Rapamycin extends life span in mice, yet paradoxically causes lipid dysregulation and glucose intolerance through mechanisms that remain incompletely understood. Whole-body energy balance can be influenced by beige/brite adipocytes, which are inducible by cold and other stimuli via β-adrenergic signaling in white adipose depots. Induction of beige adipocytes is considered a promising strategy to combat obesity because of their ability to metabolize glucose and lipids, dissipating the resulting energy as heat through uncoupling protein 1. Here, we report that rapamycin blocks the ability of β-adrenergic signaling to induce beige adipocytes and expression of thermogenic genes in white adipose depots. Rapamycin enhanced transcriptional negative feedback on the β3-adrenergic receptor. However, thermogenic gene expression remained impaired even when the receptor was bypassed with a cell-permeable cAMP analog, revealing the existence of a second inhibitory mechanism. Accordingly, rapamycin-treated mice are cold intolerant, failing to maintain body temperature and weight when shifted to 4°C. Adipocyte-specific deletion of the mTORC1 subunit Raptor recapitulated the block in β-adrenergic signaling. Our findings demonstrate a positive role for mTORC1 in the recruitment of beige adipocytes and suggest that inhibition of β-adrenergic signaling by rapamycin may contribute to its physiological effects.

Rapamycin extends life span in model organisms ranging from yeast to mice; however, its potential utility in humans remains hampered by side effects, including alterations in glucose and lipid metabolism (1). Rodents treated with rapamycin develop insulin resistance, glucose intolerance, and hyperlipidemia, often associated with changes in adiposity (2,3). In humans, the hyperlipidemic effects of rapamycin are well known (4,5), and there is a growing recognition that the drug can predispose patients to the onset of diabetes (6). The canonical target of rapamycin is mammalian/mechanistic target of rapamycin complex (mTORC)1, a kinase that regulates growth and metabolism in response to many nutrients and growth factors. However, chronic treatment with rapamycin also causes the inhibition of a second complex, mTORC2, in cells and in vivo (7–9). mTORC2 plays a direct role in the insulin signaling cascade, and mice lacking mTORC2 in the whole body display hepatic insulin resistance and glucose intolerance that is unaffected by rapamycin treatment (7). Rapamycin can further promote insulin resistance in skeletal muscle through an mTORC1-dependent mechanism (10). Interestingly, the metabolic effects of the drug evolve over time, such that animals treated for 20 weeks appear to regain insulin sensitivity, yet remain glucose intolerant (11). The causes of hyperlipidemia and changes in adiposity in rapamycin-treated animals are not fully understood. To further elucidate the metabolic effects of rapamycin in vivo, we examined its influence on the induction of beige adipocytes and thermogenic gene expression in white adipose tissue (WAT).

Adipose tissue comprises discrete depots of two main types: WAT, serving as a reservoir for excess energy, and brown adipose tissue (BAT), which generates heat in a
process known as nonshivering thermogenesis (12). Brown adipocytes are multiloculated and have a high density of mitochondria that express uncoupling protein (UCP)1. UCP1 allows protons to permeate the inner mitochondrial membrane in a manner uncoupled from ATP production, resulting in the dissipation of energy as heat. Brown adipocyte-like cells termed “beige” or “brite” (brown in white) adipocytes are inducible in WAT and were shown via lineage tracing and transcriptional profiling to represent a third class of adipocytes that are distinct from the cells found in BAT (13,14). Beige adipocytes are unevenly distributed among WAT depots, being prominent in depots such as inguinal WAT (iWAT) and virtually absent in other depots such as epididymal WAT (eWAT), and are strongly recruited upon stimulation with cold or β-adrenergic agonists. Similar to brown adipocytes, beige adipocytes are rich in UCP1 and are proposed to contribute to whole-body glucose and lipid homeostasis through their thermogenic capacity (15). Importantly, rodents that lack beige adipocyte function are predisposed to metabolic dysfunction (16), and conditions that induce beige adipocytes protect against weight gain (17,18). As a chronic imbalance between calorie intake and expenditure is the fundamental cause of obesity, expansion and activation of beige adipocytes is an attractive strategy to combat obesity and alleviate associated disorders including type 2 diabetes and cardiovascular disease (19–22).

