTORC1. The canonical Wnt pathway involves binding of extracellular Wnt proteins to cell surface receptors of the Frizzled family and subsequent inhibition of glycogen synthase 3 (GSK3). Inhibition of GSK3 stabilizes β-catenin and leads to its nuclear translocation to activate transcription of a large number of growth promoting genes. A recent study showed that TSC2 is a substrate of GSK3 and hence, that active Wnt signaling releases the GSK3-dependent inhibition of mTORC1 (Inoki et al., 2006).

The lipid second messenger phosphatidic acid (PA) is a critical mediator for mitogenic activation of mTOR signaling (Fang et al., 2001). The mechanism involves synthesis of PA by phospholipase D1 and 2 (PLD1/2), that is activated by the mitogenic signals (Fang et al., 2003; Ha et al., 2006; Kam and Exton, 2004).

Amino acids availability also regulates the activity of mTORC1. Withdrawal of amino acids results in a rapid dephosphorylation of 4E-BP and S6K, whereas an increase in amino acids increases the activity of S6K (Hara et al., 1998). In particular, leucine and arginine seem to have the most profound effect on mTORC1, since withdrawal of either one of these amino acids inhibits mTORC1 almost to the same extent as withdrawal of all amino acids (Hara et al., 1998). The effect of amino acids on mTOR signaling is dominant to that of growth factors, since it has been shown that in the absence of amino acids insulin does not activate mTOR, but not vice versa (Hara et al., 1998). Amino acids impinge on the mTOR signaling pathway independently (downstream) of the TSC proteins (Roccio et al., 2005; Smith et al., 2005). There are several indications that amino acids signal to mTORC1 via rheb: amino acids withdrawal impairs mTORC1-Rheb binding (Long et al., 2005b), and mTORC1 inhibition in amino acid starved cells can be rescued to full levels by overexpression of Rheb (Long et al., 2005a; Long et al., 2005b). Recent data suggest that

amino acids signal to mTORC1 via another mechanism that is independent of the TSC-Rheb axis: hVps34 (vacuolar protein sorting 34), a class III PI3 kinase, was identified as a positive regulator of mTORC1 that is repressed by amino acid starvation and is insulin insensitive (Byfield et al., 2005; Nobukuni et al., 2005). Another recently identified positive regulator of mTORC1 in response to amino acids is MAP4K3 (mitogen-acivated protein kinase kinase kinase kinase 3) (Findlay et al., 2007). However, the mechanism by which these proteins sense amino acids availability and transmit the signal to mTORC1 remains to be elucidated.

The effect of fatty acids on mTORC1 is much less studied than the effect of amino acids. Two studies indicate that mTORC1 might be activated by fatty acids. The first showed that mTOR activity was reduced in the hearts of rats that were treated with niacin to lower the plasma level of free fatty acids (Crozier et al., 2003). The second showed that incubation of liver cells with palmitate results in activation of mTORC1 (Mordier and Iynedjian, 2007). In both cases the molecular mechanism remains unknown.

mTORC1 can also sense the cellular energy status, via AMPK. Decrease in the cellular energy status is reflected by a rise in the AMP:ATP ratio within the cell, which activates AMPK by direct AMP binding. Active AMPK turns on catabolic ATP-generating pathways such as fatty acid oxidation and glycolysis, and shuts off ATP-consuming anabolic processes such as translation and fatty acid synthesis. AMPK phosphorylates and activates TSC2, resulting in inhibition of mTORC1 (Inoki et al., 2003b). One of the two TSC2 sites that are phosphorylated by AMPK serves as a priming event for subsequent phosphorylation of TSC2 by GSK3 (Inoki et al., 2006). In addition to high AMP:ATP ratio, AMPK can also be activated by phosphorylation by LKB1. This phosphorylation also leads to activation of TSC2 (Corradetti et al., 2004; Shaw et al., 2004).

Cellular stresses such as hypoxia inhibit mTORC1. REDD1 and 2 (Regulated in Development and DNA Damage Response genes 1 and 2) are negative regulators of mTORC1 in response to hypoxia, which limits ATP synthesis and therefore leads to energy

deprivation. REDD1/2 are transcriptionally upregulated during energy deprivation and inhibit mTORC1 by acting upstream of TSC1/2 (Brugarolas et al., 2004; Reiling and Hafen, 2004). The mechanism via which REDD1/2 activate TSC1/2 involves the dissociation of TSC2 from inhibitory 14-3-3 proteins (DeYoung et al., 2008). Hypoxia can inhibit mTORC1 even in the presence of constitutive Akt activation (DeYoung et al., 2008). Hypoxia likely inhibits mTORC1 also through the AMPK pathway, since prolonged hypoxia leads to ATP depletion and activation of AMPK.

Substrates/readouts

In budding yeast, the only known direct target of TORC1 is Sch9p, a protein that belongs to the family of AGC kinases (protein kinase A/protein kinase G/protein kinase C) (Urban et al., 2007). Other less direct effectors of TORC1 include TAP42/TIP41 (Jacinto et al., 2001; Jiang and Broach, 1999), GLN3 (Bertram et al., 2000), NPR1 (Schmidt et al., 1998), SLM1/2 (Audhya et al., 2004) and FHL1 (Martin et al., 2004). TORC1 in yeast regulates cellular processes such as ribosome biogenesis, translation, transcription, nutrient import and autophagy (Wullschleger et al., 2006). In mammals, mTORC1 regulates similar cellular processes as in yeast. Known direct phosphorylation targets are the AGC kinase S6K, and the translation inhibitor 4E-BP (also known as PHAS) (Brunn et al., 1997; Burnett et al., 1998).

4E-BP has 3 isoforms; all are small proteins (10-12 kD) that participate in the regulation of the rate limiting step in translation, i.e. recruitment of the small ribosomal subunit to mRNA during translation initiation. The hypophosphorylated form of 4E-BP binds to and sequesters the translation initiation factor eIF4E, preventing it from binding the mRNA 5' cap and thereby blocking cap-dependent translation. mTOR phosphorylates 4E-BP, causing it to dissociate from eIF4E and thus promoting translation initiation. 4E-BP is phosphorylated by mTOR on multiple sites in a hierarchical manner: phosphorylation on Thr70 and Ser65 promotes the dissociation of 4E-BP from eIF4E, and requires priming phosphorylation on Thr37 and Thr46 (Gingras et al., 1999; Gingras et al., 2001; Mothe-Satney et al., 2000).

S6K and other AGC kinases have a unique activation pattern, which requires phosphorylation of an activation loop that connects the N- and C-lobes of the kinase domain. This can be done by autophosphorylation or by another kinase such as PDK1. In addition, AGC kinases are phosphorylated on a hydrophobic motif found in the C-terminus of the kinase domain. This phosphorylation stabilizes the protein and is important for docking of PDK1 to the AGC kinase. S6K is phosphorylated by mTORC1 on 4 sites: Thr229, Thr389, Ser404 and Ser411 (Ali and Sabatini, 2005; Burnett et al., 1998; Isotani et al., 1999; Pearson et al., 1995). The main phosphorylation site is Thr389, which is located in the hydrophobic motif and is a priming site for phosphorylation of S6K by PDK1. Mutation of Thr389 to alanine ablates kinase activity, whereas mutation to glutamic acid confers constitutive kinase activity and rapamycin resistance (Pearson et al., 1995). Active S6K phosphorylates the ribosomal protein S6 that activates translation.

Both S6K and 4E-BP contain a five amino acid sequence called TOS (TOR signaling) motif, that is required for binding to raptor (Nojima et al., 2003; Schalm and Blenis, 2002). 4E-BP contains also a RAIP motif, termed after the four amino acids it comprises (Choi et al., 2003). This motif is important for the insulin response of 4E-BP, and is conserved among the sequences of 4E-BP1 and 4E-BP2 but is absent from the insulin-refractory 4E-BP3. The RAIP motif is important for phosphorylation of 4E-BP by mTORC1, and perhaps also for binding to raptor (Beugnet et al., 2003; Choi et al., 2003).

S6K has an important regulatory function on the insulin signaling pathway. As mentioned previously, Insulin stimulation elicits serine/threonine phosphorylation of IRS. However, prolonged exposure to insulin results in degradation of IRS and renders the cells refractory to further insulin stimulation. Degradation of IRS occurs in response to phosphorylation by S6K at several sites including Ser302, Ser307, and Ser1101 (Harrington et al., 2004; Haruta et al., 2000; Shah et al., 2004; Tremblay et al., 2007; Ueno et al., 2005a; Ueno et al., 2005b; Um et al., 2004; Wang et al., 2007a; Werner et al., 2004). IRS is also phosphorylated by mTOR on Ser636/639 (Ozes et al., 2001). Since insulin signaling

is what activates mTOR and S6K in the first place, the phosphorylation of IRS by S6K constitutes a so called “negative feedback loop”.

Associated diseases

Aberrant mTOR signaling is frequently involved in tumor formation, either malignant or benign tumors called hamartomas. The best example of mTOR-related hamartoma disease is TSC, caused by loss of function mutations in the TSC1/2 proteins. Loss of function mutations in other proteins that negatively regulate or are negatively regulated by mTOR, such as 4E-BP1, PTEN or LKB1, are also associated with different types of tumors. Overexpression of proteins that positively regulate or are positively regulated by mTOR, such as rheb, S6K1, PI3K or Akt, is also found in many types of cancers (Wullschleger et al., 2006). Rapamycin and its analogs are currently being tested as therapeutic anti-cancer agents.

Rapamycin and its analogs are already used as immunosuppressive drugs, to prevent graft rejection after transplants. This is attributable to rapamycin’s inhibitory effect on T cells (Dumont et al., 1990).

Because of the negative feedback loop between S6K and IRS, the mTOR signaling pathway is also implicated in glucose metabolism. Short term rapamycin treatment improves glucose uptake in humans under prandial-like hyperinsulinemia conditions (Krebs et al., 2007). In rats, rapamycin reverses the insulin resistance that is induced by hyperinsulinemia (Ueno et al., 2005b). TSC knockout MEFs are insulin resistant, similarly to HEK293 cells that overexpress rheb (Shah et al., 2004). The well known anti-diabetic drug metformin acts at least in part via inhibition of IRS degradation by the LKB1-AMPK-mTOR pathway (Dowling et al., 2007; Tzatsos and Kandror, 2006). The fatty acid palmitate induces insulin resistance in liver cells, by a mechanism that involves activation of mTORC1 and increased phosphorylation of IRS1 (Mordier and Iynedjian, 2007). On the other hand, long term rapamycin treatment in mice and in people causes hyperlipidemia, that at least in mice is accompanied by increased glucose intolerance (Cunningham et al.,

2007; Morrisett et al., 2002). The mechanism by which rapamycin induces hyperlipidemia remains to be elucidated.

Adipose tissue

Fat storage

Almost all vertebrates and especially mammals store excess energy for later use as fat. Fat is stored in adipose tissue, mainly in the form of triacylglycerols (TGs). TGs are lipid molecules, formed by condensation of one glycerol and three fatty acid molecules. The length of the fatty acid varies between 3-24 carbons, mostly 16-18. The fatty acids can be saturated, mono- or poly-unsaturated. Storage of fat in the form of TGs is highly efficient, since they contain a very high amount of energy/mass, and since due to their hydrophobicity no water molecules are required in their surroundings, and therefore they can be packed very densely. Within the adipocyte, TGs are stored in structures termed lipid droplets or vesicles. In white adipose tissue (WAT), the TGs are stored in a single large lipid droplet, whose weight is 85-90% of the adipocyte weight. The neutral lipid core of lipid droplets is surrounded by a monolayer of phospholipids. White adipose cells are very large, hundreds to thousands of times larger than red blood cells, fibroblasts and immune system cells. Adipocytes can store very large amounts of TGs, and have the capacity to grow 20-30 fold their normal diameter, corresponding to several thousand fold increase in volume. In addition to adipocytes, adipose tissue contains a matrix of conjunctive tissues (collagen and reticular fibers), nerve fibers, vascular stroma, lymph nodes, immune cells (leukocytes, macrophages), fibroblasts, and preadipocytes (undifferentiated adipose cells) (Fonseca-Alaniz et al., 2007).

Upon a fatty meal, TGs, cholesterol and fat soluble vitamins are packaged together with carrier lipoproteins into particles termed chylomicrons (Illustration 2). The lipoproteins solubilize the lipids, and bear cell-targeting properties. Between meals, TGs can also be endogenously synthesized in the liver, and are packaged together with

cholesterol and carrier lipoproteins into particles termed very low density lipoprotein (VLDL). TGs in chylomicrons and VLDLs are released from the intestine and liver into the bloodstream. Chylomicrons that are depleted of TGs by the target organs (mainly adipose tissue, cardiac and skeletal muscles) are known as chylomicron remnants, and are taken up by the liver. The remaining VLDLs after some of the TG content has been distributed to target tissues are called intermediate density lipoproteins (IDL). Further depletion of IDL from TGs results in low density lipoprotein (LDL) particles. These particles travel to all peripheral tissues and supply them with cholesterol. The liver also secretes high density lipoprotein (HDL) particles, that are named so because they contain the highest ratio of protein:lipid compared to the other lipoprotein particles. These particles travel in the blood and collect and carry excess cholesterol and free fatty acids (FFAs) back to the liver.

In order for TGs to be absorbed by the target organs, they have to be broken down by lipoprotein lipases (LPLs) (illustration 2). LPLs are secreted by the target organs, and are found on the linings of blood vessels. Their activity is stimulated by insulin. Once adipose LPL has broken down the TGs into glycerol and FFAs, adipose tissue can uptake the FFAs from the blood. Adipose tissue can also produce FFAs from glucose. This is done via glycolysis into acetyl CoA, which is then acylated into malonyl-CoA by acetyl-CoA carboxylase. Malonyl-CoA and acetyl-CoA are further acylated by fatty acid synthase, to create a FFA. However, most of the TGs in adipose tissue come from uptake of external FFAs and not from *de novo* synthesis. In order to form TGs for storage, adipose tissue first activates the FFAs by attaching a CoA molecule, and then conjugates them to glycerol-3-phosphate. The glycerol-3-phosphate molecule comes from the reduction of the glycolytic intermediate dihydroxyacetone phosphate. Thus, adipose tissue consumes FFAs and glucose and converts them into TGs. The condensation of FFAs and glycerol into a TG is a multistep enzymatic process (reviewed in (Shi and Burn, 2004)). The attachment of a CoA molecule to the FFA is done by acyl-CoA synthase. Glycerol-3-phosphate and one FFA-CoA are then conjugated by glycerol-3-phosphate acyltransferase, and the second FFA-CoA is conjugated by sn-1-acylglycerol-3-phosphate acyltransferase. The phosphate group of glycerol-3-phosphate is then removed by phosphatidic-acid

phosphohydrolase. Conjugation of the third FFA-CoA is done by diacylglycerol acyltransferase. TGs that were partly broken down can be recycled back into TGs at the step of monoacylglycerol or diacylglycerol. This process occurs at the ER, from which the TG-containing lipid droplets finally emerge.

Fat mobilization

When the body requires energy, a signaling cascade is activated, that leads to degradation of TGs into glycerol and fatty acids by a multistep enzymatic process. The glycerol and fatty acids are released into the bloodstream and transported to target tissues. This process is called lipolysis, and will be described in detail below. The glycerol that is released from adipocytes travels to the liver, where it is integrated into gluconeogenesis. The fatty acids travel to target tissues, in which they are broken down by β-oxidation into acetyl CoA, that enters the citric acid cycle. The main tissues that rely on β-oxidation for energy are the liver and muscle. However, to some extent, all tissues except the brain (Yang et al., 1987) use fatty acids for energy.

Breakdown of TGs to fatty acids and glycerol requires several lipases (Granneman and Moore, 2008): first, adipose triglyceride lipase (ATGL) degrades TGs into diacylglycerols (Kershaw et al., 2006; Zimmermann et al., 2004). Second, the rate limiting step of lipolysis happens when hormone-sensitive lipase (HSL) degrades the diacylglycerols into monoacylglycerols (Belfrage et al., 1978; Haemmerle et al., 2002), that are finally broken into a glycerol and a fatty acid molecule by monoglyceride lipase (Fredrikson et al., 1986).

Stimulation of lipolysis occurs when hormones such as catecholamines or glucagon bind to G protein-coupled receptors. This activates adenylyl cyclase, which produces cAMP. Lipolysis can also be induced by adenylyl cyclase pharmacological activators such as forskolin or isoproterenol. Insulin activates phosphodiesterase 3B that lowers cAMP levels, thereby inhibiting lipolysis (Shakur et al., 2001). cAMP activates protein kinase A (PKA), that phosphorylates and activates hormone sensitive lipase (HSL) (Egan et al., 1992). Another PKA substrate that has key functions during lipolysis is a protein called

perilipin A (Marcinkiewicz et al., 2006; Zhang et al., 2003a). Perilipin A is bound to lipid droplets (Blanchette-Mackie et al., 1995; Greenberg et al., 1991) and, in its basal, non-phosphorylated state, functions to sequester lipases from the TGs inside the lipid droplets (Brasaemle, 2007). Upon phosphorylation of HSL and perilipin A by PKA, HSL is activated and recruited to lipid droplets, where it comes in very close proximity and most likely forms a complex with perilipin A (Clifford et al., 2000; Egan et al., 1992; Granneman et al., 2007; Miyoshi et al., 2006; Sztalryd et al., 2003). In addition to recruitment and docking of HSL, perilipin A also controls the recruitment of ATGL to lipid droplets during lipolysis. Under basal conditions perilipin A binds and sequesters a coactivator of ATGL, a protein called CGI-58 (Granneman et al., 2007; Lass et al., 2006; Subramanian et al., 2004; Yamaguchi et al., 2004). During lipolytic stimulation CGI-58 rapidly dissociates from phosphorylated perilipin A and is free to activate ATGL (Granneman et al., 2007; Subramanian et al., 2004; Yamaguchi et al., 2004). In addition, under basal conditions ATGL can be found both in the cytoplasm and bound to lipid droplets (Bartz et al., 2007; Granneman et al., 2007; Notari et al., 2006; Villena et al., 2004; Zimmermann et al., 2004). During lipolytic stimulation, part of the cytoplasmic ATGL translocates to lipid droplets (Granneman et al., 2007). Perilipin A-dependent activation and translocation of HSL and ATGL to lipid droplets is required and sufficient to induce lipolysis.

Endocrine functions

Adipose tissue is not just an inert fat depot, but also an endocrine organ that participates in the regulation of whole body energy homeostasis. The hormones secreted by adipose tissue – adipokines – are involved in many metabolic processes. The following examples for adipokines that are involved in metabolism were collected from (Fonseca-Alaniz et al., 2007) and (Ronti et al., 2006):

Immune/stress responses: IL-6, TNFα, adipsin, adiponectin, CRP, leptin, resistin, metallothionein, MIF, prostaglandins.

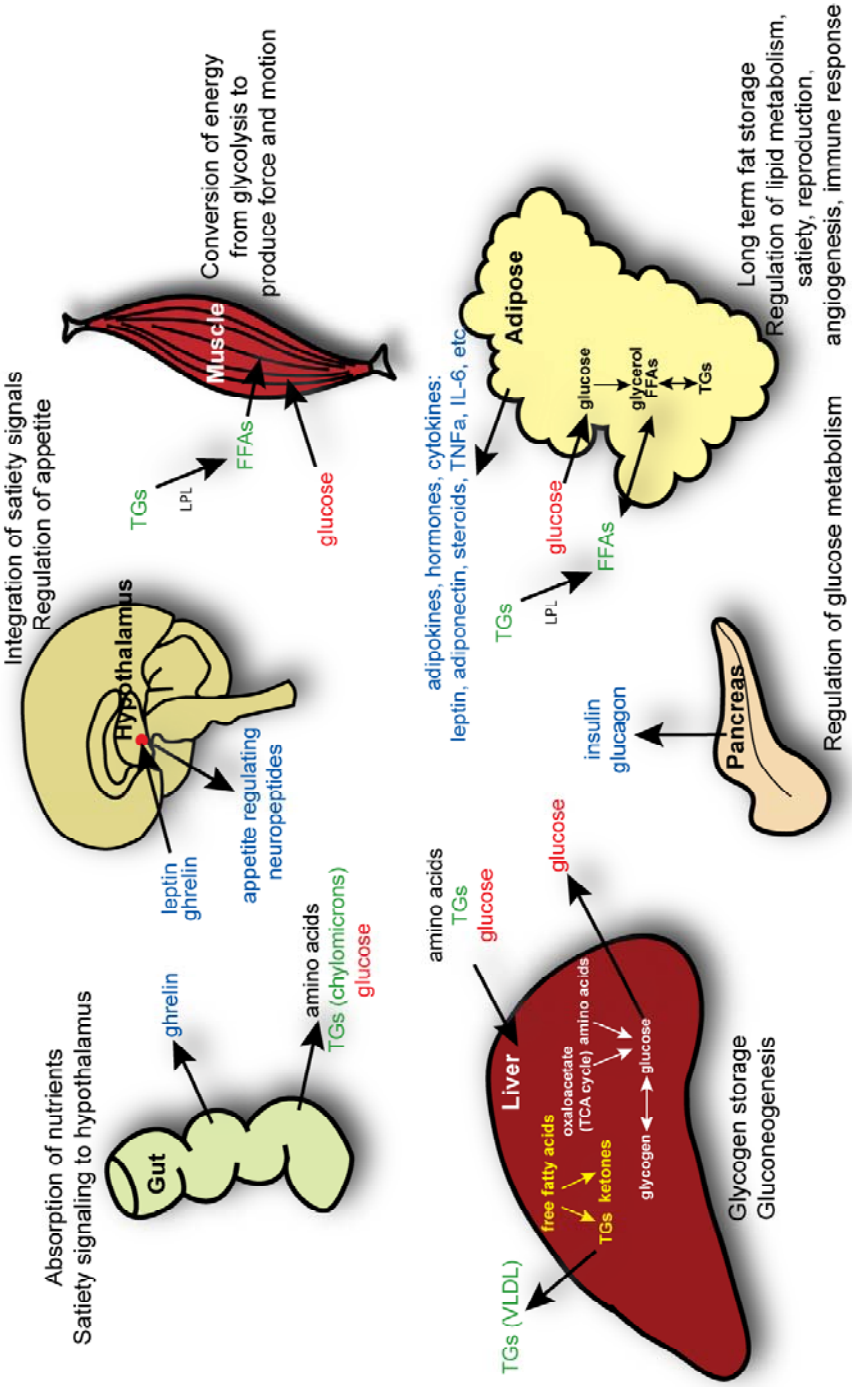


Illustration 2. Schematic view of the interplay between metabolic tissues. *Prepared together with Alexandre Soulard and Nadine Cybulski.*

Vascular homeostasis: angiotensinogen, PAI-1, tissue factor, VEGF, monobutyrin, prostaglandins.