In the current study, we tested whether rapamycin affects whole-body metabolic homeostasis by influencing the induction of beige adipocytes and thermogenic gene expression. We show that pretreatment with rapamycin strongly attenuates the induction of beige fat–associated genes, including Ucp1, by the β3-adrenergic receptor agonist CL316,243 (CL). These effects are recapitulated in primary adipocytes and by genetic ablation of mTORC1 in adipocytes in vivo. Moreover, rapamycin-treated mice failed to maintain body temperature, weight, and blood glucose when exposed to cold and were glucose intolerant when challenged. Our data reveal that rapamycin suppresses the induction of beige adipocytes in WAT and impairs thermogenesis, which may contribute to its detrimental effects on metabolism.

RESEARCH DESIGN AND METHODS

Animals and Treatments

All experiments were approved by the Institutional Animal Care and Use Committees at the University of Pennsylvania. Eight- to 10-week-old male C57BL/6N mice from Taconic were injected once daily with 2 mg/kg i.p. rapamycin (553210; Calbiochem) suspended in 0.9% NaCl and 2% ethanol at a concentration of 0.5 mg/mL or vehicle for 2 weeks and 1 mg/kg body wt β3-adrenergic receptor agonist CL (Sigma). CL was diluted to 0.1 mg/mL in saline and injected at a volume of 10 μL/g body wt 24 h prior to sacrifice. For acute experiments, rapamycin was injected 1 h before injection with CL. For dietary rapamycin treatment, 6- to 8-week-old male or female mice were fed ad libitum a diet containing 42 ppm encapsulated rapamycin (Rapamycin Holdings International) in LabDiet 5LG6 (PMI Nutrition International) or a matched control diet for 2 weeks at room temperature as described (23) before injection with CL. Another group of mice were fed the 42 ppm rapamycin diet or matched control for 2 weeks at thermoneutrality (TN) (30°C) before the cold groups were moved to 4°C (2–3 animals per cage). A glucose tolerance test was performed after 72 h of cold exposure, and the mice then were allowed to recover in their respective TN or 4°C housing conditions for 24–48 h prior to sacrifice. Adipocyte-specific Raptor knockout mice (Raptorfl/flAdipoCre) were generated by crossing Raptor floxed mice (24) with adiponectin-Cre mice (25).

Preadipocyte Isolation and Differentiation

iWAT depots were isolated from wild-type C57BL/6N mice at 6–8 weeks of age. Tissues were minced and digested with Collagenase D (Roche) at 6.1 mg/mL and Dispase II (Roche) at 2.4 mg/mL in DMEM at 37°C for 30–40 min. The digested mixture was then mixed with an equal volume of DMEM containing 10% FBS and penicillin/streptomycin, passed through a 100-μm filter, and centrifuged at 500g for 5 min. The stromal vascular fraction was resuspended in DMEM with 10% FBS and penicillin/streptomycin and grown until 90–95% confluent. The media was then replaced with differentiation medium (DMEM with 10% FBS, 20 mmol/L HEPES, penicillin/streptomycin, 20 nmol/L insulin, 1 nmol/L T3, 125 μmol/L indomethacin, 500 nmol/L dexamethasone, 0.05 mmol/L isobutylmethylxanthine [T5516; Sigma], and 1:1,000 Primocin) for 48 h before being returned to DMEM with 10% FBS, 20 mmol/L HEPES, and penicillin/streptomycin for 5–6 days until fully differentiated. An immortalized cell line derived from the stromal vascular fraction of iWAT from wild-type mice of a mixed 128sv/C57B6 genetic background was used and differentiated following the protocol above. Fully differentiated adipocytes were treated with 500 nmol/L rapamycin (Selleckchem) for 24 h or H-89 (Cayman Chemical) for 1 h before addition of either 1 μmol/L CL or 1 mmol/L cAMP analog (8-Br-cAMP) (Sigma) for 24 h.

Glucose Tolerance Tests

Mice were injected with 1.5 g/kg i.p. d-glucose after a 5-h fast. Tail vein blood glucose was measured with the AlphaTrakII meter over 2 h.

Quantitative Real-Time RT-PCR Analysis

Total RNA was extracted using TRizol. RNA (1 μg) was used to generate cDNA with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Reactions were run on a 7900HT Fast Real-Time PCR System (Applied Biosystems) using SYBR Green Master Mix (Applied Biosystems) with results normalized to 36B4 or Tbp.

Western Blot Analysis

Cells and tissue samples were lysed in radioimmunoprecipitation assay buffer supplemented with phosphatase...
(Roche) and protease inhibitors (Roche). Protein concentration was determined by the Pierce BCA Protein Assay Kit. Total protein was resolved by SDS-PAGE on 8–16% Tris-glycine gradient gel or 10% resolving gels (Bio-Rad) and transferred to polyvinylidine fluoride membranes (Bio-Rad). Membranes were blocked with 5% nonfat dry milk in Tris-buffered saline with Tween (50 mmol/L Tris, pH 7.6, 150 mmol/L NaCl, and 0.1% Tween 20) and incubated overnight at 4°C in 3% nonfat dry milk in Tris-buffered saline with Tween with the following primary antibodies at 1:1,000: UCP1 (ab10983; Abcam), phospho-S6 ribosomal protein (2215; Cell Signaling Technology) and S6 ribosomal protein (2217; Cell Signaling Technology), phospho-cAMP-dependent protein kinase (PKA) substrate (9624; Cell Signaling Technology), phospho-CREB (9198; Cell Signaling Technology), β3-adrenergic receptor (R&D Systems), and β-actin (Abcam). Immunolabeling was detected using the Femto ECL reagent (Pierce). Quantification was performed using Quantity One and normalized to β-actin (Bio-Rad).

**Histology/Immunohistochemistry**

Isolated adipose tissue was fixed in 4% paraformaldehyde in PBS for 24 h prior to embedding in paraffin. Sections were deparaffinized in xylene, rehydrated through graded ethanol, and stained with hematoxylin–eosin. Sections were incubated with anti-UCP1 antibody (1:1,000) (ab10983; Abcam) at 4°C overnight and then with biotinylated anti-rabbit secondary antibody (1:1,000) (ab10983; Abcam) at 4°C overnight and then incubated overnight at 4°C in 3% nonfat dry milk in Tris-buffered saline with Tween with the following primary antibodies at 1:1,000: UCP1 (ab10983; Abcam), phospho-S6 ribosomal protein (2215; Cell Signaling Technology) and S6 ribosomal protein (2217; Cell Signaling Technology), phospho-cAMP-dependent protein kinase (PKA) substrate (9624; Cell Signaling Technology), phospho-CREB (9198; Cell Signaling Technology), β3-adrenergic receptor (R&D Systems), and β-actin (Abcam). Immunolabeling was detected using the Femto ECL reagent (Pierce). Quantification was performed using Quantity One and normalized to β-actin (Bio-Rad).

**Statistical Analysis**

All data are presented as mean ± SEM and analyzed using Prism (GraphPad). Statistical significance was determined using the unpaired two-tailed Student t test for single comparisons and one-way ANOVA with post hoc t tests for multiple variables.

**RESULTS**

**Two Weeks of Dietary Rapamycin Attenuates CL-Induced Thermogenic Gene Expression in White Adipose Tissue**

To test whether rapamycin affects the recruitment of beige adipocytes in WAT, we fed male C57BL/6N mice for 2 weeks with a diet containing 42 ppm rapamycin that was previously shown to increase longevity (26) and then injected the β3-adrenergic receptor agonist CL (1 mg/kg) 24 h before sacrifice. Thermogenic genes Ucp1, Cidea, Elov13, and Pgc-1α were robustly induced by CL at the mRNA level in iWAT and eWAT. This effect was significantly reduced by rapamycin (Fig. 1A and B). Similar results were obtained when rapamycin was injected intraperitoneally (7) (Fig. 1C). In contrast, rapamycin did not affect the CL-induced increase in Ucp1 expression in BAT (Fig. 1D). The CL-induced increase in UCP1 protein was also blocked in WAT from mice fed dietary rapamycin (Fig. 1E and F). CL injection caused activation of mTORC1 signaling, as evidenced by S6 (Ser240/244) phosphorylation, and this effect was blocked in rapamycin-fed mice. To establish whether these changes resulted in physiologically relevant effects on respiratory capacity, we measured oxygen consumption in adipose tissue homogenates from mice treated with CL in the presence or absence of rapamycin. We found that CL treatment enhanced the respiratory capacity of iWAT and decreased the respiratory capacity in rapamycin-treated mice (Fig. 1G). While there was a slight trend toward decreased respiratory capacity in BAT of rapamycin-treated mice, this effect did not reach statistical significance (Fig. 1H). Histologically, CL injection induced the formation of multilocular beige adipocytes in the iWAT within 24 h, as shown in the inset in Fig. 1I, an effect that was suppressed by rapamycin (Fig. 1I). In contrast, rapamycin did not prevent CL-induced inflammation, a well-known side effect (27), in the eWAT (Fig. 1J) or depletion of lipid droplets from BAT (Fig. 1K). Together, these results indicate that rapamycin suppresses the CL-mediated induction of beige adipocytes in WAT.