Glucose metabolism: leptin, adiponectin, resistin, TNFα, IL-6, visfatin, steroids, apelin.

Appetite regulation: leptin.

Lipid metabolism: LLP, steroids, CETP, ApoE, LPL, RBP4, ZAG.

Adipose cell size affects the nature of secreted adipokines. The adipokine secretome of larger adipocytes is shifted toward more proinflammatory cytokines (Skurk et al., 2007).

Two of the most studied adipokines are leptin and adiponectin. Leptin is a small protein of 16KDa, that is secreted almost exclusively from adipose tissue in response to feeding (Frederich et al., 1995; Weigle et al., 1997). Leptin receptors are expressed mainly in the hypothalamus. In the hypothalamus, leptin negatively regulates appetite (Halaas et al., 1995; Pelleymounter et al., 1995) by activating the expression of anorexigenic neuropeptides such as proopiomelanocortin (POMC) (Bariohay et al., 2005; Golden et al., 1997; Kristensen et al., 1998; Meister, 2000; Sahu, 1998). Leptin also inhibits the expression of orexigenic neuropeptides such as agouti-related protein (AgRP) (Arvaniti et al., 2001; Kumano et al., 2003; Lopez et al., 2000; Meister, 2000; Sahu, 1998; Schwartz et al., 1996). Apart from its role in reducing appetite, leptin also participates in reproduction, immune responses, hematopoiesis, angiogenesis, osteogenesis, wound healing, and regulation of the neuroendocrine system during starvation (Ahima et al., 1996; Fantuzzi and Faggioni, 2000; Lord et al., 1998; Mantzoros et al., 1997; Takeda et al., 2002).

Adiponectin (also called Adrp30) is another adipokine, that positively regulates insulin sensitivity (Berg et al., 2001; Combs et al., 2002; Hotta et al., 2001; Steffes et al., 2004; Weyer et al., 2001). Adiponectin receptors are found on skeletal muscle and liver cells (Yamauchi et al., 2003). Adiponectin acts through activation of AMPK and PPARα, which

results in stimulation of fatty-acid oxidation and decreased triglyceride content in skeletal muscle and liver (Yamauchi et al., 2002).

Both adiponectin and leptin signaling act at least in part via mTORC1: Adiponectin negatively regulates the mTOR signaling pathway via activation of AMPK. As a result, IRS1 phosphorylation by S6K is reduced, and the systemic outcome is enhanced insulin sensitivity (Huypens, 2007; Tomas et al., 2002; Wang et al., 2007a; Yamauchi et al., 2002). Leptin can activate or inhibit AMPK in different organs (Minokoshi et al., 2004; Minokoshi et al., 2002), but it also inhibits PTEN and thereby positively regulates mTOR. Therefore the systemic effect of leptin on insulin sensitivity is negative (Huypens, 2007).

Adipocytes do not only excrete hormones, but also carry hormone receptors on their surface to receive signals. Signals can be autocrine, such as from IGF1, HGF or TGFβ that regulate proliferation and differentiation of preadipocytes and adipocyte apoptosis, or endocrine, such as from insulin, glucagon, catecholamines, T3 and T4, and many others (Fonseca-Alaniz et al., 2007).

Adaptive thermogenesis

White adipose tissue is widespread subcutaneously and is an excellent thermal insulator. In addition, another type of adipose tissue known as brown adipose tissue (BAT), actively participates in adaptive thermogenesis. BAT oxidizes FFAs and dissipates the energy in the form of heat. BAT is especially important for thermoregulation in small and hibernating mammals (Golozoubova et al., 2001), where it is found in the interscpaular region. In humans, BAT is abundant in newborns but is largely replaced by WAT in adults. The main depots of BAT in humans are in the supraclavicular and the neck regions, with some additional paravertebral, mediastinal, para-aortic, and suprarenal localizations (Nedergaard et al., 2007). Some brown fat cells can also be found interspersed within WAT, both in rodents and humans (Cousin et al., 1992; Garruti and Ricquier, 1992; Guerra et al., 1998; Lean et al., 1986; Oberkofler et al., 1997). Brown fat cells were also found interspersed between muscle bundles in mice (Almind et al., 2007). BAT contains many

densely packed mitochondria, and its brown color comes from high expression of cytochrome oxidase.

Heat production in BAT is achieved by short-circuiting of the mitochondrial proton gradient that is generated by the respiratory chain. Instead of producing ATP, the energy from the proton gradient is discharged as heat. Uncoupling of the proton gradient from ATP production is done by a protein called uncoupling protein 1 (UCP1 or thermogenin), that is found in the inner membranes of mitochondria. UCP1 is a nucleotide-binding protein, and its activity is inhibited by purine nucleoside di- or triphosphate and is activated by FFAs (Nicholls, 2001). The mechanism by which UCP1 acts is not clear (Echtay, 2007; Garlid et al., 2000): one model suggests that UCP1 is a proton transporter, and that the protons' pathway is buffered with fatty acid head groups. The second model is that UCP1 transports fatty acid anions from the matrix to the cytosol of the mitochondria. These fatty acid anions then grab protons from the cytosol, to become neutral fatty acids. Then, the neutral fatty acids flip-flop rapidly and spontaneously toward the side of the matrix, release the proton and are transported back to the cytosolic side by UCP1, and so on. The directionality of the transport is maintained by the high, inside-negative membrane potential of the mitochondrial inner membrane.

Two other uncoupling proteins are expressed in BAT, UCP2 and UCP3. However, these proteins are expressed in much lower levels compared to UCP1 (100-1000 fold less), and do not transfer protons unless specifically stimulated, for example by hydroxynonenal (Brand and Esteves, 2005).

One of the key regulators of BAT is the transcriptional coactivator PPARγ coactivator 1α (PGC1α). PGC1α stimulates the transcription of genes involved in the brown adipocyte differentiation process and mitochondrial biogenesis, such as nuclear respiratory factor 1 and 2, which leads to the transcription of mitochondrial transcription factor A, as well as other nuclear-encoded mitochondria subunits of the electron transport chain complex such as β-ATP synthase, cytochrome c and cytochrome c oxidase IV (Liang and Ward, 2006; Puigserver et al., 1998). Importantly, PGC1α also regulates thermogenesis, since its

activity is induced by cold temperature (Puigserver et al., 1998), and it induces expression of UCP1 (Barbera et al., 2001). To demonstrate the importance of PGC1α for thermogenesis, mice deleted for *PGC1α* were unable to maintain core body temperature following exposure to cold (Leone et al., 2005; Lin et al., 2004). Mice deleted for *UCP1* were also cold-sensitive (Enerback et al., 1997).

Two other genes were used during my PhD research work as markers to distinguish BAT from WAT: *type 2 deiodinase* (*dio2*), and *cell death-inducing DNA fragmentation factor-α-like effector A* (*cidea*). Dio2 is expressed in BAT, brain and pituitary, and its expression is induced by catecholamines and cold exposure (Silva and Larsen, 1983). It functions to accelerate thyroxine (T4) to 3,3',5-triiodothyronine (T3) conversion (Silva and Larsen, 1983). Cidea is expressed in BAT but not in WAT and other tissues in mice (Lin and Li, 2004; Zhou et al., 2003). Its exact function is unknown, but it interacts with UCP1, and in mice deficient for cidea the expression of UCP1 is increased (Zhou et al., 2003). In humans, however, cidea is also expressed in WAT (Dahlman et al., 2005; Nordstrom et al., 2005).

Adipogenesis

Adipocytes are derived from mesenchymal stem cells, through a complex transcriptional cascade (reviewed in (Farmer, 2006)). Adipogenesis of WAT has been extensively studied using model cell lines such as the fibroblast-derived 3T3-L1 and 3T3-F442A, and mouse embryonic fibroblasts (MEFs).

The most important transcription factor for adipogenesis is peroxisome proliferator-activated receptor γ (PPARγ). The *PPARγ* gene is transcribed from three different promoters, resulting in three different transcripts (Fajas et al., 1997; Fajas et al., 1998). These are translated into three isoforms – PPARγ1 is expressed ubiquitously but in low levels, PPARγ2 is highly expressed exclusively in adipose tissue, and contains 30 additional amino acids in the N-terminus. PPARγ3 is identical to PPARγ1, and is expressed in high levels in adipose tissue and macrophages. No functional differences between the

isoforms are known to date. PPARγ belongs to the nuclear hormone receptor superfamily of transcription factors, and functions as a heterodimer with the retinoid X receptor (RXR). This heterodimer binds to a DNA consensus sequence in promoters of target genes and enhances or represses transcription. The transcription transactivation occurs via recruitment of coactivators, mainly from the p160 steroid receptor coactivator family (SRC), CBP/p300, and the p300/CBP-associated factor (p/CAF)/GCN family, as well as other coactivators such as PGC1α. Known corepressors are retinoblastoma, the silencing mediator for retinoid and thyroid hormone receptors (SMRT), and nuclear receptor corepressor (NCoR) (Desvergne and Wahli, 1999; Miard and Fajas, 2005). As most nuclear hormone receptors, PPARγ is also activated by ligand. Endogenous ligands include many fatty acids and fatty acid derivatives, however these bind with low affinity and activate PPARγ to a lower extent as compared to synthetic ligands. The best example of an endogenous ligand is 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (PGJ2) which has been shown to directly bind PPARγ and induce adipogenesis (Forman et al., 1995). However, it is assumed that a mechanism other than fatty acid binding is responsible for full PPARγ activation. Synthetic ligands of PPARγ such as rosiglitazone, troglitazone or pioglitazone are used widely in therapy of type 2 diabetes, since they enhance insulin sensitivity.

Expression of PPARγ is induced during adipogenesis, where it controls transcription of several important adipose-specific genes, among them *adipocyte fatty acids binding protein 4* (*fabp4*, a.k.a. *aP2*), *fatty acid transport protein*, *LPL*, *Acyl-CoA synthase* and others (Desvergne and Wahli, 1999). PPARγ is regarded as a master regulator of adipogenesis, since induction of PPARγ activity in preadipocytes or even in NIH-3T3 fibroblasts is sufficient to stimulate adipogenesis (Chen et al., 1998; Kletzien et al., 1992; Lehmann et al., 1995; Sandouk et al., 1993; Tontonoz et al., 1994b). Tissue cultures of adipogenic cell lines or stem cells lacking PPARγ fail to differentiate into adipocytes (Kubota et al., 1999; Rosen et al., 1999). Knockout of PPARγ in mice results in embryonic lethality due to defects in placental development. By aggregating these embryos with tetraploid PPARγ$^{+/+}$ embryos, which supplemented a placenta for the knockout embryos, one mouse was born, and it suffered total lack of adipose tissue (Barak et al., 1999).

Heterozygous deletion of PPARγ results in mice with smaller adipocytes and reduced fat mass, that are resistant to high fat diet (HFD)-induced obesity (Kubota et al., 1999). These data indicate that PPARγ is required for adipogenesis *in vivo*. Knockout of *PPARγ* specifically in adipose tissue of mice showed that PPARγ is also essential for the survival of mature adipocytes *in vivo* (He et al., 2003; Imai et al., 2004; Jones et al., 2005). PPARγ is also required for the maintenance of mature adipocytes *in vitro* (Kim and Chen, 2004; Tamori et al., 2002).

Upstream of PPARγ in the adipogenesis transcriptional cascade is the transcription factor CCAAT/enhancer binding protein α (CEBPα). Ectopic expression of CEBPα induces adipogenesis (Freytag et al., 1994), but only in the presence of PPARγ (Rosen et al., 2002). Once PPARγ expression is induced by CEBPα, these two transcription factors help maintain each other's expression (Wu et al., 1999).

Further upstream are two other transcription factors named CEBPβ and CEBPδ. These two are expressed earlier in adipogenesis than CEBPα and PPARγ, and in cell culture function to induce their expression (Cao et al., 1991; Christy et al., 1991; Clarke et al., 1997; Yeh et al., 1995b). However, it appears that *in vivo* CEBPβ and CEBPδ have a different function, since knockout of these genes in mice did not affect the levels of PPARγ or CEBPα, although it did result in impaired adipogenesis (Tanaka et al., 1997). It has been suggested that CEBPβ and CEBPδ affect the production of PPARγ endogenous ligands (Hamm et al., 2001). The expression of CEBPβ and CEBPδ is induced by another transcription factor that is expressed very early during adipogenesis, called cAMP regulatory element-binding protein (CREB) (Zhang et al., 2004). Another transcription factor, Krox20, is activated early in adipogenesis and promotes expression of CEBPβ, and later on cooperates with CEBPβ to facilitate terminal differentiation (Chen et al., 2005).

I have described here the canonical transcriptional cascade of WAT adipogenesis. Recently, numerous other transcription factors and signaling pathways have been implicated in adipogenesis regulation, that are beyond the scope of this introduction. A detailed description can be found, for example, in (Farmer, 2006).

It has been previously suggested that mTOR controls adipocytes by positively regulating the activity of PPARγ (Kim and Chen, 2004). Kim and Chen (2004) have shown that mTORC1, and specifically the kinase activity of mTOR, are required for adipogenesis *in vitro*. They also showed that rapamycin treatment does not change the level of PPARγ expression, but reduces the expression of a reporter gene driven by a PPARγ controlled promoter. This effect of rapamycin, as well as the effect on adipogenesis, can be reversed by addition of the PPARγ synthetic ligand troglitazone.

In order to differentiate cellular models for WAT, the cells are incubated two days post confluence in medium containing bovine serum, and then stimulated with a glucocorticoid (usually dexamethasone), a cAMP production activator (usually the chemical phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX)), and a high concentration of insulin. This mix is kept on the cells for two-three days, and is then replaced with medium containing serum and insulin for another six days. During this period, the cells become larger and round, express adipogenic markers, and accumulate TGs in lipid droplets. Glucocorticoids and cAMP signaling are required during adipogenesis, for example to induce the transcription of CREB and CEBPβ (Cao et al., 1991; Yeh et al., 1995b; Zhang et al., 2004). Insulin also participates in the transcriptional cascade of adipogenesis, and later on promotes uptake of glucose and FFAs from the medium.

The knowledge about BAT adipogenesis is much more limited, due to lack of suitable model cell lines. Only one cell line called HIB1B has been described (Klaus et al., 1994; Tontonoz et al., 1994a), but has not been well studied to date. Differentiation of these cells is achieved by incubation of confluent cells in medium containing serum, insulin and T3 for one week (Klaus et al., 1994).

It is known that PPARγ is required for BAT differentiation (Barak et al., 1999; Rosen et al., 1999), but in contrast to WAT, CEBPα is not required for BAT differentiation (Linhart et al., 2001). Several cofactors of PPARγ have been implicated in determination of BAT versus WAT differentiation: one of the most important transcriptional coactivators in BAT

is the previously mentioned PGC1α (Lin et al., 2005; Uldry et al., 2006). This transcription factor is almost not expressed in WAT. Other transcriptional cofactors of PPARγ that promote BAT differentiation are retinoblastoma (Hansen et al., 2004a; Hansen et al., 2004b; Scime et al., 2005), p107 (Scime et al., 2005), receptor interacting protein 140 (RIP140) (Christian et al., 2005; Kiskinis et al., 2007; Leonardsson et al., 2004; Powelka et al., 2006), and steroid receptor coactivator-1 (SRC1) (Picard et al., 2002). Recently, prdm16, a protein of unknown function, has been described as an early determinant of BAT differentiation (Seale et al., 2007).

Associated diseases

Excess or deficiency of adipose tissue are both strong risk factors for development of type 2 diabetes (Guilherme et al., 2008). Type 2 diabetes develops as a result of two factors: peripheral insulin resistance (the main contributor to this is skeletal muscle, as it consumes most of the blood glucose), and failure of the pancreas β-cells to produce sufficient levels of insulin to overcome the peripheral insulin resistance. Weight loss and gain are associated with decreased and increased muscular insulin resistance, respectively (Freidenberg et al., 1988; Sims et al., 1973), suggesting that adipose tissue takes part in the regulation of muscle insulin sensitivity. Adipose tissue controls peripheral insulin sensitivity by several mechanisms. First, as mentioned above, adipocytes secrete various adipokines such as leptin, adiponectin, TNFα, and resistin, that affect insulin sensitivity. Second, adipose tissue sequesters FFAs from the blood circulation. Increased concentration of FFAs in the blood can cause peripheral insulin resistance (Kelley et al., 1993; Santomauro et al., 1999) and aberrant insulin secretion from β-cells (Boden, 1997). Impairment of adipose tissue functions, for example as a result of a genetic or acquired lipodystrophy on the one hand or a long term high fat diet on the other hand, results in elevated FFAs in the blood, steatosis, and changes in the adipokine profile. Large adipocytes secrete higher amounts of inflammatory cytokines that promote macrophage infiltration. The inflammatory cytokines that are secreted by adipocytes and macrophages induce lipolysis and inhibit TG synthesis, contributing

further to elevated concentrations of FFAs in the blood. Thus, normal adipose function is an influential factor in maintenance of proper insulin secretion and sensitivity.

Obesity is also associated with an increased risk for cardiovascular diseases and hypertension. The pathophysiology of this process is not entirely understood. The surplus of adipose tissue causes elevated cadiac workload, that is linked with enlargement and fibrillation of the heart (Poirier et al., 2006). Adipokines such as leptin have also been suggested to play a role in the development of obesity-related hypertension and cardiovascular diseases (Mathew et al., 2007).

Obesity is a major risk factor for obstructive sleep apnea, occurring in up to 50% of obese men (Vgontzas et al., 1994; Young et al., 1993).

Recently, obesity has emerged as a risk factor also for cancer incidence and mortality. Obesity is significantly associated with higher death rates from cancer of the esophagus, colon and rectum, gallbladder, pancreas, kidney, stomach, prostate, breast (postmenopausal), uterus, cervix, and ovary (McMillan et al., 2006). This increased risk for cancer is most likely a result of an increase in sex steroids, pro-inflammatory cytokines, insulin, and IGF1 in obesity.

In this thesis, I first present evidence confirming that mTORC1 is required for the differentiation and maintenance of adipocytes *in vitro.* In tissue culture, inhibition of mTORC1 caused a decrease in the expression of adipose transcription factors, which led to a decreased expression of genes related to fat metabolism and storage. This caused de-differentiation of the cells, that manifested as loss of intracellular triglycerides. I further focused my research on the key adipogenic transcription factor PPARγ, and tried to elucidate the molecular mechanism by which mTORC1 regulates its activity. The results suggested that rapamycin treatment acts to inhibit PPARγ downstream of its ligands.

To examine the role of mTORC1 in adipose tissue *in vivo*, we generated mice that were deleted for the specific and essential mTORC1 component raptor. Since a non-conditional, full body knockout of *raptor* in mice is embryonic lethal (Guertin et al., 2006), we used the cre/loxP system to delete the *raptor* gene specifically in adipose tissue. The *raptor*$^{ad-/-}$ mice had substantially less adipose tissue than control littermates, but lean tissue mass was unaffected. Leanness was despite unaffected caloric intake, lipolysis, and absorption of lipids from the food. The white adipose tissue of *raptor*$^{ad-/-}$ mice showed a marked increase in several brown fat markers including enhanced expression of genes encoding mitochondrial uncoupling proteins. Leanness of the *raptor*$^{ad-/-}$ mice is attributed to elevated energy expenditure due to mitochondrial uncoupling. In addition, *raptor*$^{ad-/-}$ mice were protected against diet-induced obesity and hypercholesterolemia, and exhibited improved insulin sensitivity. These results suggest that mTORC1 is a regulator of adipose metabolism and thereby controls whole body energy homeostasis.

Materials and methods

Plasmids, Antibodies and Reagents

Adenoviruses encoding RNAi against raptor were created by cloning the previously described RNAi sequence and H1 promoter (Jacinto et al., 2004) from pSuper into pAd-DEST (Invitrogen). Empty pAd-DEST vector was used as control. The HA-hPPARγ in pCI expression vector was constructed on the backbone of pCI-HA. The human PPARγ2 fragment was cloned by PCR from hPPARγ in pSG5 (Deeb et al., 1998), using the following oligonucleotides that contained NotI restriction sites: forward 5' GCG GCC GCA TGG GTG AAA CTC TGG GAG AT 3' reverse 5' CGC CGG CGG ATC ATG TTC AGG AAC ATC TAG AG 3'. The PCR fragment was ligated to pCI-HA that was digested with NotI. Antibodies for raptor were from Bethyl. Antibodies for RXR, pRb, NCoR and PPARγ were from Santa Cruz. Antibodies for actin were from Chemicon. Rapamycin was from LC Laboratories and was used at a concentration of 100nM. Isoproterenol was from Sigma and was used at a concentration of 10µM. Insulin, dexamethasone and IBMX were from Sigma. JetPEI was from Polyplus transfections.

Mice

Mice were maintained in a temperature-controlled (20°C) facility with a 12h light/dark cycle, and were given free access to food and water. All experiments were performed in accordance with the regulatory standards of the *Kantonales Veterinaeramt* of *Kanton Basel-Stadt*, permit no. 2175. Experiments were performed on male mice that were 4-5 times backcrossed, and littermates that did not carry the *cre* allele were used as controls. C57BL/6J mice expressing the cre recombinase under the adipose-specific promoter of *fabp4/aP2* were purchased from Jax. *raptor*$^{+/-}$ mice were generated by crossing *raptor*^{floxed} mice with mice expressing cre under control of the human cytomegalovirus minimal promoter (Schwenk et al., 1995). To compare body weight and food intake, 8 week old WT or KO mice were fed chow diet (4.5% fat, Provimi Kliba) or a high fat diet

(60% calories from fat, D12492, Harlan) for 13 weeks. Body and food weight were measured twice a week, and food consumption was calculated.

Histology

For H&E stainings, WAT pieces were fixed in 4% formaldehyde overnight at room temperature immediately after sacrifice. Tissues were paraffinized and sectioned by microtome, and the slides were stained with hematoxilin and eosin (Sigma) according to the standard protocol. For Oil Red O staining of liver, tissues were flash frozen at -150°C, overhanging in a tragacanth-based gel (7% tragacanth in water). Tissues were sectioned by cryostat, and the slides were stained with oil red o and hematoxilin according to the standard protocol.

Cell Culture

3T3-L1 cells stably expressing the adenovirus receptor were kindly provided by Dr. David J. Orlicky (Orlicky et al., 2001). Cell were maintained in DMEM containing 10% fetal bovine serum, and differentiated according to the normal protocol (Kim and Chen, 2004). MEFs were freshly isolated on day E12.5, cultured for one passage, and either used immediately or frozen for later use. MEFs were differentiated using the same protocol as for 3T3-L1 cells.