**Acute Rapamycin Injection Partially Blocks CL-Induced Thermogenic Gene Expression**

Acute rapamycin treatment inhibits mTORC1, whereas chronic treatment has also been shown to suppress mTORC2 (7–9). To determine the role of impaired mTORC1 signaling in the inhibition of beiging, we administered rapamycin to mice as a single injection 1 h before CL treatment, a time point at which only mTORC1 activity would be affected. CL induced an increase in thermogenic gene expression that was partially suppressed by rapamycin treatment in both iWAT and eWAT (Fig. 2A and B). Although the effect on Ucp1 mRNA was not statistically significant in iWAT, UCP1 protein was reduced by the acute dose of rapamycin (Fig. 2C and D). Continued suppression of mTORC1 was confirmed by the lack of S6 phosphorylation, whereas phosphorylation of Akt at
Figure 1—Dietary rapamycin (Rapa) attenuates CL-induced thermogenic gene expression in WAT. A and B: Expression of thermogenic genes, *Ucp1*, *Elovl3*, *Cidea*, and *Pgc-1α*, in iWAT (A) and eWAT (B) of mice given rapamycin or control (Ctr) diet for 2 weeks before CL injection (n = 4–7). C: Expression of thermogenic genes, *Ucp1*, *Elovl3*, and *Cidea*, in eWAT of mice treated with rapamycin intraperitoneally (IP-Rapa) for 2 weeks and then injected with CL (n = 4–5). D: *Ucp1* gene expression in BAT of mice fed rapamycin or control diet for 2 weeks before CL injection (n = 4–5). E: Densitometric analysis of the UCP1 protein expression in iWAT of mice on rapamycin or control diet for 2 weeks prior to CL injection. F: UCP1 protein expression and mTORC1 signaling (pS6) in iWAT of mice fed dietary rapamycin for 2 weeks before CL injection. G and H: *O2* consumption by whole-tissue homogenates of iWAT (G) and BAT (H) (n = 4–7). I–K: Representative hematoxylin-eosin–stained sections of iWAT (I), eWAT (J), and BAT (K) from mice fed control or rapamycin diet for 2 weeks before CL injection. Scale bar: 200 μm. Values shown are mean ± SEM. *P < 0.05 compared with control, #P < 0.05 compared with CL, %P < 0.05 compared with rapamycin.
Ser\textsuperscript{473} (an mTORC2 site) was not affected (Fig. 2C). These experiments indicate that inhibition of mTORC1 contributes to the effect of rapamycin on the beiging of WAT.

**Rapamycin Modulates Acute Responses to CL**

We observed that 24 h of CL treatment consistently resulted in negative transcriptional feedback on the β3-adrenergic receptor (Adrb3) in multiple adipose depots and that rapamycin exacerbated this effect (Fig. 3A). It is possible that the suppressive effects of rapamycin on thermogenic gene expression might reflect a decrease in ADRB3, and CL acutely induces lipolysis and increases serum glucose and insulin in a manner dependent on the expression of Adrb3 in adipose tissue (28). To determine whether rapamycin modulated these effects, we measured serum FFAs over the first hour after CL injection. We found that CL-induced lipolysis was significantly attenuated by rapamycin treatment (Fig. 3B). CL has also been shown to induce an acute increase in insulin and a fall of glucose below baseline levels. This was observed in control animals injected with CL, but rapamycin-treated animals displayed an exaggerated glucose spike (Fig. 3C) even with a normal or slightly greater insulin response (Fig. 3D). These acute responses to CL are consistent with partially attenuated ADRB3/PKA signaling in combination with preexisting insulin resistance and glucose intolerance in the rapamycin-treated group.

**Rapamycin Attenuates Thermogenic Gene Expression in Primary Adipocytes**

We next determined whether the suppression of CL-induced thermogenic gene expression by rapamycin was cell autonomous. Fully differentiated primary adipocytes were pretreated with rapamycin for 24 h before stimulation with CL for an additional 24 h. CL strongly induced expression of Ucp1 and Elovl3, and pretreatment with rapamycin decreased this response (Fig. 4A). To test whether the inhibitory effect of rapamycin occurred at the level of receptor expression, we treated cells with a cAMP analog (1 mmol/L 8-Br-cAMP) to bypass ADRB3. Rapamycin suppressed thermogenic gene expression in response to increased cAMP, indicating that an inhibitory mechanism acts downstream of the receptor (Fig. 4B).
Additionally, negative feedback on ADRB3 expression after CL or cAMP treatment was recapitulated in vitro (Fig. 4C and D). To further investigate the interaction between mTORC1 and β-adrenergic signaling, we tested whether PKA activation was affected by rapamycin treatment using an iWAT-derived cell line. CL stimulation increased phosphorylation of PKA substrates, CREB, and S6. In the presence of rapamycin, CL-stimulated phosphorylation of PKA substrates and CREB occurred normally; however, phosphorylation of S6 was completely prevented when PKA was inhibited (Fig. 4E). These findings place mTORC1 downstream of PKA.

**Two Weeks of Rapamycin Partially Suppresses Cold-Induced Beige Fat Gene Expression**

Next, we tested whether rapamycin blocks induction of thermogenic genes in response to cold. Mice were fed control or rapamycin diets and adapted to TN (30°C) for 2 weeks before exposure to 4°C for 96 h. Cold exposure induced expression of Ucp1, Elovl3, and Cidea in iWAT (Fig. 5A) and eWAT (Fig. 5B). Induction of thermogenic genes by cold in iWAT and eWAT was decreased with rapamycin treatment (Fig. 5A and B). As with CL treatment, rapamycin did not prevent the cold-induced increase in Ucp1 expression in BAT (Fig. 5C). UCP1 protein was induced in iWAT by cold exposure, and the effect was attenuated by rapamycin treatment (Fig. 5D and E). This correlated with reduced phosphorylation of S6 as well as mTOR itself at Ser2448. Attenuation of PGC-1α transcription by rapamycin suggested that mitochondrial biogenesis might be decreased in adipose tissue, consistent with reduced expression of several mitochondrial proteins in the presence of rapamycin (Fig. 5F). Exacerbation of cold-induced negative feedback on ADRB3 expression was evident in iWAT, eWAT, and BAT of rapamycin-treated animals (Fig. 5G), and a decrease in ADRB3 protein was confirmed in iWAT (Fig. 5D and H). Despite partial induction of UCP1 in rapamycin-treated animals, phosphorylation of S6 was completely blocked, suggesting that mTORC1 signaling amplifies, but is not essential for, the beiging response during cold exposure or that its requirement is only transient.

The effects of cold on iWAT morphology are attenuated by rapamycin. At TN, the adipocytes in iWAT of both control and rapamycin-treated mice contain large unilocular lipid droplets. As expected, the iWAT of cold-exposed

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**Figure 2**—Acute rapamycin (Rapa) treatment partially blocks CL-induced thermogenic gene expression. A and B: Expression of thermogenic genes, Ucp1, Elovl3, Cidea, and Pgc-1α, in iWAT (A) and eWAT (B) of mice given an intraperitoneal injection of 2 mg/kg rapamycin or saline 1 h before CL injection. C: UCP1 protein expression and mTORC1 signaling (pS6) in iWAT from this experiment. pAKT, phosphorylated Akt. D: Densitometric analysis of the UCP1 protein expression. Values shown are mean ± SEM. n = 4–6. *P < 0.05 compared with control (Ctr), #P < 0.05 compared with CL, %P < 0.05 compared with rapamycin. hr, hour.
mice exhibited many multilocular beige adipocytes interspersed between the unilocular cells (Fig. 5I). While cold exposure still induced distinct morphological changes in the presence of rapamycin, there was a clear decrease in the number of multilocular cells compared with cold controls (Fig. 5I).