Blood Tests

Blood was drawn from the vena cava immediately after sacrifice, and plasma was prepared. The following kits were used to determine metabolic parameters: triglyceride determination kit (Sigma), total cholesterol kit (Roche), free fatty acids determination kit (WAKO), leptin ELISA kit (R&D systems), adiponectin ELISA kit (AdipoGen), insulin ELISA kit (Mercodia).

Energy Expenditure

To measure voluntary activity, mice were housed individually in cages containing running wheels, and allowed to acclimatize for 7 days. Running distance was measured over the next 7 days. Oxygen consumption and carbon dioxide output were measured simultaneously with an open circuit calorimeter (Labmaster (TSE)). Mice were housed in individual Labmaster chambers for 21h (12am-9am) with free access to food and water. Measurements were taken at 1h intervals between 4pm-10am. The light/dark was 7am/7pm, ambient temperature was 29°C. For the cellular oxygen consumption assay, primary adipocytes from epididymal WAT were isolated by mincing the tissue, followed by a 50min collagenase digestion (2mg type II collagenase (Sigma) / g tissue) at 37°C in 20ml KREBS buffer. Undigested tissue was filtered on a 70µm cell strainer and washed 3 times with 5ml of cold KREBS buffer. The adipocyte fraction was isolated by collecting the upper phase after a 10min centrifugation at 500rpm at 4°C, and subsequently washed once in 25ml cold KREBS buffer and once in 25ml respiration solution (2.77mM CaK_2EGTA, 7.23mM K_2EGTA, 6.56mM $MgCl_2$, 3mM K_2HPO_4, 20mM taurine, 0.5mM DTT, 50mM K methane-sulfonate, 20mM imidazole pH 7.1, 5mM glutamate, 2mM malate, 2 mg/ml fatty acid-free bovine serum albumin) at pH 7.0. After the final centrifugation, adipocytes were counted and diluted in respiration solution at 1 million cells/ml. Respiratory rates were determined on 3 million cells using Clark electrodes (Strathkelvin Instruments) in oxygraphic chambers at 22°C with continuous stirring. 5min before the start of the experiment, cells were permabilized with 50µg/ml saponin. After the experiment, cells were collected and genomic DNA was extracted and quantified for normalization using qPCR (primer pair 5'-CTACAGATGTGGTAAAGGTCCGC-3', 5'-GCAATGGTCTTGTAGGCTTCG-3').

IP Glucose Tolerance Test

Mice after 10 weeks of chow or HFD were fasted overnight, followed by an IP injection of 2g/kg glucose. Basal blood glucose was measured before the injection, using an Aviva

glucometer (Accu-chek, Roche). Glucose levels were then measured at the indicated time points after the injection.

qPCR

RNA was isolated from 3T3-L1 cells with the RNeasy mini kit (Qiagen). Mouse tissue RNA was isolated as follows: approximately 100mg tissue was homogenized by 30sec bead beating in lysing matrix D tubes (Q-Biogene) containing 1mL TRIZOL reagent (Invitrogen). Samples were further treated according to the TRIZOL manufacturer's instructions. After the chloroform centrifugation, samples were mixed with 600µL 70% ethanol, and the extraction was continued with the RNeasy kit (Qiagen). 1µg total RNA was reverse transcribed using Superscript II reverse transcriptase (Invitrogen). qPCR was done using the power SYBR green mix (Applied Biosystems). qPCR Primer pairs for *PPARγ*, *CEBPα*, *SREBP*, *aP2*, *FAS*, *LPL* and *resistin* were previously described (Fu et al., 2005). Primer pairs for *cidea*, *prdm16* and *dio2* were previously described (Seale et al., 2007). Primer pair for *HSL* was: 5'-CAGTGTGACCGCCAGTTC-3' (forward), 5'-ACCTCAATCTCAGTGATGTTCC-3' (reverse).

Cell Size

Adipocyte cell circumference was determined using the ImageJ software. At least 35 adipocytes from random sections of each mouse were quantified. Cell volume ($4/3\pi R^3$) was calculated from the cell circumference (πR^2).

Lipolysis assay

Glycerol release from cells was monitored as a measure of lipolysis (triglyceride lysis). Differentiated 3T3-L1 cells were incubated with rapamycin or isoproterenol for the indicated periods of time. To measure glycerol release, medium was replaced with DMEM without serum for 30min, and glycerol content of the medium was measured using the free glycerol reagent (Sigma). For the mice, freshly isolated epididymal WAT pieces of approximately 30mg were washed in PBS and incubated for 12h in DMEM. Then

WAT was removed and the glycerol content of the medium was measured. The assay was done in triplicates.

Kinase assay

Kinase assays were performed as described (Jacinto et al., 2004). HEK293 cells (in 10cm dishes; about 90% confluent) were placed on ice. Extracts from cells corresponding to half a 10cm plate were used for each immunoprecipitation condition. Cells were washed twice with 1 ml of cold PBS and scraped in lysis buffer (0.6ml per 10cm plate; 40mM HEPES, 120mM NaCl and 0.3% CHAPS) supplemented with protease and phosphatase inhibitors (Roche complete protease inhibitor cocktail, 10mM NaF, 10mM NaN_3, 10mM NaPPi, 10mM β-glycerophosphate, 10mM p-nitrophenylphosphate, 0.5mM benzamidine HCl, 1.5mM Na_3VO_4 and 20µg/ml heparin). Crude extracts, typically 350µl for each immunoprecipitation, were cleared with two 3' spins at 600g, and pre-cleared with a mixture of protein-A-, and protein-G-coupled Sepharose for 20'. Pre-cleared lysates were subjected to immunoprecipitation with 1.5µl of anti-HA (for mock immunoprecipitations), anti-raptor (36T; Hall laboratory), or 3µl of anti-mTOR (FRAP N19; Santa Cruz) for 30' at 4 °C, followed by the addition of 6µl of protein-A- and 6µl of protein-G-coupled sepharose, and incubation for an additional 90'. Beads were washed 3 times with 1ml of lysis buffer and resuspended in 30µl kinase assay buffer (40mM HEPES, 120mM NaCl, 0.3% CHAPS, 10mM NaF, 10mM NaN_3, 10mM β-glycerophosphate, 10mM p-nitrophenylphosphate, 4mM $MnCl_2$, 10mM dithiothreitol, 1X Roche Protease inhibitor cocktail lacking EDTA, 1µg 4E-BP1 (Stratagene), 80µM cold ATP and 10µCi ^{32}P-ATP, and 1.67% DMSO. 500nM GST−FKBP12 were used. Reactions were started with the addition of the hot and cold ATP. Tubes were maintained at 30°C for 20' and the reactions were terminated with the addition of 8µl of 5X protein sample buffer. Proteins were then resolved on an SDS−PAGE gel. Radioactivity was detected by autoradiography, and total 4E-BP1 and PPARγ were detected by staining with ponceau.

2D gels

Differentiated 3T3-L1 were treated or not with rapamycin, and harvested in lysis buffer (7M urea, 2M thiourea, 4% CHAPS, 20mM DTT). Extracts were cleared by a 30' spin, isoelectric focusing (IEF) buffer (IPG buffer, pH 3-10 NL, GE Healthcare) and bromophenol blue were added, and 1200µg of proteins in 400µl buffer were loaded on IEF strips (Immobiline Drystrip pH 3-10 NL, GE Healthcare). IEF was done as follows: 12h rehydration (no voltage), 1h at 500V, 1h at 1000V, 48h at 8000V. Each IEF strip was then mounted on top of a 10% SDS PAGE gel, and gels were run at 150V and transferred to nitrocellulose membranes. Membranes were immunoblotted with antibodies to detect PPARγ.

Mass spectrometry analysis of PPARγ phosphorylation sites

HEK293 cells in 15-cm dishes were transfected with 12µg HA-hPPARγ in pCI using the JetPEI reagent. Two days later, cells were treated with 100nM rapamycin or DMSO for 30' and harvested in lysis buffer as described for kinase assays. Crude extracts from 2 dishes for each immunoprecipitation were cleared with two 3' spins at 600g, and pre-cleared with protein-A-coupled Sepharose for 20'. Pre-cleared lysates were subjected to immunoprecipitation with 100µl of anti-HA coupled beads for 2h at 4°C. Beads were washed 3 times with 1ml of lysis buffer and resuspended in 100µl 2X sample buffer without β-mercaptoethanol. Samples were boiled for 5', and separated on 8% SDS-PAGE gels. Gels were then stained with the proteosilver plus kit (Sigma), and the bands corresponding to PPARγ were analyzed for potential phosphorylation sites by mass spectrometry.

Immunofluorescence

For PPARγ staining, 3T3-L1 cells were differentiated in 12-well dishes containing coverslips, and treated with 100nM rapamycin or DMSO for the indicated periods of time. Cells were then fixed in a 3.7% PFA solution at RT for 7', permeabilized in PBS-0.1% triton for 1', blocked with PBS-5% BSA at RT for 30', and stained with a PPARγ antibody diluted

1:50 in blocking solution for 1h, followed by secondary anti-mouse antibody conjugated to C3y diluted 1:200 in blocking solution for 1h. After each step the cells were washed 4X 5' with PBS, the final wash contained DAPI diluted 1:1000. For RXR staining, a similar protocol was used with the following changes: PBS-10% FBS was used for blocking, and the RXR antibody was diluted 1:200.

Statistical analysis

All graphs show average ± SEM unless otherwise indicated. Statistical significance was calculated using unpaired, two-tailed Student's t-test. * p value ≤ 0.05, ** p value ≤ 0.01.

Results

mTORC1 is required for adipogenesis and adipose maintenance *in vitro*

As shown previously, long term rapamycin treatment prevents adipogenic differentiation of 3T3-L1 cells in culture (Bell et al., 2000; Cho et al., 2004; El-Chaar et al., 2004; Gagnon et al., 2001; Kim and Chen, 2004; Yeh et al., 1995a). Since long term rapamycin treatment can disrupt mTORC2 as well as inhibit mTORC1 (Sarbassov et al., 2006), we genetically knocked down raptor, in undifferentiated 3T3-L1 pre-adipocytes, to determine the role specifically of mTORC1 in adipogenesis (Figure 1A). The cells in which raptor was knocked down were then treated to induce adipogenesis and examined for lipid accumulation by Oil Red O staining (Figure 1B). Inactivation of mTORC1 by either rapamycin treatment or by knockdown of raptor prevented 3T3-L1 cells from differentiating into Oil Red O-staining cells, indicating that mTORC1 is indeed required for adipogenesis. To test further the requirement for mTORC1 in adipogenesis, we differentiated heterozygous *raptor*$^{+/-}$ mouse embryonic fibroblasts (MEFs) derived from mice lacking one copy of the *raptor* gene (see Materials and methods). Heterozygous MEFs were used for this experiment because homozygous *raptor* knockout MEFs could not be obtained due to very early embryonic lethality, as observed previously (Guertin et al., 2006). The *raptor*$^{+/-}$ MEFs differentiated into adipocyte-like cells but with significantly reduced efficiency compared to MEFs from wild type littermates (Figure 1C), providing further support for a role of mTORC1 in adipogenesis.

It has also been shown previously that culturing differentiated 3T3-L1 cells in the presence of rapamycin causes loss of fat, suggesting that mTORC1 is also required for maintenance of adipocytes (Kim and Chen, 2004). We confirmed this observation by measuring the triglyceride content of differentiated 3T3-L1 cells that were treated with rapamycin for 6 days after differentiation. Differentiated 3T3-L1 cells that were incubated with rapamycin for 6 days lost on average 46% of their triglyceride content (Figure 1D). Rapamycin treatment or siRNA-mediated raptor knockdown also caused loss

of lipid stores as visualized by Oil Red O staining (Figure 1E). The above results confirm that mTORC1 is required for both establishment and maintenance of mature adipocytes.

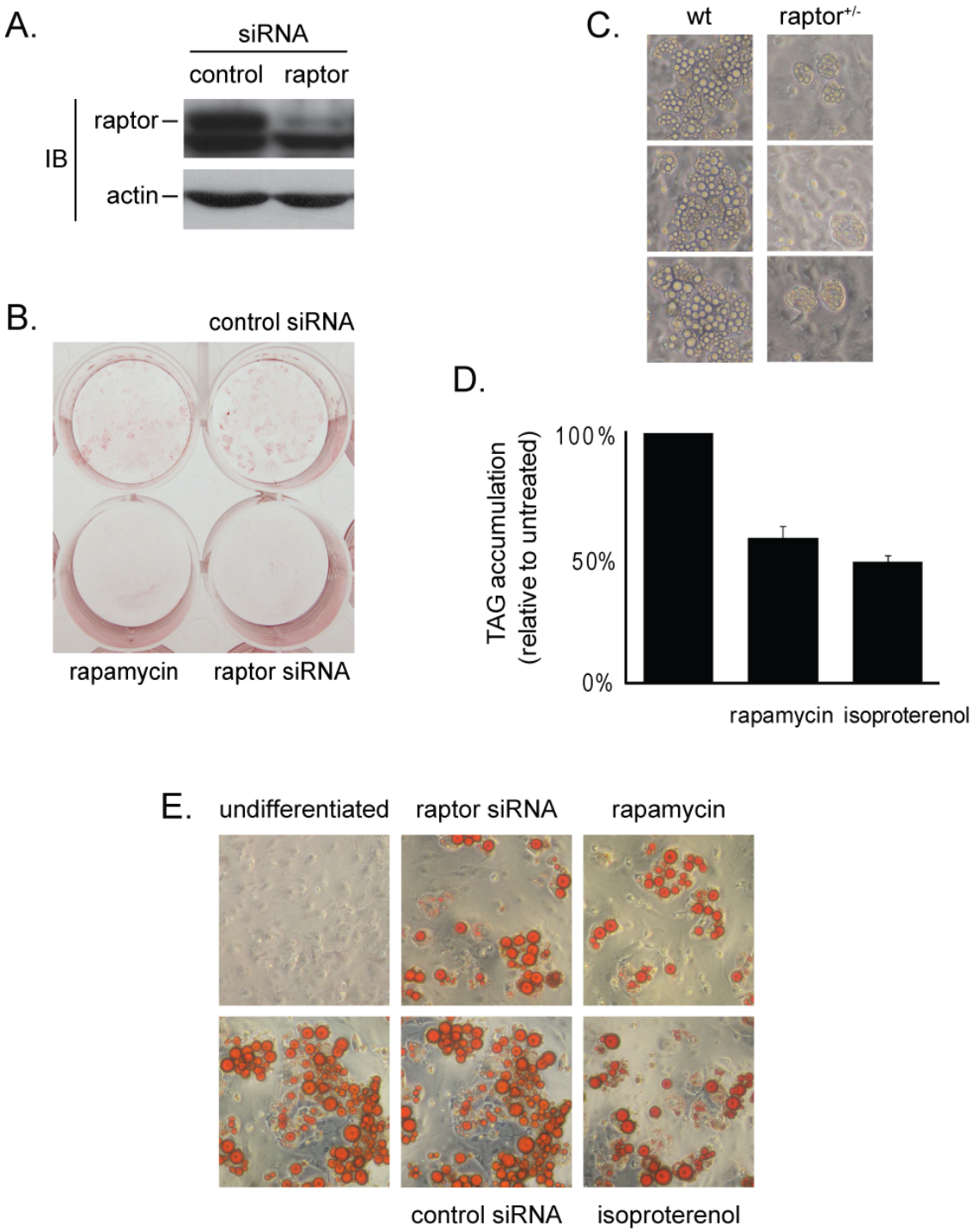


Figure 1. mTORC1 is required for adipogenesis and adipose maintenance in vitro. A. *To test the efficiency of raptor knockdown, 3T3-L1 pre-adipocytes were infected with control or* raptor *siRNA-encoding adenovirus for 6h, then washed and incubated for another 48h. Protein extracts were prepared, followed by immunoblotting to detect raptor and actin.*

B. *3T3-L1 pre-adipocytes were treated with rapamycin, or infected with control or* raptor *siRNA-encoding virus as described in A, or left untreated. Cells were differentiated for 8 days and stained with Oil Red O. Rapamycin was added together with the differentiation mix, and maintained during the differentiation period.* ***C.*** *MEFs were isolated at day E12.5 and cultured for 2 passages, then differentiated for 8 days. Representative pictures were taken from 3 knockout embryos and 3 wild type littermates.* ***D.*** *3T3-L1 pre-adipocytes were differentiated for 8 days, and then treated with rapamycin or isoproterenol or left untreated for another 6 days. Triglyceride content was measured as described in "Materials and methods". Isoproterenol was used as a positive control to induce lipolysis (n=3).* ***E.*** *3T3-L1 pre-adipocytes were untreated (upper left panel) or differentiated for 8 days (lower left panel). Differentiated cells were then transduced with control or* raptor *siRNA-encoding viruses (upper and lower middle panels, respectively), or treated with rapamycin (upper right panel) or isoproterenol as a positive control for lipolysis (lower right panel) for another 6 days. Lipids were visualized by Oil Red O staining.*

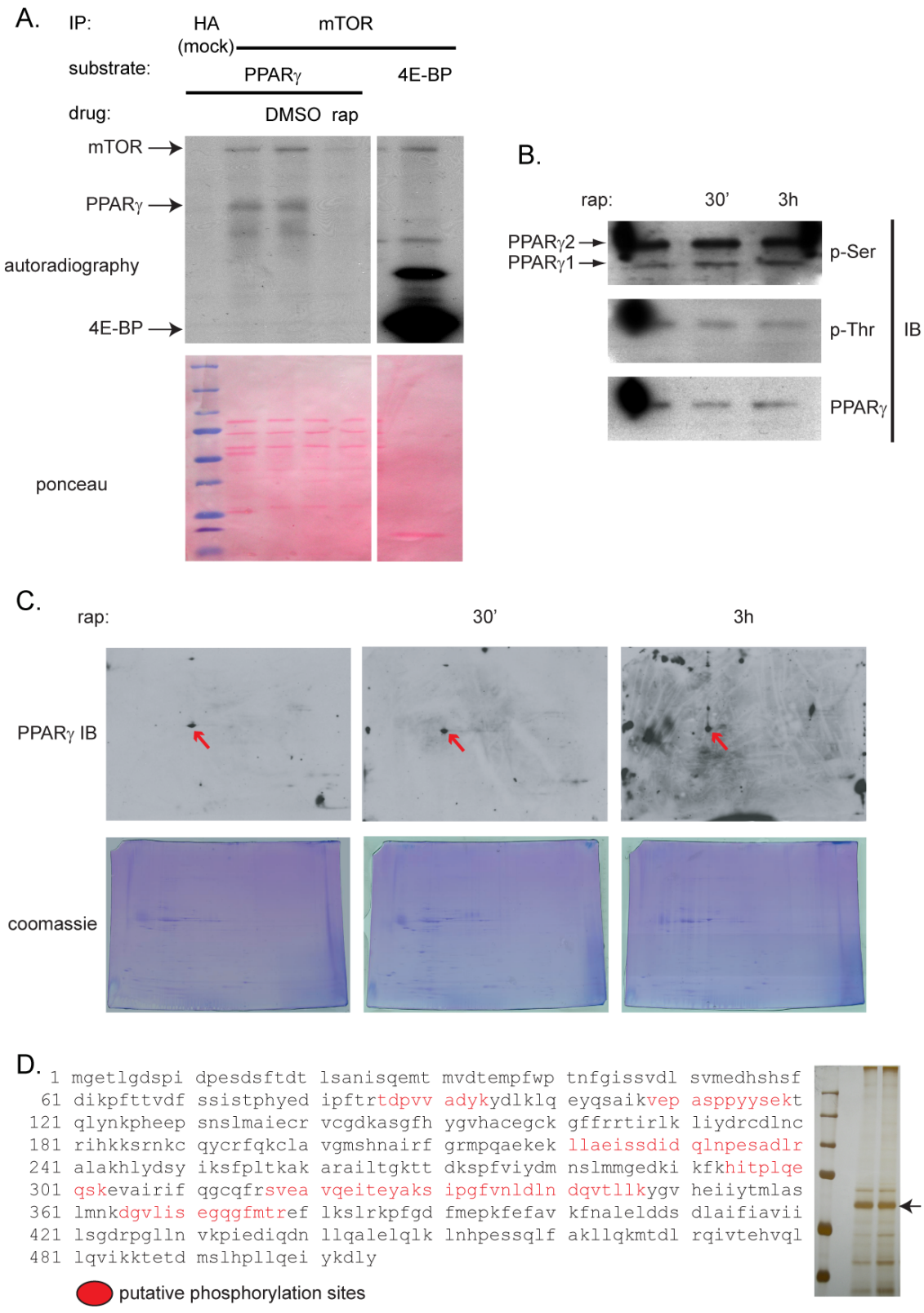
mTORC1 does not phosphorylate PPARγ

Previous studies have suggested that rapamycin's inhibitory effect on adipocytes is a result of inhibition of the key adipogenic transcription factor PPARγ (Gagnon et al., 2001; Kim and Chen, 2004). We decided to examine more closely the possible routes in which mTORC1 might affect the activity of PPARγ. We considered several possibilities: since mTOR is a kinase, we tested whether it could directly or indirectly phosphorylate PPARγ. We also examined less direct effects, such as a localization change or a change in the levels or binding of the cofactors of PPARγ.

To test whether mTOR can directly phosphorylate PPARγ, we performed an *in vitro* kinase assay with mTORC1 as a kinase and purified PPARγ as a substrate. Analysis of the results showed that mTORC1 can phosphorylate PPARγ in a rapamycin-dependent manner (Figure 2A), although very weakly compared to phosphorylation of 4E-BP. A similar experiment with raptor IP instead of mTOR resulted in a similar outcome (data not shown). Although the intensity of the band corresponding to PPARγ on the autoradiography image is similar to that of the autophosphorylation of mTOR, the ponceau staining shows that the quantity of PPARγ protein is much higher than that of

the mTOR protein, therefore the phosphorylation of PPARγ by mTORC1 is also weaker than the autophosphorylation of mTOR.