UCP1 was undetectable in most regions of the iWAT in both control and rapamycin-treated mice at TN (Fig. 5J), whereas cold-exposed mice showed a robust increase in UCP1 that was most prominent in the regions of beige fat. Interestingly, UCP1 staining even within regions containing multilocular adipocytes appeared to be decreased by rapamycin treatment (Fig. 5J).

**Rapamycin Decreases Cold Tolerance**

While all mice that were transferred to 4°C exhibited a decrease in body temperature, this effect was exaggerated by prior rapamycin treatment (Fig. 6A). Similarly, cold-exposed rapamycin-treated mice lost a higher percentage of body weight during the cold challenge compared with control mice (Fig. 6B).

Cold exposure has been reported to improve glucose tolerance in rats (29,30). To test whether this holds true in mice and whether the effect is influenced by rapamycin treatment, we performed glucose tolerance tests after 72 h of cold challenge. Cold exposure reduced fasting glucose levels in control mice, and the effect was more pronounced in rapamycin-treated animals (Fig. 6C). Thus, we adjusted for baseline levels to determine the glucose excursion (Fig. 6D). Area under the glucose curve is significantly improved by cold in control mice yet remains unchanged by cold in rapamycin-treated mice (Fig. 6D). Interestingly, while rapamycin-treated animals tend to be less glucose tolerant than controls even at TN (P = 0.12) (Fig. 6D), the effect is far stronger at 4°C (P = 0.002) (Fig. 6D). Rapamycin did not affect food intake at TN; however, the hyperphagic response to cold was attenuated (Fig. 6E). Notably, the dose of rapamycin may have increased slightly in the cold-exposed group owing to the increase in food intake by ~16%. In addition, it has been reported that impairment of nonshivering thermogenesis

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**Figure 3**—Rapamycin (Rapa) modulates the acute response to CL. **A**: Expression of β3-adrenergic receptor (Adrb3) in iWAT, eWAT, and BAT of mice given dietary rapamycin 2 weeks before CL injection. **B–D**: Mice given control (Ctr) or rapamycin diet for 2 weeks were injected with CL at time 0, as denoted by the arrow. FFA (B), glucose (C), and insulin (D) were monitored over the next hour after CL injection. Values shown are mean ± SEM. n = 4. *P < 0.05 compared with control, #P < 0.05 compared with CL, %P < 0.05 compared with rapamycin.
can lead to weight loss as a result of the energetic requirements of compensatory shivering (31), although others have challenged this view (32). To determine whether such a mechanism contributes to weight loss in rapamycin-treated mice, we measured the plasma creatine kinase activity (a surrogate marker for shivering) and found that while control mice show an increase in the cold, rapamycin-treated mice fail to show a commensurate increase (Fig. 6). These experiments demonstrate that rapamycin treatment renders mice intolerant to cold and diminishes the beneficial effects of cold on glucose homeostasis.

**Adipocyte-Specific Ablation of mTORC1 Impairs β-Adrenergic Signaling**

While our acute rapamycin experiments (Fig. 2) suggested a requirement for mTORC1 in beiging, a previous study concluded that mice with adipocyte-specific loss of the mTORC1 subunit Raptor had enhanced beiging under basal conditions (33). To clarify the role of mTORC1 in this system, we generated Raptor^fl/fl^Adipo^Cre^ mice (Fig. 7A). Loss of Raptor in adipocytes prevented induction of Ucp1 and other thermogenic genes in response to CL injection (Fig. 7B and D). In fact, expression of Ucp1 in these mice was lower after CL treatment than before, yet we also confirmed the observation that basal expression of the thermogenic genes is elevated in the absence of mTORC1 activity. Similarly, we found that while Raptor^fl/fl^Adipo^Cre^ mice display increased basal level serum FFAs, they fail to upregulate lipolysis in response to CL treatment (Fig. 7E). Histologically, mice with adipocyte-specific deletion of Raptor displayed variation in adipocyte size and localized regions of remodeling in response to CL treatment (Fig. 7F) in the absence of Ucp1 induction.