In contrast to the weak ability of mTORC1 to phosphorylate PPARγ *in vitro*, we could not identify changes in phosphorylation of PPARγ following rapamycin treatment in tissue culture. We immunoprecipitated PPARγ from differentiated adipocytes, and then immunoblotted with antibodies to detect phosphorylated serine or threonine. No change in the phosphorylation of PPARγ was observed following rapamycin treatment (Figure 2B). We further tested the migration of PPARγ in a 2D gel. Total protein extract from differentiated 3T3-L1 treated or not with rapamycin was separated by isoelectric focusing followed by SDS-PAGE, and immunoblotting with antibodies to detect PPARγ. The migration pattern of PPARγ was similar under all conditions (Figure 2C). Finally, we used a mass spectrometer to identify putative phosphorylation sites on PPARγ: we transfected HEK cells with a tagged version of PPARγ, treated or not with rapamycin, and immunoprecipitated PPARγ. We could identify several putative phosphorylation sites on PPARγ, including the well-known Ser112 site. However, there was no difference between the phosphorylation sites identified from rapamycin treated or non-treated cells (Figure 2D). We also failed to detect an interaction between mTORC1 components and PPARγ by coimmunoprecipitation (data not shown). We therefore conclude that in tissue culture PPARγ is not a phosphorylation target of mTORC1, nor is it phosphorylated in an mTORC1-dependent manner.


A.
IP:
HA
(mock)
mTOR
substrate:
PPARγ
4E-BP
drug:
DMSO rap
mTOR
PPARγ
autoradiography
4E-BP
ponceau
B.
rap:
30'
3h
PPARγ2
PPARγ1
p-Ser
p-Thr
PPARγ
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coomassie
D.
1 mgetlgdspi dpesdsftdt lsanisqemt mvdtempfwp tnfgissvdl svmedhshsf
61 dikpfttvdf ssistphyed ipftrtdpvv adykydlklq eyqsaikvep asppyysekt
121 qlynkpheep snslmaiecr vcgdkasgfh ygvhacegck gffrrtirlk liydrcdlnc
181 rihkksrnkc qycrfqkcla vgmshnairf grmpqaekek llaeissdid qlnpesadlr
241 alakhlydsy iksfpltkak arailtgktt dkspfviydm nslmmgedki kfkhitplqe
301 qskevairif qgcqfrsvea vqeiteyaks ipgfvnldln dqvtllkygv heiiytmlas
361 lmnkdgvlis egqgfmtref lkslrkpfgd fmepkfefav kfnaleldds dlaifiavii
421 lsgdrpglln vkpiediqdn llqalelqlk lnhpessqlf akllqkmtdl rqivtehvql
481 lqvikktetd mslhpllqei ykdly
putative phosphorylation sites

Figure 2. Analysis of the effect of rapamycin on PPARγ phosphorylation. A. *mTOR precipitates from HEK293 cells were split equally into 5 tubes and re-suspended in kinase assay buffer. DMSO or rapamycin (100nM) and FKBP12 were added to the reaction tubes as indicated. The autoradiographs in the top panel depict mTOR, PPARγ and 4E-BP1 phosphorylation. The bottom panel is a picture of ponceau staining, to visualize protein quantities. Several lanes that represent irrelevant conditions were digitally removed from the picture.* ***B.*** *3T3-L1 cells were differentiated and then treated with rapamycin for the indicated periods of time. Protein extracts were prepared using the procedure described for kinase assay in "materials and methods", and PPARγ was immunoprecipitated. Immunoprecipitates were immunoblotted to detect phosphorylated serine (p-Ser) or threonine (p-Thr), or PPARγ.* ***C.*** *3T3-L1 cells were differentiated and then treated with rapamycin for the indicated periods of time. Protein extracts were prepared, separated by 2D gels and immunoblotted with antibodies to detect PPARγ.* ***D.*** *HEK293 cells were transfected with HA-hPPARγ in pCI for two days, followed by treatment with 100nM rapamycin or DMSO for 30', and immunoprecipitation with anti-HA antibodies. Immunoprecipitates were separated by SDS-PAGE, and the bands corresponding to PPARγ were analyzed for potential phosphorylation sites by mass spectrometry. The amino acid sequence of human PPARγ is shown, with the potential phosphorylation sites marked in red. On the right is a picture of the silver stained gel, with an arrow indicating PPARγ.*

Inhibition of mTORC1 does not affect the level or localization of PPARγ and its cofactors

Since PPARγ must be in the nucleus to function, and rapamycin reduces its transcriptional transactivation activity, we considered the possibility that rapamycin treatment might cause a change in the intracellular localization of PPARγ. To test this possibility, we differentiated 3T3-L1 cells, treated them with rapamycin or DMSO, and examined the intracellular localization of PPARγ by immunofluorescence staining. However, PPARγ remained in the nucleus under rapamycin treatment (Figure 3A). Similar results were obtained with PPARγ's obligatory interaction partner RXR (Figure 3B). As previously reported, short term rapamycin treatment of 3h did not reduce the level of PPARγ protein (Kim and Chen, 2004). In addition, we found no changes in the levels of expression of RXR, and the two cofactors NCoR and retinoblastoma (Figure 3C). Therefore, changes in localization or level of expression of PPARγ or the above mentioned cofactors cannot account for the reduction in PPARγ activity following rapamycin treatment.

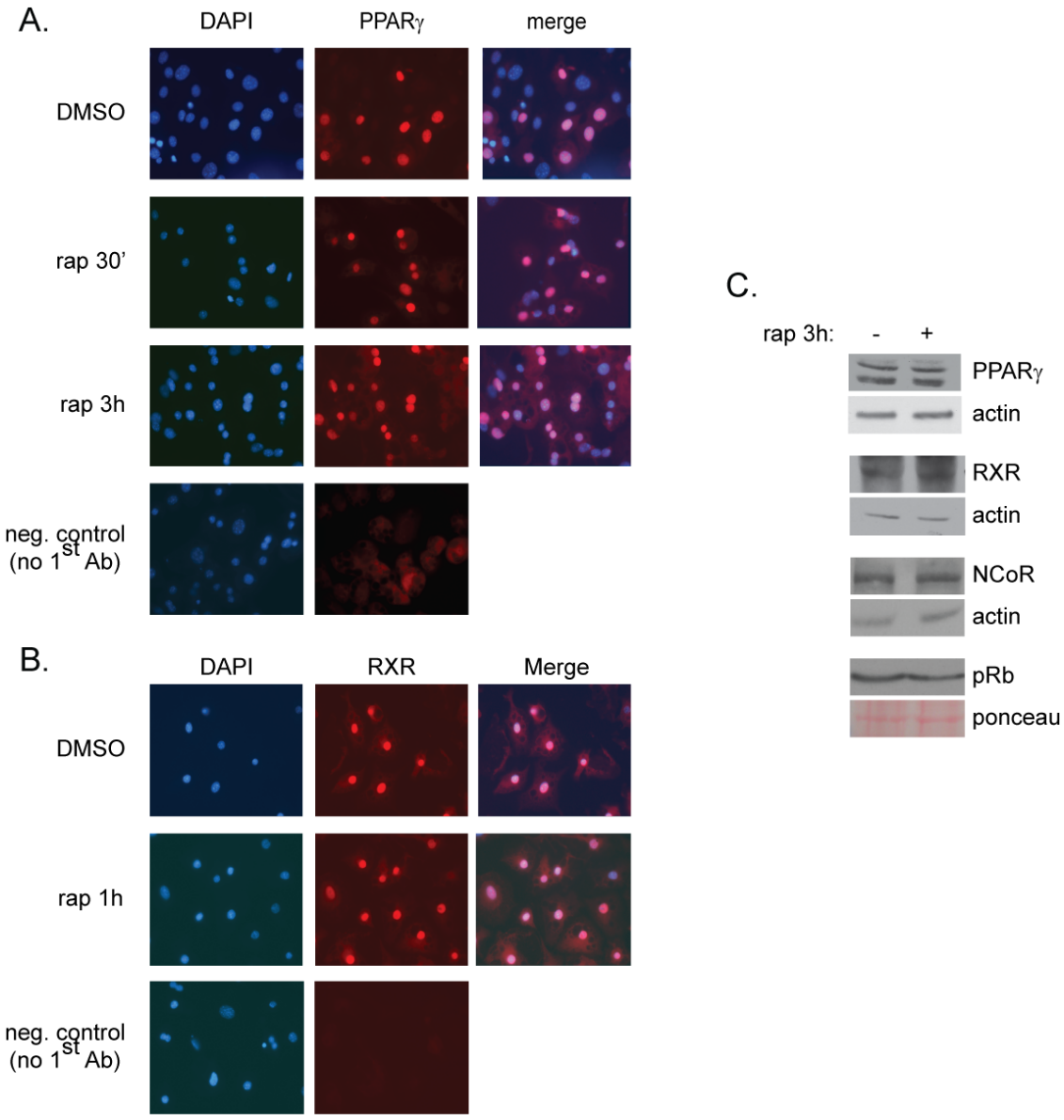
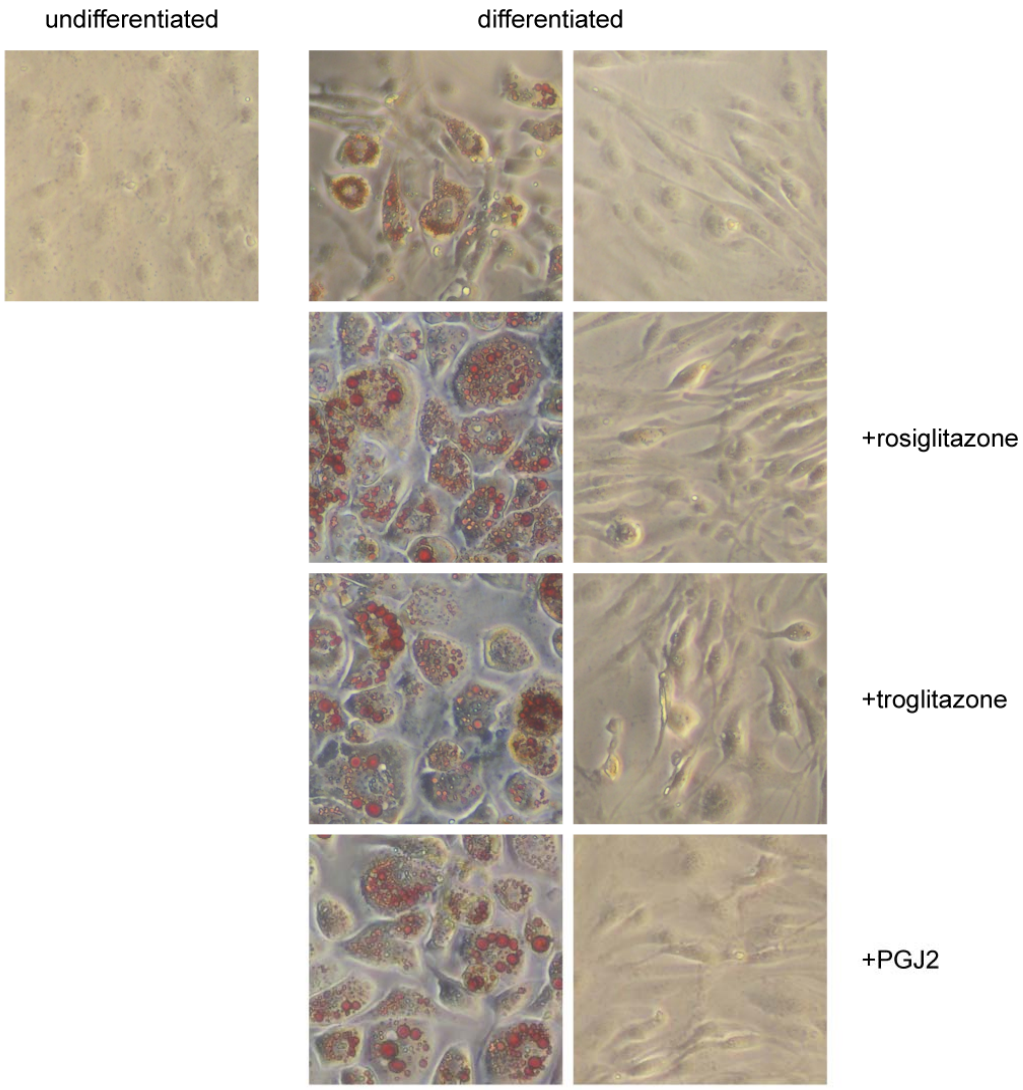


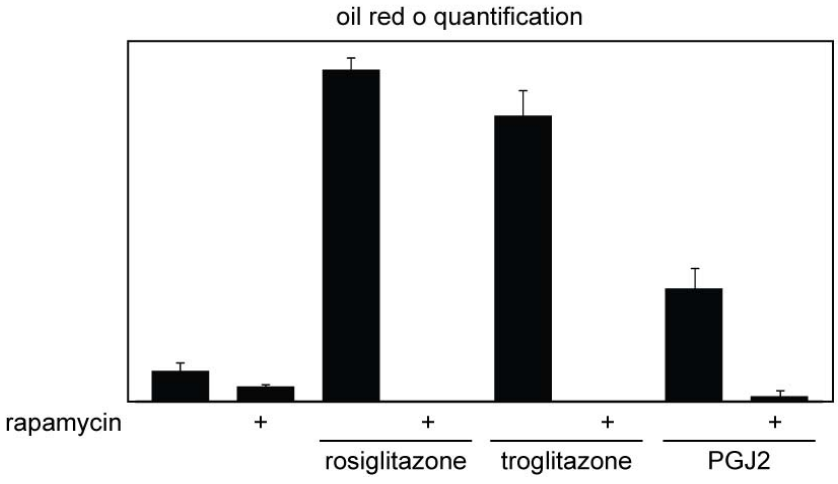
Figure 3. Analysis of PPARγ and RXR localization and expression levels. A. *Differentiated 3T3-L1 cells were treated with 100nM rapamycin or DMSO for the indicated periods of time. Cells were then stained with a PPARγ antibody and DAPI. Pictures were merged using Adobe photoshop.* ***B.*** *Differentiated 3T3-L1 cells were treated with 100nM rapamycin or DMSO for the indicated periods of time. Cells were then stained with an RXR antibody and DAPI. Pictures were merged using Adobe photoshop.* ***C.*** *Differentiated 3T3-L1 cells were treated with 100nM rapamycin or DMSO for 3h. Protein extracts were prepared using the procedure described for kinase assay in "materials and methods", and*

immunoblotted with antibodies to detect PPARγ, RXR, NCoR or retinoblastoma (pRb). Actin or ponceau staining were used as a loading control.

Inhibition of mTORC1 decreases the activity of PPARγ in 3T3-L1 cells downstream of the PPARγ ligands

PPARγ is a nuclear receptor, which is known to be activated by several synthetic and endogenous ligands. To test whether rapamycin acts to inhibit PPARγ upstream or downstream of the PPARγ ligands, we differentiated 3T3-L1 cells in the presence of either rapamycin, each of the PPARγ ligands rosiglitazone, troglitazone or PGJ2, or a combination of rapamycin and the ligands, and tested the effect on differentiation. As can be seen in figure 4, the PPARγ ligands strongly increased the proportion of differentiated cells, whereas rapamycin blocked the differentiation. In the presence of both rapamycin and a PPARγ ligand differentiation was still blocked, indicating that rapamycin acts downstream of the PPARγ ligands.


undifferentiated
differentiated
+rosiglitazone
+troglitazone
+PGJ2
rapamycin
-
+


oil red o quantification
rapamycin
+
+
+
+
rosiglitazone
troglitazone
PGJ2

[51]

Figure 4. Differentiation of 3T3-L1 cells with rapamycin in combination with PPARγ ligands. *3T3-L1 cells were induced to differentiate in the presence of the normal mix, as well as rapamycin (100nM), rosiglitazone (10μM), troglitazone (10μM) or PGJ2 (5μM) as indicated. After differentiation cells were stained with oil red o. Microscope pictures were taken and then oil red o was extracted back from the cells with isopropanol. OD_{540} of the extracted solution was measured and is shown in the graph below the pictures.*

The next part of the results will focus on the phenotypic characterization of mice baring an adipose-specific knockout of *raptor*. These results, as well as the data included in the first figure of this thesis, will be submitted for publication in Cell Metabolism in the next few days.

Generation and validation of adipose-specific *raptor* knockout mice (*raptor*$^{ad-/-}$)

To elucidate the role of mTORC1 in adipose tissue, we utilized the cre/loxP system to generate mice in which raptor, an essential and specific component of mTORC1, was deleted exclusively in adipose tissue. A 'floxed' *raptor* allele was created in embryonic stem cells of the 129S1/SvlmJ mouse strain by introducing a loxP site into the introns flanking *raptor* exon 6, using a *neo* cassette (G418 resistance) flanked by frt sites as a selectable marker (Figure 5A). Stem cells in which homologous recombination had occurred were selected by G418 resistance, and recombination was confirmed by southern blot analysis (data not shown). The targeted stem cells were injected into blastocysts of C57BL/6J mice to obtain chimeric floxed mice. After germline transmission, the mice were crossed to C57BL/6J mice expressing flp recombinase to remove the *neo* cassette. Progeny lacking the neo cassette (*raptor*^{floxed}) were further back crossed to C57BL/6J mice. Mice used for all phenotyping experiments had been backcrossed 4 or 5 times. To knock out *raptor*, homozygous *raptor*^{floxed} mice were crossed with heterozygous *raptor*$^{ad+/-}$ mice expressing cre recombinase under control of the adipose-specific, *fabp4/aP2* gene promoter (He et al., 2003). Cre recombinase under control of the *aP2*

promoter is expressed relatively late in adipogenesis (Tontonoz et al., 1994b), leading to knockout of *raptor* only in mature adipocytes. Thus, the generated *raptor*$^{ad-/-}$ mice could be used to study the role of mTORC1 in the maintenance of mature adipocytes, but not in adipogenesis.

We confirmed by western blot analysis that raptor protein was indeed missing specifically in white and brown adipose tissue of *raptor*$^{ad-/-}$ mice (Figure 5B). We also examined, by RT-PCR, expression of the cre recombinase and confirmed that cre was expressed in white and brown adipose tissue, but not in liver, muscle, kidney, intestine, pancreas or spleen (data not shown). *raptor*$^{ad-/-}$ mice were born at the expected Mendelian ratio. *raptor*$^{ad-/-}$ females showed reduced fertility, giving birth on average to 3.9±0.5 pups per litter as compared to 6.79±0.6 and 6.8±0.5 pups per litter for floxed females that did not express cre (*raptor*^{floxed}) and for wild type C57BL/6J females, respectively. The time between litters was also slightly longer for *raptor*$^{ad-/-}$ females. *raptor*$^{ad-/-}$ males showed similar fertility to that of *raptor*^{floxed} and C57BL/6J males.

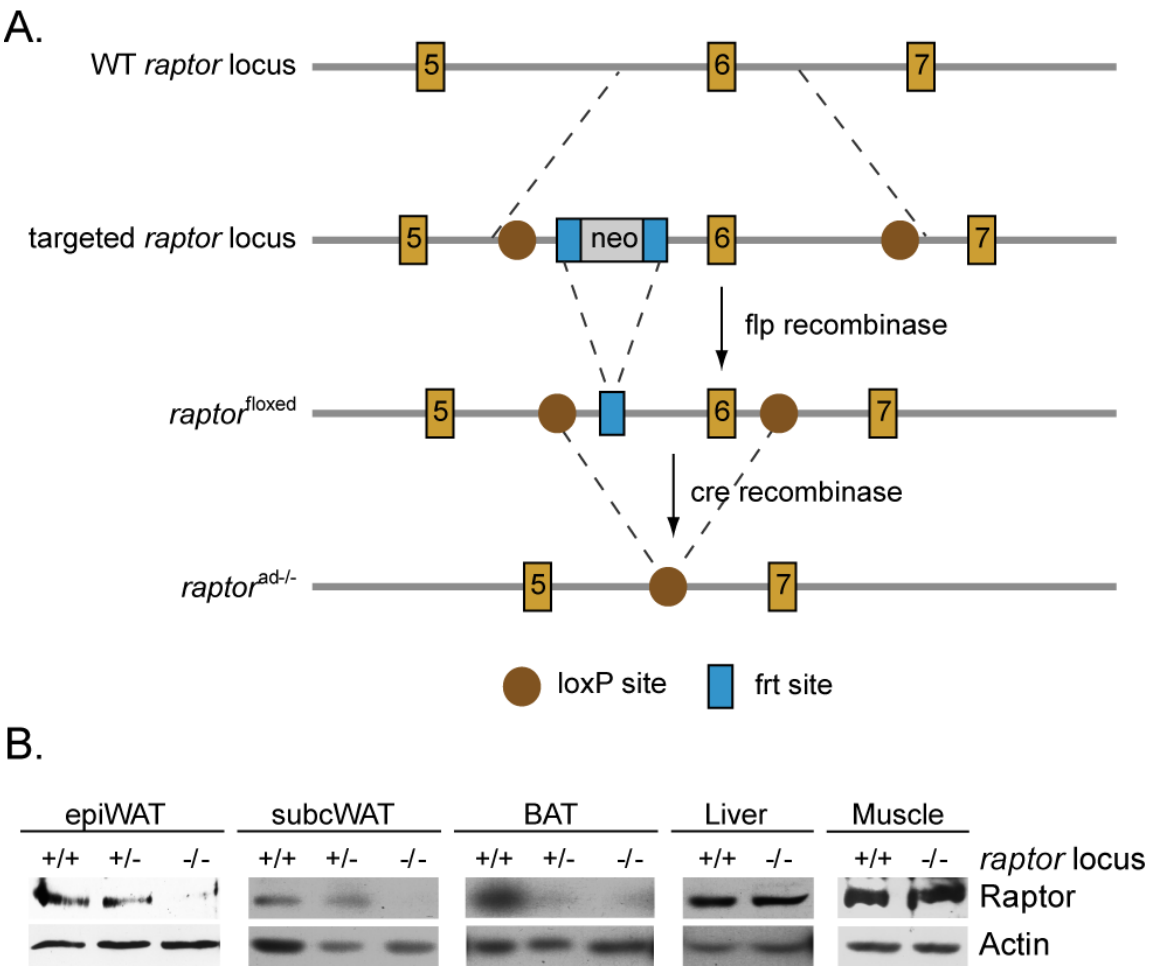


Figure 5. Generation and validation of raptor knockout. ***A.*** *Scheme describing the generation of* raptor *knockout.* ***B.*** *Epididymal WAT, inguinal WAT, BAT, liver, and soleus muscle were isolated, flash-frozen in liquid nitrogen and stored at -80°C. For protein extraction, approximately 50mg frozen tissue was homogenized by polytron, in 800µl lysis buffer (Tris pH7.5, 2mM EDTA, 2mM EGTA, 0.5M mannitol, 1% triton, phosphatase and protease inhibitors). Extracts were spun down and the fat layer and cell debris were removed. 20-40µg proteins were loaded on SDS-PAGE and blotted with raptor or actin antibodies as indicated.*

raptor$^{ad-/-}$ mice are lean and resistant to diet-induced obesity

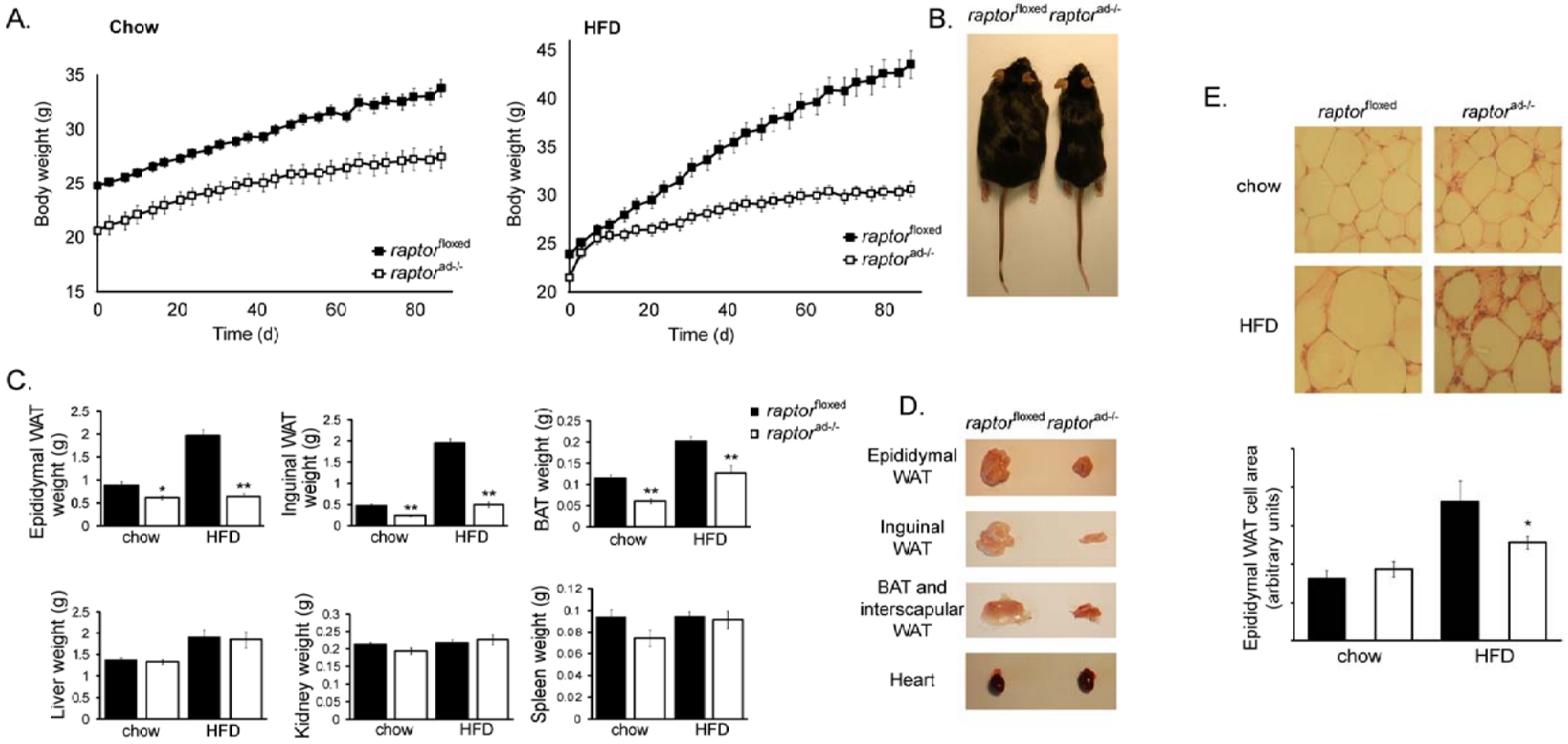
As a first analysis of the effect of *raptor*$^{ad-/-}$ on adipose tissue, we monitored the weight of *raptor*$^{ad-/-}$ mice and littermate controls (*raptor*^{floxed}) over 13 weeks, between the ages of 8 and 21 weeks. On a chow diet, *raptor*$^{ad-/-}$ mice weighed 18% less than *raptor*^{floxed} mice (Figure 6A left graph), but both gained weight at a similar rate. After 13 weeks, both the *raptor*$^{ad-/-}$ and *raptor*^{floxed} mice had gained 33% of their initial weight. When challenged with a high fat diet (HFD), *raptor*$^{ad-/-}$ mice initially gained weight more rapidly than control *raptor*^{floxed} mice (Figure 6A, right graph). However, while the control mice continued to gain weight throughout the time course of the experiment, *raptor*$^{ad-/-}$ mice gained little to no weight after the initial 2 - 3 week period on the HFD. After 13 weeks on the HFD, *raptor*^{floxed} mice had gained 85% of their starting weight whereas *raptor*$^{ad-/-}$ mice had gained only 41% of their starting weight (Figure 6A,B).

To determine the cause of the lower body weight of *raptor*$^{ad-/-}$ mice, we examined individual fat pads and organs. The major fat pads of *raptor*$^{ad-/-}$ mice were significantly lighter and smaller than those of *raptor*^{floxed} mice (Figure 6C,D). This difference in the fat pads was more pronounced for the mice on a HFD (Figure 6C,D). HFD-fed *raptor*$^{ad-/-}$ mice had 70% less WAT than similarly fed *raptor*^{floxed} mice (Figure 6C). The knockout and control mice displayed no significant difference in the weight of lean organs such as the liver, kidney, spleen, and heart, on either the chow or high fat diet, indicating that the effect of the knockout was specific to adipose tissue. This adipose-specific effect of the knockout was confirmed by DEXA scan analysis (data not shown). Thus, an adipose-specific knockout of mTORC1 results in leaner mice and confers resistance to diet-induced obesity.

raptor$^{ad-/-}$ mice have smaller and fewer WAT cells

To determine whether the reduced amount of fat in *raptor*$^{ad-/-}$ mice is due to fewer fat cells and/or smaller fat cells, we measured the circumference of individual adipocytes in

epididymal WAT of *raptor*$^{ad-/-}$ and *raptor*^{floxed} mice (Figure 6E). The adipocytes from knockout and control mice on a chow diet were similar in size, suggesting that the lower amount of WAT in *raptor*$^{ad-/-}$ mice was due to fewer adipocytes. Adipocytes from *raptor*^{floxed} mice on a high fat diet displayed an expected, approximately two-fold increase in size (Kubota et al., 1999; Lemonnier, 1972), whereas adipocytes from *raptor*$^{ad-/-}$ mice on a HFD displayed only a slight increase in size. Calculating and comparing the volumes of adipocytes from *raptor*^{floxed} and *raptor*$^{ad-/-}$ mice on a HFD indicated that *raptor*$^{ad-/-}$ adipocytes were approximately half the size of the *raptor*^{floxed} adipocytes. Although *raptor*$^{ad-/-}$ adipocytes were significantly smaller, this decrease was not sufficient to account for the observed overall reduction in adipose tissue (Figure 6C), suggesting again that a *raptor* knockout also reduces adipocyte number. The reduced weight of a *raptor* knockout mouse is likely due to a combination of smaller and fewer WAT cells. There was no obvious morphological difference in BAT due to either diet or *raptor* knockout (data not shown), although the *raptor*$^{ad-/-}$ mice contained less overall BAT. Thus, mTORC1 is required to maintain adipose tissue.


A.
Chow
HFD
Body weight (g)
Time (d)
raptor^floxed
raptor^ad-/-
B.
raptor^floxed raptor^ad-/-
C.
Epididymal WAT weight (g)
Inguinal WAT weight (g)
BAT weight (g)
Liver weight (g)
Kidney weight (g)
Spleen weight (g)
chow
HFD
D.
raptor^floxed raptor^ad-/-
Epididymal WAT
Inguinal WAT
BAT and interscapular WAT
Heart
E.
raptor^floxed
raptor^ad-/-
chow
HFD
Epididymal WAT cell area (arbitrary units)

[57]

Figure 6. Knockout mice are lean and resistant to diet-induced obesity. ***A.*** *8 week old mice were fed a chow diet (left graph) or a high fat diet (HFD, right graph) for 13 weeks. Body weight was monitored biweekly (n=15-20).* ***B.*** *Representative pictures of mice that were on a HFD for 13 weeks.* ***C.*** *Weight of epididymal WAT, inguinal WAT, BAT, liver, kidney and heart was measured in 21 weeks old mice (n=13-18 for chow diet, n=6-9 for HFD).* ***D.*** *Representative pictures of fat pads and heart from mice that were on a HFD for 13 weeks.* ***E.*** *Top panel: representative pictures of H&E staining of epididymal WAT sections from* raptor^{floxed} *and* raptor$^{ad-/-}$ *mice fed a chow or HFD. Bottom panel: quantification of WAT cell circumference (n=7-10).*

raptor$^{ad-/-}$ mice have better metabolic parameters

We next investigated the effect of the *raptor* knockout on whole animal metabolism. First, we performed an IP glucose tolerance test to determine the ability of the mice to clear glucose from the blood (Figure 7A). On a chow diet, the basal glucose level of the *raptor*$^{ad-/-}$ mice was lower than that of *raptor*^{floxed} mice (4.72±0.3 versus 6.36±0.52mM, respectively). Upon IP injection of glucose, the knockout mice had a normal glucose response and clearance rate. On the HFD, basal glucose levels were, as expected, higher than on the chow diet, but slightly lower in *raptor*$^{ad-/-}$ mice compared to *raptor*^{floxed} mice. HFD-fed *raptor*$^{ad-/-}$ and *raptor*^{floxed} mice both exhibited reduced rates of glucose clearance. However, the glucose clearance rate of the *raptor*$^{ad-/-}$ mice was markedly better compared to the *raptor*^{floxed} mice, indicating that the knockout mice were more glucose tolerant than control mice. We then determined plasma insulin levels. Insulin levels were lower in *raptor*$^{ad-/-}$ mice compared to *raptor*^{floxed} mice (Figure 7B). The lower insulin level combined with the observed lower basal glucose level and better glucose clearance rate suggest that the *raptor*$^{ad-/-}$ mice were more insulin sensitive. This enhanced insulin sensitivity of the *raptor*$^{ad-/-}$ mice was despite lower adiponectin plasma levels in these mice (Figure 7C).

Plasma levels of cholesterol showed a similar trend as observed for glucose tolerance (Figure 7D). Levels were similar for the *raptor*$^{ad-/-}$ and *raptor*^{floxed} mice on the chow diet, and were substantially increased for *raptor*^{floxed} mice on the HFD. However, *raptor*$^{ad-/-}$

mice maintained normal cholesterol levels even on the HFD. The *raptor* knockout had no effect on plasma levels of triglycerides and free fatty acids, on either the chow or high fat diet (Figure 7E and F, respectively). Taken together, the above results suggest that inactivation of mTORC1 in adipose tissue improves whole animal metabolism, in particular when mTORC1-deficient mice are challenged by a HFD.

As described above, the *raptor*$^{ad-/-}$ mice displayed reduced adiposity. Since an alteration in fat metabolism often results in aberrant accumulation of fat in the liver (steatosis), we examined the livers of the knockout and control mice by Oil Red O staining and by quantification of liver triglyceride content (Figure 7G). On the chow diet there was a small but significant reduction in the amount of triglycerides stored in livers of *raptor*$^{ad-/-}$ compared to *raptor*^{floxed} mice. The HFD caused extensive and similar steatosis in the livers of *raptor*$^{ad-/-}$ and *raptor*^{floxed} mice.

[09]

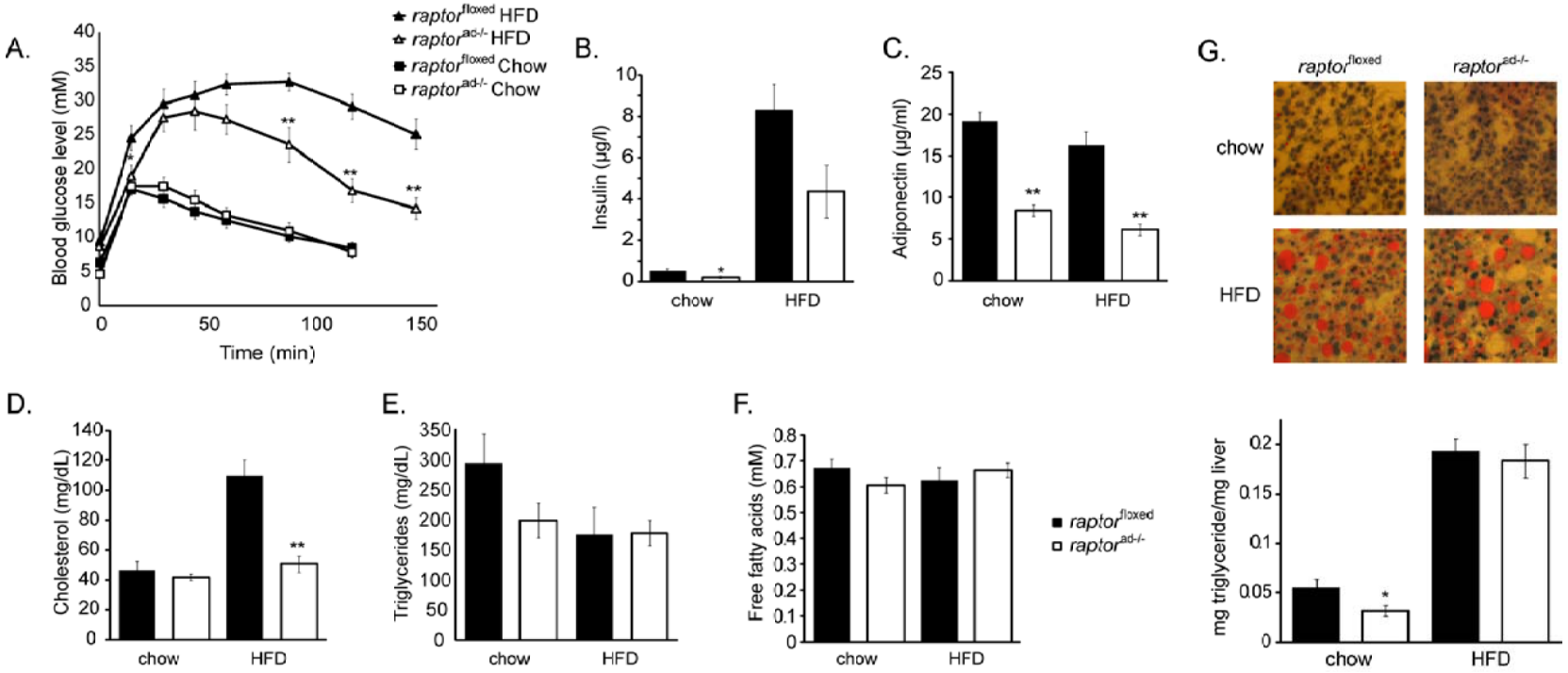

A.
raptor floxed HFD
raptor ad-/- HFD
raptor floxed Chow
raptor ad-/- Chow
Blood glucose level (mM)
Time (min)
B.
Insulin (µg/l)
chow
HFD
C.
Adiponectin (µg/ml)
chow
HFD
G.
raptor floxed
raptor ad-/-
chow
HFD
D.
Cholesterol (mg/dL)
chow
HFD
E.
Triglycerides (mg/dL)
chow
HFD
F.
Free fatty acids (mM)
chow
HFD
raptor floxed
raptor ad-/-
mg triglyceride/mg liver
chow
HFD

Figure 7. Metabolic parameters. ***A.*** *IP glucose tolerance test (n=7-10).* ***B-F.*** *Plasma values (n=10).* ***G.*** *Top panel: representative pictures of Oil Red O and hematoxylin staining of liver sections from raptor^{floxed} and raptor$^{ad-/-}$ mice fed a chow or HFD. Bottom panel: quantification of triglyceride content (n=10).*

Food intake or absorption, physical activity, adipose-specific lipolysis or expression of fat genes do not account for the leanness of *raptor*$^{ad-/-}$ mice

What is the underlying mechanism that causes *raptor*$^{ad-/-}$ mice to be lean and resistant to diet-induced obesity? Adipose tissue is an endocrine organ that interacts with other metabolic organs to regulate behavior and whole body metabolism. The observed leanness could therefore be due to the interaction of adipose tissue with other organs. To examine this possibility, we first measured feeding behavior. *raptor*$^{ad-/-}$ and *raptor*^{floxed} mice consumed similar amounts of calories per day, on both the chow and high fat diets (Figure 8A). Similar food intake was despite markedly reduced plasma leptin levels in *raptor*$^{ad-/-}$ mice, suggesting enhanced leptin sensitivity (Figure 8B). Second, as another form of behavior, we investigated physical activity. We examined the voluntary activity of *raptor*$^{ad-/-}$ and *raptor*^{floxed} mice. Surprisingly, *raptor*$^{ad-/-}$ mice were less active than *raptor*^{floxed} mice, as measured by activity on running wheels. On average, *raptor*$^{ad-/-}$ mice ran approximately 2000 meters/day whereas *raptor*^{floxed} mice ran approximately 4500 meters/day. Third, we examined fecal lipid content to determine if the observed leanness could be due to a defect in food absorption. There was no difference in the fecal lipid content of *raptor*$^{ad-/-}$ and *raptor*^{floxed} mice (Figure 8C), indicating that *raptor*$^{ad-/-}$ mice do not have a defect in absorption of lipid from food. Thus, the leanness of *raptor*$^{ad-/-}$ mice was not due to decreased food intake, increased physical activity or reduced lipid absorption.

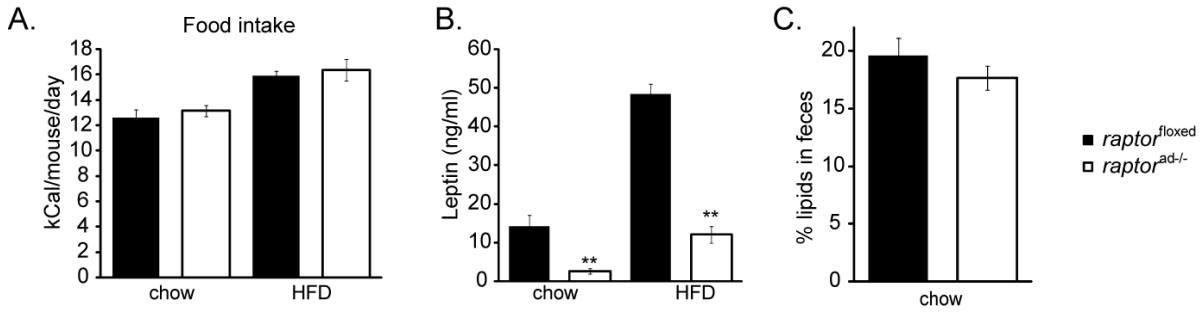
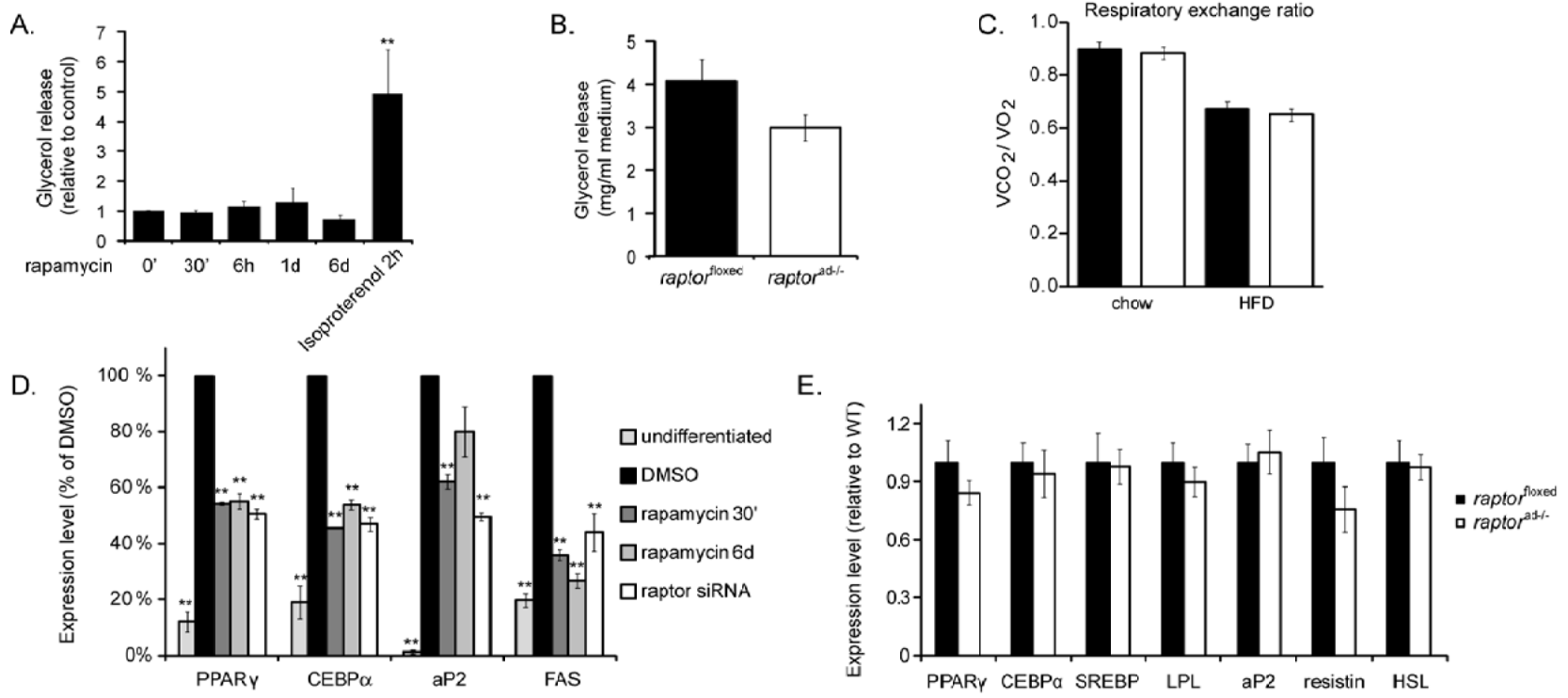


Figure 8. Food intake, leptin levels and fecal lipid content. A. *8 week old mice were fed a chow or high fat diet for 13 weeks. Food intake was measured biweekly (n=15-20).* ***B.*** *Leptin plasma levels were measured using an ELISA kit (n=10).* ***C.*** *To measure lipid absorption in the digestive system, feces were collected over 24h, and fat was separated by methanol:chloroform extraction. Extracted lipids were weighed, and the percentage of feces fat (W/W) was calculated (n=7-11).*

An adipose maintenance defect could also be due to increased lipolysis or to de-differentiation resulting from decreased expression of adipose-specific fat storage genes. To test these possibilities, we first measured lipolysis in differentiated 3T3-L1 cells treated or untreated with rapamycin (Figure 9A) and in epididymal WAT pads of *raptor*$^{ad-/-}$ and *raptor*^{floxed} mice (Figure 9B). In neither system did we detect a significant difference in glycerol release from the cells upon inactivation of mTORC1. We also examined perilipin A phosphorylation, another indicator of lipolysis, and found that it was not induced in epididymal WAT of *raptor*$^{ad-/-}$ mice or in rapamycin-treated 3T3-L1 cells (data not shown). Finally, we determined the respiratory exchange ratio of *raptor*$^{ad-/-}$ and *raptor*^{floxed} mice, as a measure of the utilized energy source. As expected, HFD-fed mice had a lower ratio than chow-fed mice, indicating greater utilization of fat versus carbohydrates as an energy source. However, the *raptor*$^{ad-/-}$ and *raptor*^{floxed} mice had a similar ratio (Figure 9C), indicating similar utilization of carbohydrates and fat as energy sources. Thus, the leanness of *raptor*$^{ad-/-}$ mice was not due to enhanced lipolysis.

We then analyzed the expression pattern of several well known adipose-specific genes that mediate fat storage, in particular, the genes encoding the transcription factors PPARγ, CEBPα and SREBP, the fatty acid binding protein aP2, fatty acid synthase (FAS), lipoprotein lipase (LPL), resistin, and hormone sensitive lipase (HSL). In 3T3-L1 cells, inhibition of mTORC1, either by rapamycin treatment or by *raptor* siRNA, caused a significant decrease in all genes examined (Figure 9D), suggesting that inactivation of mTORC1 causes mature adipocytes to "de-differentiate" due to lack of expression of the genes necessary for lipid production and storage. These results are in agreement with a previous study which showed that the activity of PPARγ is reduced following rapamycin treatment (Kim and Chen, 2004). However, unexpectedly, similar expression levels were detected for the genes listed above in epididymal WAT derived from *raptor*$^{ad-/-}$ and *raptor*^{floxed} mice (Figure 9E). This result suggests that although inactivation of mTORC1 leads to a similar phenotype in cell culture and mice, i.e. loss of fat, the underlying molecular mechanism for this phenotype is probably more complex *in vivo* where interactions between many different cell types and other external factors participate to determine WAT metabolism. However, the leanness of *raptor*$^{ad-/-}$ mice appears not to be due to changes in expression of common lipid metabolizing genes.

Macrophage infiltration into WAT increases with obesity (Weisberg et al., 2003) and contributes to the secretion of inflammatory cytokines that are associated with changes in insulin sensitivity and other metabolic parameters (Weisberg et al., 2006; Xu et al., 2003). To determine the extent of macrophage infiltration into the epididymal WAT of *raptor*$^{ad-/-}$ and *raptor*^{floxed} mice, we measured the expression level of *mac1*, a macrophage marker, in cDNA extracts made from WAT (including any infiltrated macrophages). As expected, *mac1* expression was increased in response to a HFD and the resulting obesity. However, there was little to no difference in *mac1* expression between *raptor*$^{ad-/-}$ and *raptor*^{floxed} mice on either a chow or high fat diet. Thus, leanness was not due to a change in metabolism resulting from a difference in macrophage infiltration.


A.
Glycerol release (relative to control)
rapamycin
0'
30'
6h
1d
6d
Isoproterenol 2h
B.
Glycerol release (mg/ml medium)
raptor^floxec
raptor^ad-/-
C.
Respiratory exchange ratio
VCO2/VO2
chow
HFD
D.
Expression level (% of DMSO)
undifferentiated
DMSO
rapamycin 30'
rapamycin 6d
raptor siRNA
PPARγ
CEBPα
aP2
FAS
E.
Expression level (relative to WT)
raptor^floxed
raptor^ad-/-
PPARγ
CEBPα
SREBP
LPL
aP2
resistin
HSL

[64]

Figure 9. Lipolysis, respiratory exchange ratio and gene expression analysis. ***A.*** *3T3-L1 cells were differentiated and then treated or not treated with rapamycin for the indicated periods of time. Glycerol release was measured during the last 30min of treatment. Isoproterenol was used as a positive control to induce lipolysis (n=4-6).* ***B.*** *Glycerol release was measured from epididymal WAT that was isolated and incubated in DMEM for 12h (n=7).* ***C.*** *The respiratory exchange quotient was calculated as CO_2 production/O_2 consumption during indirect calorimetry measurements.* ***D.*** *3T3-L1 cells were differentiated and then treated or not with rapamycin for the indicated periods of time. RNA was prepared and reverse transcribed, and qPCR was used to measure expression levels of the indicated genes (n=3-5). All genes were normalized to actin. Expression levels in differentiated cells that were not further treated were considered as 100%, and expression levels for all treatments were calculated relative to this untreated control. p value ≤0.01 for all genes except aP2 on day 6.* ***E.*** *Epididymal WAT was isolated, RNA was prepared and reverse transcribed, and qPCR was used to measure expression levels of the indicated genes (n=9-10).*

raptor$^{ad-/-}$ mice have higher energy expenditure due to an increase in uncoupled respiration in WAT

Leanness of the *raptor*$^{ad-/-}$ mice could be due to increased energy expenditure. To investigate energy expenditure, we measured oxygen consumption by indirect calorimetry. *raptor*$^{ad-/-}$ mice displayed an increase in oxygen consumption as compared with *raptor*^{floxed} mice (Figure 10A), suggesting that the knockout mice indeed expend more energy. We then analyzed, using a Clark electrode, the oxygen consumption by isolated white adipocytes derived from *raptor*$^{ad-/-}$ and *raptor*^{floxed} mice. Adipocytes from *raptor*$^{ad-/-}$ mice displayed a significant increase in oxygen consumption compared to control adipocytes (Figure 10B). Thus, *raptor*$^{ad-/-}$ caused an increase in mitochondrial respiration in WAT.

Why is respiration increased in WAT of *raptor*$^{ad-/-}$ mice? The observed increase in oxygen consumption by isolated adipocytes, as described above, was abolished by the addition of ADP. This effect of ADP, which stimulates coupled respiration and inhibits uncoupled respiration (Nicholls, 2001), suggested that the increase in oxygen consumption was due to uncoupled mitochondrial respiration. To investigate this

possibility further, we examined expression of mitochondrial uncoupling genes in WAT from *raptor*$^{ad-/-}$ and *raptor*^{floxed} mice. We observed a strong increase in the expression of the *uncoupling protein 1* (*UCP1*) gene, and to a lesser extent in expression of *UCP2* and other brown fat markers such as *type 2 deiodinase* (*dio2*) and *cidea* in WAT of *raptor*$^{ad-/-}$ mice (Figure 10C). Thus, the leanness of *raptor*$^{ad-/-}$ mice appears to be due to increased energy expenditure resulting from mitochondrial uncoupling in WAT.

No difference in oxygen consumption was detected in brown adipocytes isolated from raptor$^{ad-/-}$ and raptor^{floxed} mice (data not shown). In agreement with this finding, the level of *UCP1* expression in BAT was unaffected by *raptor* knockout (Figure 10D). However, expression of *PPARγ*, as well as *UCP2* and *UCP3,* was reduced (Figure 10D). Thus, the decreased BAT mass in raptor$^{ad-/-}$ mice might be due to de-differentiation resulting from decreased expression of the key adipogenic transcription factor PPARγ.

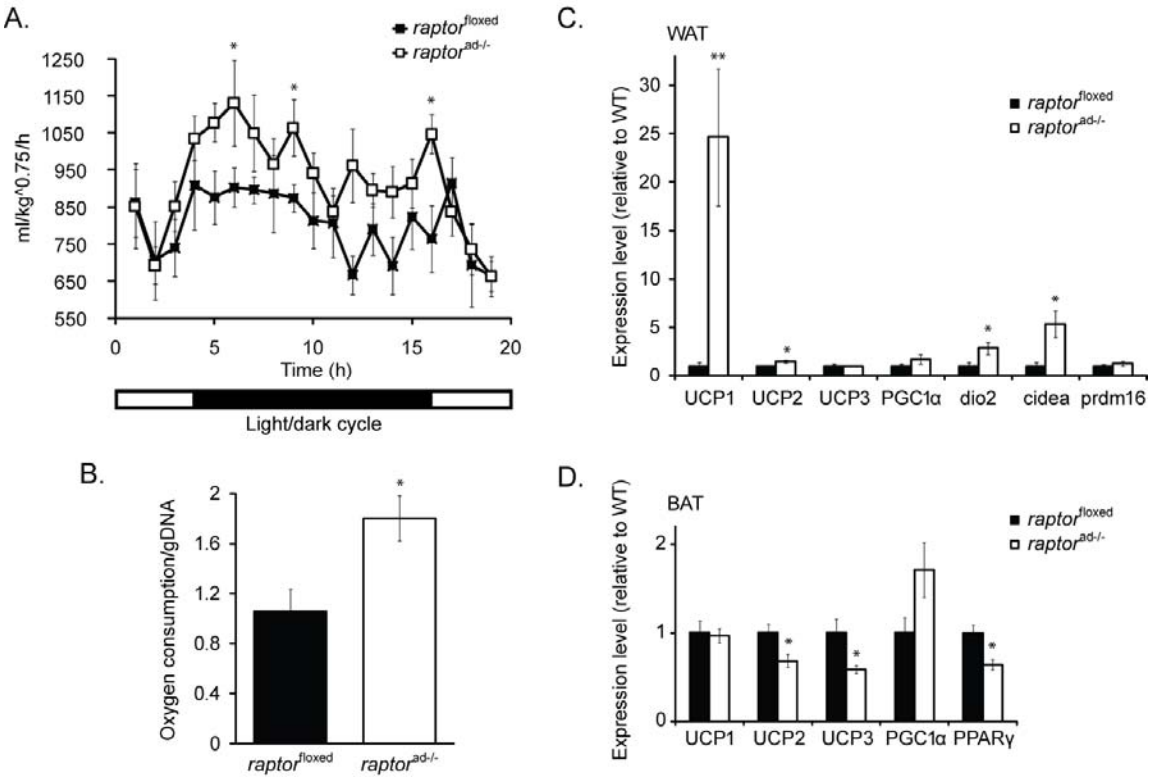


Figure 10. Energy expenditure and gene expression in WAT and BAT. A. *O_2 consumption by 14-15 week old mice that remained on chow or high fat diet during the previous 5-6 weeks.* ***B.*** *O_2 consumption in primary white adipocytes isolated from raptor^{floxed} or raptor$^{ad-/-}$ mice (n=4), as measured with a Clark electrode. O_2 consumption was normalized to the amount of genomic DNA (gDNA).* ***C-D.*** *Epididymal WAT or BAT were isolated, RNA was prepared and reverse transcribed, and qPCR was performed to measure expression levels of the indicated genes (n=9-10).*

Discussion

In this thesis, I describe the generation and phenotypic characterization of the first conditional *raptor* knockout mice. Knockout of *raptor* specifically in adipose tissue (*raptor*$^{ad-/-}$) resulted in mice that were lean and resistant to diet-induced obesity. *raptor*$^{ad-/-}$ mice also exhibited an overall better metabolic profile than control mice, including enhanced glucose tolerance and resistance to diet-induced hypercholesterolemia. Furthermore, we present evidence that the leanness of *raptor*$^{ad-/-}$ mice was due to elevated energy expenditure as a consequence of uncoupled mitochondria in white adipose tissue. These results suggest that mTORC1 in adipose plays an important role in the control of both adipose metabolism and overall body energy homeostasis.

Similar to adipose-specific *raptor* knockout, whole body knockout of S6K1, which is positively regulated by mTORC1, results in lean mice that are resistant to diet-induced obesity due to increased energy expenditure (Pende et al., 2000; Um et al., 2004). Furthermore, *S6K1* knockout mice exhibit normal food intake despite lower leptin levels, smaller WAT cells, and enhanced insulin sensitivity. Conversely, knockout of 4E-BP1 and 2, which are negatively regulated by mTORC1, results in increased obesity and hypersensitivity to diet-induced obesity due to lower energy expenditure (Le Bacquer et al., 2007). This remarkable correlation between adipose-specific knockout of mTORC1 and full body knockout of its direct effectors S6K1 and 4E-BP1/2 suggests that the effects of the *S6K1* and *4E-BP1/2* knockouts are via adipose tissue. This, in turn, provides further evidence that mTORC1 signaling in adipose plays a central role in controlling whole animal metabolism.

raptor$^{ad-/-}$ mice have lower basal glucose levels, improved glucose clearance, and reduced plasma insulin levels compared to control *raptor*^{floxed} mice, indicating that *raptor*$^{ad-/-}$ mice have enhanced insulin sensitivity. Since 70%-90% of glucose clearance from the blood is by skeletal muscle (De Fronzo, 1997), the observed improvement in insulin sensitivity is likely in muscle (and possibly other tissues). This underscores the role

of adipose and, in particular, adipose mTORC1 in controlling whole body energy homeostasis. It remains to be determined how mTORC1 signaling in adipose tissue controls insulin sensitivity in other tissues.

Previous studies on the role of mTORC1 in mitochondrial function have provided seemingly conflicting results. Observed changes in respiration by full body S6K1 or 4E-BP1/2 knockout mice suggest, in line with our results, that mTORC1 negatively controls mitochondrial respiration (or uncoupling) (Le Bacquer et al., 2007; Um et al., 2004). In contrast, rapamycin treatment or knockdown of mTOR or raptor in muscle cells or in *TSC* knockout MEFs decreases mitochondrial gene expression and oxygen consumption (Cunningham et al., 2007). These latter effects are via down-regulation of PGC1α and the transcription factor YY1, and do not involve S6K1 or Akt (Cunningham et al., 2007; Schieke et al., 2006). Taken together, our data and the above findings suggest that mTORC1 controls mitochondrial respiration either negatively or positively depending on the mTORC1 effectors that might be found in a particular tissue.

Impaired TORC1 signaling in yeast, flies and worms extends lifespan (Kaeberlein et al., 2005; Kapahi et al., 2004; Powers et al., 2006; Vellai et al., 2003). Inhibition of TOR signaling mimics nutrient deprivation and the extension of lifespan by TOR inhibition might thus be equivalent to the extension of lifespan by dietary restriction. In this context, it is interesting to note that *raptor*$^{ad-/-}$ mice have improved metabolic parameters that could lead to extended lifespan. Furthermore, *raptor*$^{ad-/-}$ enhances respiration and, at least as shown in budding yeast, impaired TORC1 signaling extends lifespan by increasing mitochondrial respiration among other effects (Bishop and Guarente, 2007; Bonawitz et al., 2007; Kaeberlein and Powers Iii, 2007). It would be of interest to determine if mTORC1 in mammals, and in particular in adipose tissue, controls lifespan.

Metformin is a widely prescribed anti-diabetic drug that increases insulin sensitivity and lowers blood glucose and lipid levels. It functions through activation of the LKB1-AMPK pathway in liver (Shaw et al., 2005; Tzatsos and Kandror, 2006). Metformin also inactivates mTORC1 downstream of AMPK (Dowling et al., 2007; Inoki et al., 2003b; Shaw

et al., 2004; Tzatsos and Kandror, 2006). Given that adipose-specific knockout of mTORC1 increases insulin sensitivity and lowers blood glucose and cholesterol levels, metformin might also exert part of its therapeutic effect via activation of AMPK and inhibition of mTORC1 in adipose tissue.

raptor$^{ad-/-}$ mice are lean due to a reduction in the size and number of adipocytes. A previous study in 3T3-L1 cells demonstrated that rapamycin blocks adipogenesis via inhibition of the key adipogenic transcription factor PPARγ (Kim and Chen, 2004). Kim et al. (2004) also reported that rapamycin strongly inhibits PPARγ activity in differentiated 3T3-L1, and that incubation of differentiated 3T3-L1 with rapamycin induces loss of fat. Our results in 3T3-L1 cells support these findings (Figure 1 and 9D). However, surprisingly, *raptor*$^{ad-/-}$ mice are lean despite the absence of an effect on expression of PPARγ and PPARγ targets (Figure 9E, unpublished for PPARγ protein level). Thus, in animals there must be a mechanism(s) in addition to mTORC1 signaling that maintains PPARγ. It remains to be determined what this mechanism might be. mTORC1 deficient mice lose fat due to enhanced energy expenditure and not due to a PPARγ deficiency.

Close examination of the weight curves shows that *raptor*$^{ad-/-}$ mice reached a maximal weight of approximately 30 grams, regardless of being on a chow or high fat diet (Figure 6A). This weight was reached at a slow, constant rate on the chow diet, or very rapidly on the high fat diet. This observed maximal weight suggests there is a limit to the amount of fat that *raptor*$^{ad-/-}$ mice can accumulate. This is also supported by the findings that the size of individual adipocytes did not increase upon HFD, and that the number of adipocytes was reduced in *raptor*$^{ad-/-}$ mice on both the chow and high fat diet (Figure 6E). The reason for such a fixed limit on the amount of fat *raptor*$^{ad-/-}$ mice can accumulate is unknown.

Rapamycin is a specific inhibitor of mTORC1, and cannot bind mTOR in the context of mTORC2 (Jacinto et al., 2004; Sarbassov et al., 2004). However, it can bind free mTOR (Brown et al., 1994; Sabatini et al., 1994; Sabers et al., 1995), thereby indirectly inhibiting the formation of mTORC2 during prolonged rapamycin treatment in many cell types

(Sarbassov et al., 2006). Many previous studies have shown that rapamycin treatment inhibits adipogenesis and lipid maintenance of 3T3-L1 cells (Bell et al., 2000; Cho et al., 2004; El-Chaar et al., 2004; Gagnon et al., 2001; Kim and Chen, 2004; Yeh et al., 1995a), and attributed this inhibition to loss of mTORC1 activity. However, these experiments require incubation of cells with rapamycin for several days, and might therefore overlook effects caused by mTORC2 inhibition. We used siRNA against raptor to specifically inhibit mTORC1, and found that under these conditions adipogenesis and adipose maintenance of 3T3-L1 cells is strongly inhibited (Figure 1). Furthermore, we used MEFs in which one allele of the *raptor* gene was genetically deleted, and found that under these conditions adipogenesis was impaired as well. Taken together, these results confirmed that mTORC1 is indeed required for the differentiation and maintenance of adipocytes in culture.

We further attempted to elucidate how mTORC1 regulates adipocytes by looking at possible interactions between mTORC1 and the key adipogenic transcription factor PPARγ. We found that PPARγ is most likely not a phosphorylation substrate of mTORC1 (Figure 2). The nuclear localization and protein levels of PPARγ, its dimerization partner RXR, or the cofactors N-CoR or retinoblastoma were not affected by rapamycin treatment (Figure 3 and data not shown). It would be interesting to further examine parameters such as retinoblastoma phosphorylation and binding to PPARγ following rapamycin treatment, since rapamycin has been reported to affect retinoblastoma in many cell types (Brennan et al., 1999; Decker et al., 2003; Francis et al., 2006; Gallo et al., 1999; Gao et al., 2004; Garcia-Morales et al., 2006; Marx et al., 1995; Nader et al., 2005; Wanner et al., 2006). Furthermore, phosphorylation of retinoblastoma during adipogenesis is inhibited by rapamycin (Usui et al., 2000), and mice with an adipose-specific knockout of *retinoblastoma* display a very similar phenotype to our adipose-specific *raptor* knockout mice (Dali-Youcef et al., 2007).

When combining rapamycin with either one of the PPARγ ligands rosiglitazone, troglitazone or PGJ2, rapamycin still inhibited adipogenesis. This suggests that rapamycin acts downstream of PPARγ ligand binding. These results contradict with previously

published results by Kim and Chen (Kim and Chen, 2004), who reported that troglitazone reversed the inhibition of adipogenesis by rapamycin. The experimental design, materials and cell line were similar in the two studies, and therefore the reason for this conflicting result is unknown.

In conclusion, mTORC1 in adipose tissue regulates whole body metabolism and energy homeostasis. Inhibition of mTORC1 results in metabolically healthier mice, with no apparent adverse effects. Thus, adipose mTORC1 is a potential target for anti-obesity and anti-diabetes drugs.

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PRAS40 and PRR5-Like Protein Are New mTOR Interactors that Regulate Apoptosis

For several months during my Ph.D. research period I was involved in a side project with my colleague Kathrin Thedieck. This project included the discovery of two new mTOR interacting partners, and the characterization of their role in the mTOR signaling pathway.

The discovery of these new interactors was by Kathrin via purification of members of the mTORCs and analysis of the interactors by mass spectrometry. The biochemical characterization was performed together by Kathrin and me. In addition, we collaborated with Man-Lyang Kim from the lab of Cécile Arrieumerlou, who prepared the siRNA and did the apoptosis analysis.

The results of this project have been published in the journal PLoS one (Thedieck et al., 2007). Since this project was not directly related to my main research theme of mTORC1 in adipose tissue, I only attach a copy of the manuscript to this thesis, without putting it into a broader context.

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PLoS one

PRAS40 and PRR5-Like Protein Are New mTOR Interactors that Regulate Apoptosis

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TOR (Target of Rapamycin) is a highly conserved protein kinase and a central controller of cell growth. TOR is found in two functionally and structurally distinct multiprotein complexes termed TOR complex 1 (TORC1) and TOR complex 2 (TORC2). In the present study, we developed a two-dimensional liquid chromatography tandem mass spectrometry (2D LC-MS/MS) based proteomic strategy to identify new mammalian TOR (mTOR) binding proteins. We report the identification of Proline-rich Akt substrate (PRAS40) and the hypothetical protein Q6MZQ0/FLJ14213/CAE45978 as new mTOR binding proteins. PRAS40 binds mTORC1 via Raptor, and is an mTOR phosphorylation substrate. PRAS40 inhibits mTORC1 autophosphorylation and mTORC1 kinase activity toward eIF-4E binding protein (4E-BP) and PRAS40 itself. HeLa cells in which PRAS40 was knocked down were protected against induction of apoptosis by TNFα and cycloheximide. Rapamycin failed to mimic the pro-apoptotic effect of PRAS40, suggesting that PRAS40 mediates apoptosis independently of its inhibitory effect on mTORC1. Q6MZQ0 is structurally similar to proline rich protein 5 (PRR5) and was therefore named PRR5-Like (PRR5L). PRR5L binds specifically to mTORC2, via Rictor and/or SIN1. Unlike other mTORC2 members, PRR5L is not required for mTORC2 integrity or kinase activity, but dissociates from mTORC2 upon knock down of tuberous sclerosis complex 1 (TSC1) and TSC2. Hyperactivation of mTOR by TSC1/2 knock down enhanced apoptosis whereas PRR5L knock down reduced apoptosis. PRR5L knock down reduced apoptosis also in mTORC2 deficient cells. The above suggests that mTORC2-dissociated PRR5L may promote apoptosis when mTOR is hyperactive. Thus, PRAS40 and PRR5L are novel mTOR-associated proteins that control the balance between cell growth and cell death.

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INTRODUCTION

TOR (Target of Rapamycin) kinase is a highly conserved, central controller of cell growth [1–3]. The fundamental importance of TOR is underscored by genetic studies showing TOR to be essential for cell growth and development; disruption of the *TOR* gene is lethal in all examined species [4–12]. In humans, dysfunctional mTOR signaling plays an important role in many if not most cancers, as well as in diseases such as tuberous sclerosis complex (TSC, #191100 OMIM) and lymphangiomyelomatosis (LAM, #606690 OMIM). TOR is found, from yeast to human, in two functionally and structurally distinct multiprotein complexes termed TOR complex 1 (TORC1) and TORC2 [13–15]. The rapamycin-sensitive mammalian TOR complex 1 (mTORC1) consists of mTOR, mLST8 and Raptor [13,16,17]. mTORC2 contains Rictor and SIN1 instead of Raptor, and is rapamycin-insensitive [14,15,18–20]. Knock out of Raptor, SIN1 or Rictor in mice is embryonic lethal, indicating that both mTORC1 and mTORC2 are essential [19–22].

mTORC1 is activated by nutrients (amino acids), anabolic growth factors (e.g., insulin and insulin-like growth factor), and cellular energy (ATP) [1–3]. The growth factor signal and energy status are transmitted to mTORC1 via the essential tumor suppressor tuberous sclerosis complex (TSC) proteins TSC1 and TSC2 [23,24]. The TSC heterodimer (TSC1-TSC2) is a GTPase activating protein (GAP) that inhibits the essential small GTPase Rheb [25,26]. Rheb-GTP binds and activates mTORC1 [27]. Akt (also known as PKB) phosphorylates and inactivates TSC2 in response to growth factors [28], whereas AMPK phosphorylates and activates TSC2 in response to low energy (high AMP) [29,30]. Nutrients impinge on mTORC1 at the level of Rheb or mTORC1 by a poorly understood mechanism involving the type III PI3K hVps34 [27,31]. The upstream regulators of the more recently identified mTORC2 are not known, but mTORC2 appears to respond at least to growth factors, possibly via TSC1-TSC2 [32].

mTORC1 and mTORC2 separately control many cellular processes that collectively determine cell growth and development. mTORC1 controls transcription, protein synthesis, ribosome biogenesis, nutrient transport, and autophagy, among other processes. mTORC1 controls protein synthesis via phosphorylation of S6 kinase (S6K) and eIF-4E binding protein (4E-BP), two key regulators of translation initiation [3,33,34]. mTORC2 controls organization of the actin cytoskeleton via small Rho-type GTPases and Protein Kinase C [14,15,35], and thereby determines the shape and possibly motility of the cell. In addition, mTORC2 phosphorylates Ser473 in the hydrophobic motif of Akt and thereby activates Akt toward substrates such as the Forkhead transcription factor FOXO and the apoptosis regulator BAD [19–21,36].

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Although upstream regulators of mTOR, at least for mTORC1, are relatively well characterized, astonishingly few direct substrates and downstream effectors of the mTORCs are known. This is particularly true for mTORC2 which was discovered only recently and, due to its rapamycin insensitivity, is not pharmacologically addressable. To identify additional regulators, substrates, and/or components of the mTORCs, we developed a highly sensitive mass spectrometry-based screen. Here we report the identification of two novel mTOR binding proteins, PRAS40 (Q96B36 Swiss-Prot) and PRR5L (Q6MZQ0 Swiss-Prot), which bind specifically to mTORC1 and mTORC2, respectively. We further characterize the roles of these two proteins in mTOR complex formation and function.

RESULTS AND DISCUSSION

PRAS40 and PRR5L bind specifically to mTORC1 and mTORC2

To identify new mTOR binding proteins, we used a 'gel-less' mass spectrometry-based method to screen for mTOR associated proteins. mTOR complexes, first purified by large scale immunoprecipitations (IPs) with antibody directed against mTOR, were digested with trypsin and after detergent removal subjected directly to two-dimensional liquid chromatography tandem mass spectrometry (2D LC-MS/MS). Within a single 2D LC-MS/MS run up to 270 different proteins were identified. To identify specific mTOR interactors, we compared mTOR and mock IPs, and chose those proteins that were present only in the mTOR IPs. Furthermore, to qualify as a specific interactor, a protein had to be identified in at least three out of four independent mTOR IP experiments. We reproducibly identified all known members of the mTOR complexes (Table 1). The sequence coverage for mTOR, Rictor, mLST8 and Raptor was about 20%, while sequence coverage for SIN1 was 13.8%. The above experiment was repeated with antibodies specific for Rictor or Raptor and, as expected, mTOR and mLST8 were found in both the Rictor and Raptor IPs, whereas SIN1 was found only in the Rictor IP.

In addition to the known mTORC partners, we also identified novel mTOR interacting proteins. The proline-rich Akt substrate PRAS40 (10.6% sequence coverage) was found in mTOR and Raptor IPs but not in Rictor IPs (Table 1). These interactions were confirmed by co-IP experiments with HeLa and HEK293 cells (Figure 1). PRAS40 is therefore a specific mTORC1 binding partner (Figure 1A). PRAS40 was originally discovered as an Akt substrate of unknown function [37]. During the preparation of this manuscript, two studies appeared suggesting that PRAS40 is an mTORC1 inhibitor [38,39]. In addition to PRAS40, we identified the hypothetical protein Q6MZQ0/FLJ14213/CAE45978 as a specific mTORC2 interactor (Table 1). Q6MZQ0 was cloned with an N-terminal GST tag and its interaction with mTORC2 was confirmed by co-IP and GST pull downs from HeLa and HEK293 cells (Figure 1B). Since Q6MZQ0 displays 39% sequence similarity with the proline rich protein PRR5, we named it PRR5-Like protein (PRR5L). PRR5L is an uncharacterized protein. The related protein PRR5, however, is highly expressed in kidney and has been suggested to be a tumor suppressor since it is down regulated in a subset of breast tumors [40].

Earlier studies on the mTORCs failed to detect PRAS40 and PRR5L possibly because both have an apparent molecular weight of approximately 40 kDa as measured by SDS-PAGE. Former searches for mTOR binding proteins relied on IPs followed by SDS-PAGE analysis. The co-migrating heavy chain of the IP antibody used in these earlier experiments might have masked PRAS40 and PRR5L. Our 2D LC-MS/MS approach also identified Transferrin Receptor 1 (P02786 Swiss-Prot), NICE-4 (Q14157 Swiss-Prot), Plectin 1 (Q6S383 Swiss-Prot), and Thymopoietin (P42166 Swiss-Prot) as potential mTOR binding proteins, but direct co-IP experiments indicated that these were non-specific binding proteins (data not shown).

PRAS40 binds mTORC1 via Raptor

Following rapamycin treatment, PRAS40 dissociated from mTOR (Figure 1A). However, released PRAS40 remained bound to Raptor which, as reported previously [41], is also released from mTOR upon rapamycin treatment. Furthermore, PRAS40 binding to mTOR was strongly reduced when Raptor was knocked down (Figure 2A). These findings indicate that PRAS40 binds mTORC1 via Raptor. We also observed that PRAS40 associated less well with a kinase dead version of mTOR (Figure 2B), suggesting that mTORC1-mediated phosphorylation of PRAS40 (see below) may affect the PRAS40- mTORC1 interaction. Our findings are in agreement with recent PRAS40 studies showing that PRAS40 binds preferentially to Raptor [38], and that the mTOR kinase domain is also involved in PRAS40 binding [39].

Table 1. mTORC1 and mTORC2 associated proteins identified by 2D-LC-MS/MS

Protein	# Identifications (out of 4 independent IPs)	Sequence Coverage	Identified in IP of			Predicted MW (Da)	Length (aa)
			mTOR	Rictor	Raptor		
mTOR	4	26.2%	x	x	x	288892	2549
Rictor	4	21%	x	x		192217	1708
Raptor	4	18.2%	x		x	149038	1335
SIN1	4	13.8%	x	x		59123	522
PRR5L	3	5.2%	x	x		40866	368
mLST8	4	21.5%	x	x	x	35902	326
PRAS40	3	10.6%	x		x	27383	256

mTOR complexes were purified by immunoprecipitation (IP) with antibody directed against mTOR, Rictor or Raptor. Immunoprecipitates were analyzed by 2D LC-MS/MS. Proteins that were found in at least three out of four mTOR IPs, but not in mock IPs, were considered specific. IPs with antibodies directed against Rictor or Raptor indicated whether a candidate was specific for mTORC1 or mTORC2, respectively.

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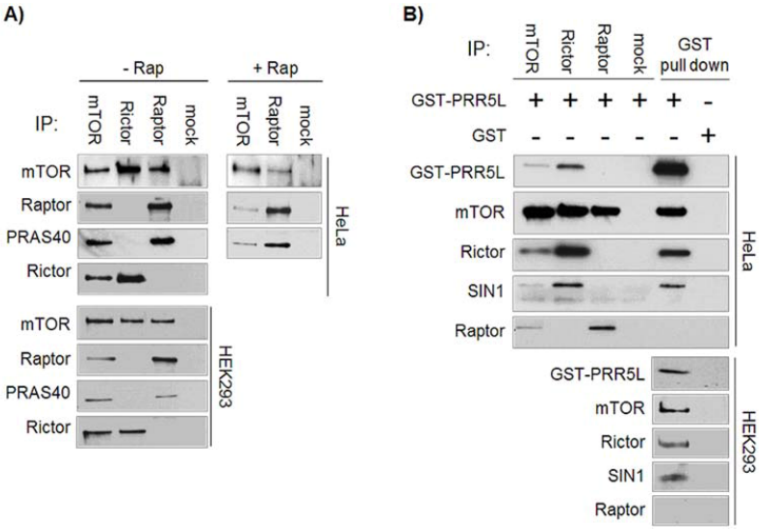

Figure 1. Confirmation of PRAS40 and PRR5L binding to mTOR. A. PRAS40 is associated specifically with mTORC1. mTOR, Rictor, Raptor and mock IPs were performed with HeLa and HEK293 extracts and analyzed by immunoblotting. PRAS40 was found specifically in mTOR and Raptor IPs. 1 h rapamycin treatment of cells dissociated a Raptor-PRAS40 subcomplex from mTOR. **B. PRR5L is associated specifically with mTORC2.** HeLa or HEK293 cells were transfected with GST-PRR5L or the empty plasmid. mTOR, Rictor, Raptor and mock IPs and GST pull downs were analyzed by immunoblotting. GST-PRR5L is detected specifically in mTOR and Rictor IPs. mTOR, Rictor and SIN1, but not Raptor, are detected specifically in GST-PRR5L pull downs.
doi:10.1371/journal.pone.0001217.g001

PRAS40 is a substrate and an inhibitor of mTORC1 *in vitro*

The above results suggested that PRAS40 is phosphorylated by mTORC1. To investigate if PRAS40 is an mTORC1 substrate, we performed *in vitro* kinase assays with mTORC1 or mTORC2 and purified PRAS40. PRAS40 was phosphorylated weakly by both mTORC1 and mTORC2. Importantly, we also found that PRAS40 inhibited mTORC1 autophosphorylation but not mTORC2 autophosphorylation (Figure 3A), suggesting that the weak phosphorylation of PRAS40 by mTORC1 might be due to PRAS40-mediated inhibition of mTORC1. To investigate whether PRAS40 inhibits mTORC1 kinase activity, we performed *in vitro* kinase assays with the known mTORC1 substrate 4E-BP and increasing concentrations of purified PRAS40. PRAS40 inhibited both mTORC1 autophosphorylation and mTORC1 phosphorylation of 4E-BP, in a concentration dependent manner (Figure 3B). In addition, PRAS40 phosphorylation inversely correlated with the concentration of PRAS40 in the kinase reaction, suggesting that PRAS40 is indeed both a substrate and an inhibitor of mTORC1 kinase activity. Our finding that PRAS40 inhibits mTORC1 kinase activity toward 4E-BP and PRAS40 is in agreement with the observation of Sancak et al. [38] and Vander Haar et al. [39] that PRAS40 inhibits mTORC1 toward S6K1. Hence, we conclude that PRAS40 is a broad mTORC1 inhibitor that inhibits mTORC1 kinase activity toward itself, 4E-BP, S6K1, and PRAS40. It remains to be determined whether mTORC1-mediated phosphorylation of PRAS40 plays a role in PRAS40's ability to bind and inhibit mTORC1.

Interestingly, we found that phosphorylation of the Akt consensus site T246 in PRAS40 is moderately reduced in Rictor knock down cells (data not shown). This suggests that mTORC2 may activate Akt toward PRAS40. This in turn suggests that mTORC2, via PRAS40, may be upstream of mTORC1. If mTORC2 is indeed upstream of mTORC1, it might be only under specific conditions or only with regard to particular mTORC1 substrates (other than S6K1), as we and others failed to detect an effect of mTORC2 disruption on S6K1 phosphorylation [14,15]. The potential regulation of mTORC1 by mTORC2 requires further investigation.

PRAS40 deficiency prevents induction of apoptosis by TNFα and cycloheximide

Constitutively active mTOR reduces apoptosis [42] whereas inhibition of mTORC1 with rapamycin induces or facilitates apoptosis in several cell lines [43–48]. We therefore reasoned that the mTORC1 inhibitor PRAS40 might promote apoptosis and that PRAS40 knock down would thus protect cells against the induction of apoptosis. To investigate this possibility, we examined the effect of PRAS40 knock down on the sensitivity of HeLa cells to apoptosis induction by TNFα in combination with cycloheximide. To monitor apoptosis, treated cells were processed for visualization of DNA and cleaved PARP. We found that apoptosis was reduced in PRAS40 knock down cells (Figure 4A), suggesting that PRAS40 is indeed pro-apoptotic. To analyze if PRAS40 promotes apoptosis via its inhibitory effect on mTORC1, we investigated if rapamycin suppressed the effect of a PRAS40 deficiency on TNFα/cycloheximide induced apoptosis in HeLa cells. Rapamycin failed to prevent the reduction in apoptosis caused by PRAS40 knock down (Figure 4B). In addition, rapamycin treatment did not affect apoptosis induction by TNFα/cycloheximide in control cells (Figure 4B), even after 6h of rapamycin treatment (data not shown). The finding that rapamycin failed to mimic the pro-apoptotic effect of PRAS40, suggests that PRAS40 mediates apoptosis independently of its

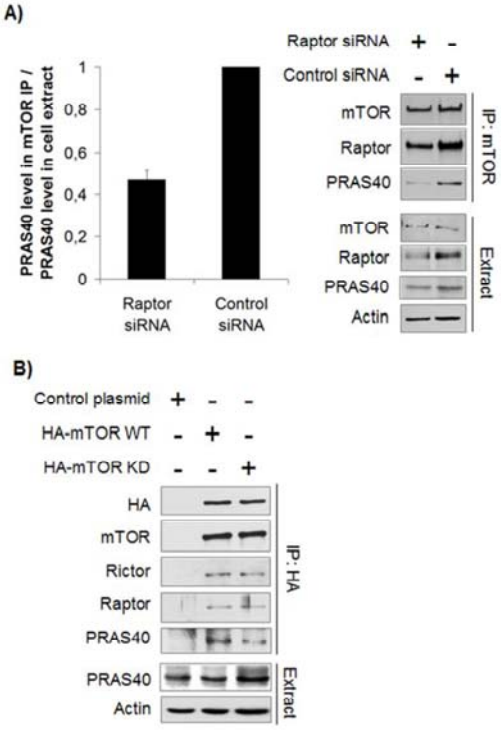

Figure 2. PRAS40 interacts with mTORC1. A. PRAS40 binds mTOR via Raptor. HEK293 cells were transfected with Raptor or control siRNA vectors and incubated for 4 days, followed by mTOR IP and immunoblotting. mTOR IPs and cell extracts were probed with antibodies directed against the indicated proteins. Since knock down of Raptor reduced the cellular amount of PRAS40, the PRAS40 signal in the IPs was quantified relative to PRAS40 levels in the corresponding extract. Quantitations were averaged over three independent experiments. Raptor knock down reduced the amount of PRAS40 associated with mTOR by 50%, as compared to control cells. **B. mTOR kinase domain is involved in PRAS40 binding.** HEK293 cells were transfected with a plasmid expressing wild type (WT) HA-mTOR or kinase dead (KD) HA-mTOR or an empty control plasmid, and incubated for 48 h followed by extract preparation and IP with an anti-HA antibody. PRAS40 levels in the extracts remained unaltered. PRAS40 association with mTORC1 containing HA-mTOR KD was moderately reduced as compared with mTORC1 containing HA-mTOR WT.
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inhibitory effect on mTORC1. However, PRAS40 T246 phosphorylation (PRAS40-pT246) appears to protect neuronal cells from apoptosis after stroke [49]. PRAS40-pT246 has also been proposed to promote cell survival in cancer cells [50]. The fact that T246 is the site via which Akt negatively regulates PRAS40's ability to inhibit mTORC1 [38] suggests that PRAS40 may indeed be pro-apoptotic via its ability to inhibit mTORC1. To explain the apparent discrepancy with our inability to induce apoptosis with rapamycin, PRAS40 may have to inhibit both mTORC1 and a second, unknown rapamycin insensitive target (or only this second target) to perform its pro-apoptotic function. mTORC1 may be the more important target in those cell lines where rapamycin induces or facilitates apoptosis [43–48].

PRR5L binds mTORC2 via Rictor/SIN1

Since we initially identified endogenous PRR5L as an mTORC2 binding protein in HeLa cells, we verified PRR5L expression in HEK293 cells by RT-PCR (Figure 5A). PRR5L is strongly expressed in both HeLa and HEK293 cells. Subsequent experiments were performed with HEK293 cells due to the higher transfection efficiency with these cells. To investigate whether PRR5L binds directly to mTOR or via other mTORC2 members, we examined PRR5L binding to mTOR in SIN1 knock down cells. As reported previously [18,19], we observed a reduction in the amount of Rictor when SIN1 was knocked down (Figure 5B), supporting the earlier suggestion that these two proteins stabilize each other. We found that the amount of mTOR in GST-PRR5L pull downs from the SIN1/Rictor deficient cells was substantially reduced compared to control cells (Figure 5B). This suggests that PRR5L binds to mTOR via Rictor and/or SIN1. To investigate whether PRR5L is required for mTORC2 integrity, we examined the binding of mTOR and SIN1 to Rictor in cells knocked down for PRR5L (Figure 5C). The amount of mTOR and SIN1 bound to Rictor, as measured by co-IP, remained unchanged in PRR5L knock down cells. We then investigated if PRR5L is required for mTORC2 kinase activity. Phosphorylation of Akt S473 and Paxillin Y118 is reduced upon mTORC2 disruption [14,15]. However, in PRR5L deficient cells the levels of Akt-pS473 and Paxillin-pY118 remained unchanged (Figure 5D). The findings that PRRL5 is not required for mTORC2 integrity or for phosphorylation of known mTORC2 targets suggest that PRR5L is not an mTORC2 upstream regulator or an integral component of mTORC2.

PRR5L is phosphorylated by mTOR *in vitro*

To determine whether PRR5L is a phosphorylation substrate for mTOR, we performed *in vitro* kinase assays with mTORC1 or mTORC2 and purified PRR5L. We found that PRR5L is phosphorylated by both mTORC1 and mTORC2, the former but not the latter phosphorylation being sensitive to rapamycin treatment (Figure 3A). Hence, PRR5L might be regulated through mTOR phosphorylation. PRR5L addition did not affect mTORC1 or mTORC2 autophosphorylation (Figure 3A), suggesting that PRR5L does not function as an mTORC2 inhibitor like PRAS40 for mTORC1. The significance of the phosphorylation of PRR5L by mTOR, in particular by mTORC1, remains to be determined. The above results taken together suggest that PRR5L is a downstream effector of mTORC2.

PRR5L dissociates from mTORC2 in TSC1/2 deficient cells

To investigate whether TSC1-TSC2 influences PRR5L binding to mTORC2, we examined GST-PRR5L pull downs from TSC1/2 deficient cells. TSC1/2 deficient cells exhibited reduced amounts of Rictor and mTOR bound to GST-PRR5L (Figure 6A). The above finding suggests that PRR5L dissociates from mTORC2 in cells with hyperactive mTOR signaling. It remains to be determined whether the effect of TSC1/2 knock down on PRR5L binding is via mTORC1 or mTORC2. TSC1/2 knock down hyperactivates mTORC1 [1–3] and possibly also mTORC2 (K.D.M and M.N.H., unpublished).

PRR5L promotes apoptosis

Hyperactive mTOR signaling, in TSC knock out MEFs, enhances induction of apoptosis by FCS starvation or TNFα/cycloheximide [51,52]. In agreement, we found that TSC1/2 knock down in human cells, in which PRR5L dissociates from mTORC2, also

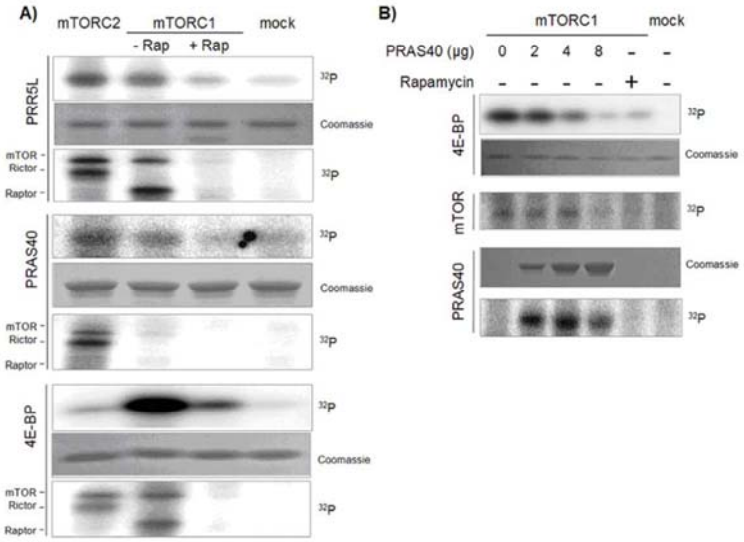

Figure 3. mTOR phosphorylates PRAS40 and PRR5L, and PRAS40 inhibits mTOR kinase activity. A. mTOR phosphorylates PRAS40 and PRR5L. Kinase assays were performed using mTORC1 or mTORC2 immunopurified from HEK293 cells, and purified PRAS40, GST-PRR5L (PRR5L) or 4E-BP as substrates. Rapamycin (100 nM) and purified FKBP12 were added directly to the reaction. Both PRR5L and PRAS40 are phosphorylated *in vitro* by both mTORCs. Phosphorylation by mTORC1 was rapamycin-sensitive. **B. PRAS40 inhibits mTORC1 kinase activity toward 4E-BP and PRAS40 itself.** Kinase assays were performed using mTORC1 immunopurified from HEK293 cells, purified 4E-BP as a substrate, and increasing concentrations of PRAS40. PRAS40 inhibits mTORC1 autophosphorylation and mTORC1 phosphorylation of 4E-BP and PRAS40, in a concentration-dependent manner.
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enhances TNFα/cycloheximide induced apoptosis (Figure 6B). To investigate whether PRR5L plays a role in apoptosis, we examined if PRR5L knock down affects TNFα/cycloheximide induced apoptosis. PRR5L knock down cells were less apoptotic compared to control cells, at various time points after TNFα/cycloheximide treatment, suggesting that PRR5L is pro-apoptotic (Figure 4A and S1). The finding that PRR5L is pro-apoptotic is consistent with the suggested role of the related protein PRR5 as tumor suppressor [40]. Furthermore, the observation that PRR5L is pro-apoptotic and is released from mTORC2 in cells with enhanced apoptosis (TSC1/2 deficient cells) suggests that released PRR5L may promote apoptosis. This in turn suggests that PRR5L is downstream of mTORC2 in mediating apoptosis. To test this possibility, we investigated whether a PRR5L deficiency still reduces apoptosis in cells knocked down for TORC2. We found that cells knocked down for both PRR5L and the mTORC2 component SIN1 were similar to cells knocked down only for PRR5L, with regard to induction of apoptosis by TNFα/cycloheximide (Figure 4C). This observation is consistent with a model in which PRR5L acts downstream of mTORC2. In particular, in response to hyperactive mTOR signaling, PRR5L may dissociate from mTORC2 to promote apoptosis. However, our data do not rule out the possibility that PRR5L controls apoptosis independently of mTORC2. It is important to note that mTORC2 also promotes cell survival via a mechanism other than tethering PRR5L. mTORC2 phosphorylates and activates Akt which then phosphorylates and inactivates the pro-apoptotic factors BAD and FOXO1/3a [19,20,36]. The above taken together suggests that either too much or too little mTOR signaling predisposes a cell to apoptosis. There seems to be a delicate balance between cell growth and cell death that may be mediated at least in part by PRR5L.

In summary, we describe two new mTOR interactors, PRAS40 and PRR5L. PRAS40 binds specifically to mTORC1 whereas PRR5L is mTORC2 specific. PRAS40 binding to mTORC1 is primarily via Raptor but also requires mTOR kinase activity. mTORC1 phosphorylates PRAS40 and this phosphorylation may contribute to the mTORC1-PRAS40 interaction. Furthermore, PRAS40 inhibits mTORC1 autophosphorylation and mTORC1 kinase activity toward its substrates 4E-BP and PRAS40. This observation extends two recent studies showing that PRAS40 inhibits mTORC1 toward its substrate S6K [38,39]. Thus, PRAS40 is an upstream negative regulator of mTORC1. We also show that PRAS40 is pro-apoptotic, but this may be an mTORC1 independent function of PRAS40. PRR5L, the new mTORC2-specific interactor, binds mTOR via SIN1 and/or Rictor. Unlike Rictor and SIN1, PRR5L is not required for mTORC2 integrity or mTORC2 kinase activity toward its downstream readouts Akt and Paxillin. Furthermore, we observed that PRR5L binding to mTORC2 is reduced in TSC1/2 deficient cells. We conclude that PRR5L dissociates from mTORC2 in cells with hyperactive mTOR signaling. We show that a TSC1/2 deficiency enhances TNFα/cycloheximide induced apoptosis. Conversely, knock down of PRR5L prevents apoptosis, even in mTORC2 deficient cells. We suggest that PRR5L is downstream of mTORC2 and is pro-apoptotic. It will be of interest to determine whether PRR5L is a tumor suppressor as suggested for the related protein PRR5 [40].

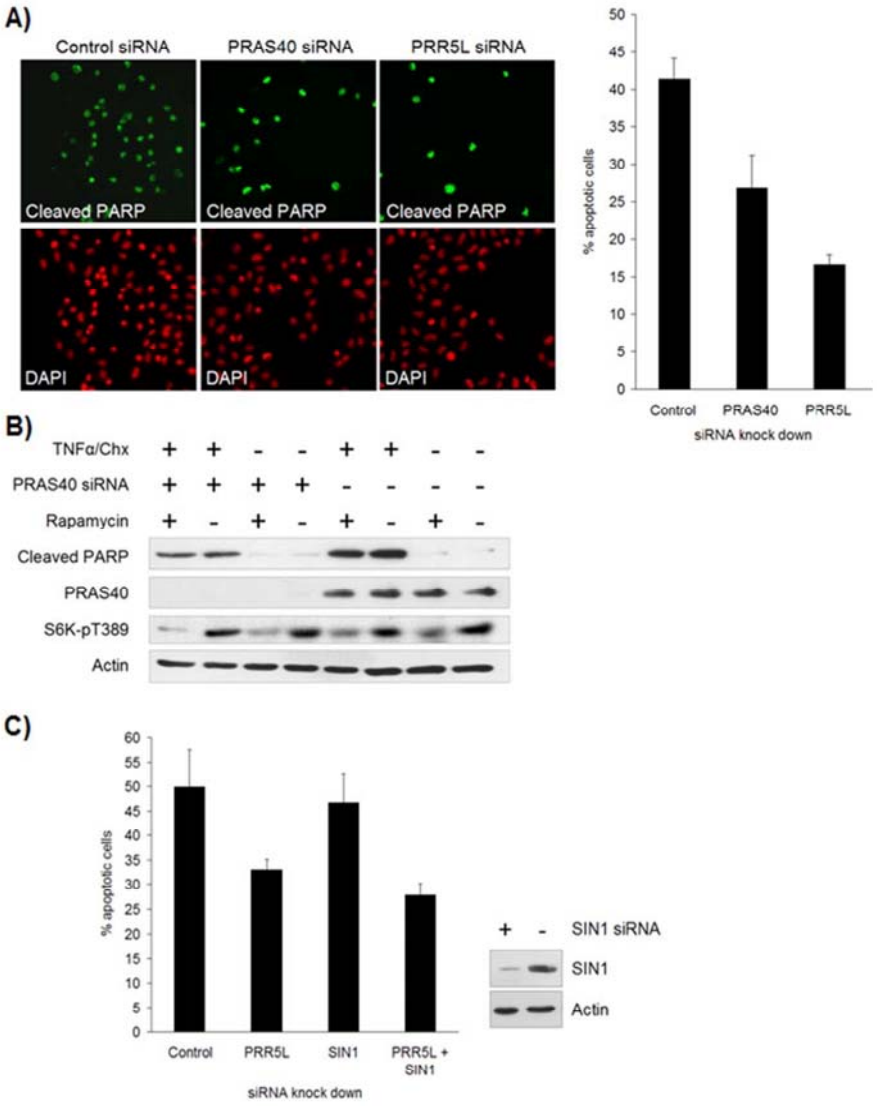

Figure 4. PRAS40 and PRR5L are pro-apoptotic. A. PRR5L and PRAS40 knock down cells are resistant to TNFα/cycloheximide induced apoptosis. HeLa cells were transfected with PRR5L, PRAS40 or control siRNA and incubated for 48 h, followed by 2 h induction of apoptosis with TNFα and cycloheximide. Cells were fixed and stained with DAPI and cleaved PARP antibody, and the percentage of apoptotic cells was quantified. **B. PRAS40's effect on apoptosis is independent of mTORC1.** HeLa cells were transfected with PRAS40 or control siRNA and incubated for 48 h, and treated with 100 nM rapamycin or carrier for 1 h before incubation with TNFα and cycloheximide for 2 h to induce apoptosis. Extracts were analyzed by immunoblotting with the indicated antibodies. **C. PRR5L deficiency protects against apoptosis in SIN1 deficient cells.** HeLa cells were transfected with diced PRR5L siRNA and/or synthetic siRNA against SIN1 as indicated, or the appropriate control siRNAs. Cells were incubated for 48 h, and apoptosis was induced with TNFα and cycloheximide for 2 h. Cells were fixed and stained with DAPI and cleaved PARP antibody, and the percentage of apoptotic cells was quantified. The efficiency of SIN1 knock down was assessed in parallel by immunoblotting (right panel).
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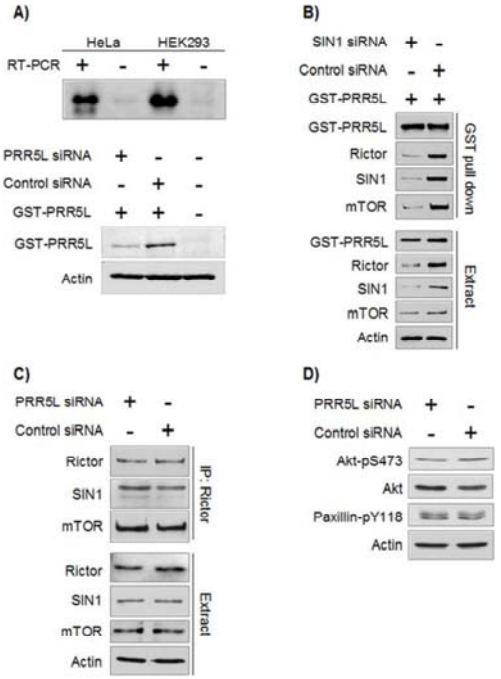

Figure 5. PRR5L binds to mTORC2 via SIN1 and/or Rictor but does not affect mTORC2 integrity or kinase activity. A. PRR5L expression in HeLa and HEK293 cells, and PRR5L knock down efficiency. Top panel: Total RNA was purified from HeLa or HEK293 cells, followed by reverse transcription and PCR with primers corresponding to PRR5L. As a negative control, reverse transcription without the transcriptase enzyme was performed. Endogenous PRR5L is expressed in both cell lines. Bottom panel: HEK293 cells were cotransfected with a GST-PRR5L vector and PRR5L siRNA or control siRNA, and incubated for 48 h. Immunoblots were performed on with antibody against GST or Actin. **B. PRR5L binds mTOR via SIN1 and/or Rictor.** HEK293 cells were cotransfected with a GST-PRR5L vector and a SIN1 siRNA vector or a control siRNA vectors, and incubated for 4 days. GST pull downs were immunoblotted with the indicated antibodies. GST-PRR5L was detected with an anti-GST antibody. mTOR binding to GST-PRR5L is weaker in the absence of SIN1 and Rictor. **C. mTORC2 remains intact in PRR5L knock down cells.** HEK293 cells were transfected with PRR5L siRNA or control siRNA and incubated for 48 h. Rictor IPs were immunoblotted with the indicated antibodies. **D. mTORC2 readouts are unaltered in PRR5L knock down cells.** HEK293 cells were transfected with PRR5L siRNA or control siRNA and incubated for 48 h. Immunoblots were performed on protein extracts with the indicated antibodies. The phosphorylation of Akt S473 and paxillin Y118 is unaltered by PRR5L knock down.
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MATERIALS AND METHODS

Screen for mTOR binding proteins and mass spectrometry

mTOR binding proteins were purified essentially as reported [14]. For each IP experiment, 6 10 cm dishes of HeLa cells at 70% confluence were used. IPs were performed with 6 μg of mTOR (Santa Cruz), Rictor or Raptor (Bethyl), or control goat (Santa Cruz) or rabbit (Bethyl) antibodies. Antibodies were bound to 300 μL magnetic Protein G coupled Dynabeads (Invitrogen). Digestion was

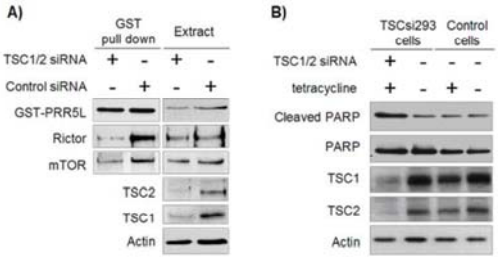

Figure 6. Analysis of TSC1/2 knock down cells. A. PRR5L is released from mTORC2 in TSC1/2 deficient cells. TSC knock down was induced in TSCsi293 cells by tetracycline treatment for 4 days. Cells were cotransfected with GST-PRR5L vector and incubated for 2 days, followed by GST pull downs and immunoblots with the indicated antibodies. GST-PRR5L was detected with an anti-GST antibody. **B. TSC1/2 knock down facilitates apoptosis.** TSCsi293 and T-REx-293 (control) cells were treated with tetracycline for 4 days. Apoptosis was induced by 1.5 h treatment with TNFα and cycloheximide. Extracts were probed with the indicated antibodies.
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performed on the beads [53] with 1 μg of Trypsin (Promega). After drying, detergents were removed by hydrophilic interaction chromatography on PolyHYDROXYETHYL TopTips (PolyLC Inc.) according to the manufacturer's instructions. Ammonium acetate remainders were removed by repeated drying of the samples.

LC-MS/MS

The peptides were analyzed by two-dimensional capillary liquid chromatography and tandem MS using a PolySULFOETHYL A ion-exchange column (0.15×50 mm, PolyLC, Columbia, MD), connected in series to a C18 trap column (Zorbax 300SB, 0.3×50 mm, Agilent Technologies, Basel, Switzerland), and to a Magic C18 separation column (0.1×100 mm, Thermo Scientific, Basel, Switzerland). The peptides were injected first onto the cation exchange column. Unadsorbed peptides were trapped on the Zorbax column and eluted onto the separation column with a linear 75 min gradient from 2 to 75% B (0.1% acetic acid in 80% acetonitrile) in solvent A (0.1% acetic acid in 2% acetonitrile). Next, peptides that had been retained by the ion-exchange column were sequentially eluted and trapped on the C18 trap column with 10 mL pulses of 50, 100, 150, 200, 250, 300, 350, 400, and 500 mM ammonium acetate, pH 3.3. Peptides eluted by each individual salt pulse were separated by the acetonitrile gradient as described above. The flow was delivered with a Rheos 2200 HPLC system (Thermo Scientific, Basel, Switzerland) at 50 mL/min. A precolumn splitter reduced the flow to approximately 500 nl/min. The eluting peptides were ionized by a Finnigan nanospray ionization source (Thermo Scientific, Basel, Switzerland). The LTQ orbitrap instrument was operated in the data-dependent mode. A survey scan was performed in the Orbitrap between m/z 400-1600 Da at 60,000 resolution. The three most abundant ions detected were fragmented in the LTQ mass spectrometer and mass analyzed in the Orbitrap at a resolution of 7,500. Singly charged ions were not subjected to fragmentation. The normalized collision energy was set to 35%. Individual MS/MS spectra were searched against the NCBI non-redundant databank using the TurboSequest software [54]. The Sequest filter parameters were as follows: Xcorr versus charge state was 1.50 for singly, 2.00 for doubly, and 2.50 for triply charged ions, respectively; the ΔCN was 0.1, and the protein probability was set to 0.01.

Plasmids and reagents

Rictor, Raptor and SIN1 siRNA constructs were previously described [14,20]. An empty pSuper-GFP-neo construct was used as a control. HA-mTOR and kinase dead HA-mTOR constructs were a kind gift from Dr. G. Thomas and were described previously [29], the control empty vector was created by cutting out the mTOR fragment using NotI and PstI.

The coding region of PRR5L was cloned from human cDNA made from HeLa cells, using the following primers: 5′ ATG ACC CGC GGC TTC G 3′ (forward, contained also restriction sites for either BamHI or SpeI), and 5′ T CAG CTG AGG GAA GCA CAG 3′ (reverse, contained also a NotI restriction site). The PCR product was digested either with BamHI and NotI or with SpeI and NotI, and cloned into pGEX-6P-1 or pEBG, respectively. Recombinant GST-PRR5L was expressed from pGEX-6P-1 and purified from *E. coli*.

The generation of the inducible TSC knock down cell line TSCsi293 from an HEK293 cell line (T-REx-293, Invitrogen), that expresses the tetracycline repressor protein TetR, and its handling were as described (K. D. M. and M. N. H., submitted). Cycloheximide was dissolved in water and used at a final concentration of 2.5 μg/mL (Calbiochem), rapamycin was dissolved in DMSO and used at a final concentration of 100 nM (LC laboratories), TNFα was dissolved in PBS containing 0.1% BSA, and used at a final concentration of 10 ng/mL (R&D systems). Purified PRAS40 was from Biosource, purified 4E-BP was from Stratagene.

RNA interference

siRNAs against PRAS40 and PRR5L and control siRNA against Luciferase were generated as described [55] using the following reagents: 5× Megascript T7 Kit (Ambion); Turbo Dicer siRNA Generation Kit (Genlantis); RNA Purification System (Invitrogen). Primers for PRAS40 were as follows: gene specific primers: forward: ttgcctccacgacatcgcac, reverse: tatttccgcttcagcttctgg, T7 primers: forward: gcgtaatacgactcactataggccacagggctgccactg, reverse: gcgtaatacgactcactataggaagtcgctggtgttaagcc. Primers for PRR5L were as follows: gene specific primers: forward: tcgtccattgtccagatgttg, reverse: agctgagggaagcacagttc, T7 primers: forward: gcgtaatacgactcactataggctcatcctgcagagtgttc, reverse: gcgtaatacgactcactataggagctccgagccctcctg. For SIN1 knock down, a synthetic pool siRNA or the appropriate control pool siRNA (Dharmacon) were used as described [19].

Cell culture and transfections

HEK293 and HeLa cells were maintained in DMEM containing 10% fetal bovine serum. Small RNAi was transfected with INTERFERin (Polyplus transfection). For combined transfection of small RNAi and DNA, jetSI-ENDO (Polyplus transfection) was used. DNA was transfected using either lipofectamin (Invitrogen) or jetPEI (Polyplus transfection). All transfections were done according to the manufacturers' instructions, for 48 h for expression or small RNAi, or for 4 days in the case of pSuper-based siRNA. Cells were harvested with lysis buffer that contained 40mM HEPES pH 7.5, 120 mM NaCl, 1 mM EDTA, 0.3% CHAPS, supplemented with protease inhibitor cocktail (Roche). In the cases where phosphorylation was to be detected, the lysis buffer was also supplemented with10 mM NaF, 10 mM NaN3, 10 mM p-nitrophenylphosphate, 10 mM sodium pyrophosphate, and 10 mM beta-glycerophosphate. Lysates were incubated for 20 minutes on ice, then cleared by a spin at 600 g for 3 minutes. Supernatants were collected and used for immunoprecipitations, GST pull downs or immunoblots.

RT-PCR

RNA was purified using the RNAeasy mini kit (Qiagen), according to the manufacturer's instructions. One microgram of total RNA was reverse transcribed using Superscript II reverse transcriptase (Invitrogen) and random nonamers (Sigma). The reverse transcription reaction was used as a template for PCR, with the PRR5L primers described above.

Immunoblotting

Protein extracts were prepared as described [14], resolved on SDS-PAGE and transferred to PVDF membranes (Immobilon-P, Millipore). Immunoblots were performed using the following antibodies: Rictor, Raptor (Bethyl); mTOR (Santa Cruz); S6K, phospho-S6K, Akt, phospho-Akt (Thr308), phospho-Akt (Ser473), PARP, cleaved PARP (Cell signaling); PRAS40 (Biosource; PRAS40 antibodies were previously described [37]); SIN1 (kind gift from Dr. Bing Su, University of Yale, CT). GST-PRR5L was detected with a GST antibody (GE Healthcare), since no antibody for PRR5L was available.

Apoptosis assay

RNAi experiments were performed by transfecting HeLa cells for 48 hours in a 96-well format. 48 hours after siRNA transfection, apoptosis was induced for the indicated times as described [56]. Apoptosis, in response to TNFα and cycloheximide treatment, was quantified by a cleaved PARP (cPARP) immunofluorescence assay (Cell Signaling). The assay was performed according to the manufacturer's instructions. Briefly, after apoptosis induction, cells were fixed with pre-chilled 100% methanol for 5min and then washed with 0.1% Triton X-100 and PBS sequentially. Cells were then incubated with anti-cPARP antibody (1/200 dilution) overnight at 4°C, washed and incubated for 1 hour with a mixed solution containing Alexa 568 goat anti-Rabbit antibody (1/500 dilution) and Hoechst (Invitrogen, 1/1000 dilution). Images were automatically taken by an ImageXpress Micro (Molecular Devices, Sunnyvale, USA). Apoptosis was quantified by automated image processing. The multi-wavelength cell scoring application module of the analysis software MetaXpress was used to quantify, at the single cell level, the intensity of cPARP staining (200 was used as the intensity above background for cPARP images). More than 6000 cells per condition were analyzed. For analysis by immunoblotting cPARP levels were detected by human or mouse specific antibodies (Cell Signaling). mTORC1 was inhibited by preincubation with 100 nM rapamycin for 1 h, and apoptosis was subsequently induced by TNFα/cycloheximide for 1.5 h in the presence of rapamycin.

Immunoprecipitation and GST pull down

Immunoprecipitations were performed as described [14]. Pull downs of GST-PRR5L were similar to immunoprecipitations, with glutathione-coupled beads (GE Healthcare).

Kinase assay

Kinase assays were performed as described [14].

SUPPORTING INFORMATION

Figure S1 Time course for apoptosis induction. HeLa cells were transfected with PRR5L or control siRNA and incubated for 48 h, followed by apoptosis induction for the indicated time spans by TNFalpha and cycloheximide. Cells were fixed and stained with cleaved PARP antibody, and the percent of apoptotic cells was quantified.

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Author Contributions

Conceived and designed the experiments: MH KT PP. Performed the experiments: KT PP MK PJ. Analyzed the data: MH KT PP MK CA. Contributed reagents/materials/analysis tools: KM AC CA. Wrote the paper: MH KT PP.

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Erklärung

Ich erkläre hiermit, dass ich diese Dissertation ‘Regulation of adipogenesis and adipose maintenance by the mammalian TOR complex 1’ nur mit der darin angegebenen Hilfe verfasst und bei keiner anderen Fakultät eingereicht habe.

Pazit Polak