**DISCUSSION**

A better understanding of the causes of the metabolic complications induced by rapamycin will be critical to designing safer alternatives or dosing regimens in order to translate its beneficial effects from rodent models to...
humans. Here, we demonstrate that rapamycin impairs the induction of thermogenic gene expression and recruitment of beige adipocytes in WAT in response to a β3-adrenergic receptor agonist or cold exposure.

Our results clearly demonstrate that signaling through mTORC1 is required for induction of thermogenic genes by CL or cold, although several reports have suggested the opposite effect. Mice lacking mTORC1 activity in adipocytes based on Ap2-Cre have been reported to have increased basal expression of thermogenic genes (33), and mice with hyperactive mTORC1 in adipocytes secondary to Grb10 deletion have impaired thermogenic gene expression that, in BAT, is partially restored by rapamycin treatment (34). Importantly, we observed a dramatic effect of rapamycin on thermogenic gene expression only upon stimulation with CL or cold, neither of which was previously tested in animals lacking adipocyte mTORC1. Interestingly, we found that mice lacking adipocyte mTORC1 activity were insensitive to β-adrenergic signaling, yet displayed mild induction of thermogenic genes in the absence of stimulation as previously reported. The mechanism accounting for this effect will be an interesting area for future work. In the case of Grb10 deletion, Liu et al. (34) have proposed that mTORC1 mediates its
own inhibition via Grb10 phosphorylation, which suggests that perhaps a wave of mTORC1 signaling may be required to initiate the beiging response, after which the signal must be appropriately terminated. Experiments in tuberous sclerosis complex 1–null animals support the contention that mTORC1 hyperactivation “whitens” brown fat in a rapamycin-sensitive manner (35). This may in part reflect differences between white/beige and brown adipocytes, since the response of Ucp1 to CL was largely intact in brown adipocytes in our studies (Fig. 1D), while clearly inhibited in WAT. The distinct effects in different adipose depots observed may also be related to the fact that beiging in WAT requires a change in cell fate either by transdifferentiation or by de novo adipogenesis. Clearly, further studies will be required to fully elucidate the role(s) of mTORC1 in this process.

Our results provide support for two different modes of inhibition of β-adrenergic signaling by rapamycin. We provide direct support for a requirement for mTORC1 activity at or below the level of PKA, and we also observe that rapamycin synergistically enhances transcriptional feedback on Adrb3 expression. Acute rapamycin treatment has a greater effect on UCP1 protein than mRNA expression, which could indicate a translational or other posttranscriptional effect. Interestingly, acute rapamycin treatment fails to block induction of Elovl3 and Cidea, whereas longer-term treatment blocks induction of all of the genes examined. While the reason for this discrepancy is not immediately clear, one possibility is that expression of Elovl3 and Cidea is not directly dependent on mTORC1 but becomes diminished only after ADRB3 protein levels fall. It is also interesting that Adrb3 expression is reduced in the BAT of mice treated with rapamycin and exposed to cold, yet induction of Ucp1 remains unimpaired (Fig. 5C). We speculate that this decrease can be tolerated because basal ADRB3 protein in BAT is very high but that more prolonged dosing or chronic stimulation might reveal a defect. In any case, it will be important to test whether chronic reductions in Adrb3 expression contribute to the consequences of rapamycin in vivo and to determine whether other β-adrenergic receptors and other tissues display similar behavior.
Figure 6—Two weeks of rapamycin (Rapa) diet results in decreased cold tolerance. 

A: Core body temperature of mice fed control (Ctr) or rapamycin diet for 2 weeks at TN (30°C) was recorded by rectal probe during the first 72 h after mice were shifted to cold conditions (4°C) (n = 14–18).

B: Changes in body weight over 72 h of cold challenge (n = 14–18).

C: Glucose levels after a 5-h fast in mice fed control or rapamycin diet at TN or after 72 h of cold exposure. D: Glucose excursion during a glucose tolerance test. Inset: Area under the glucose excursion curves (AUC) (n = 12–17).

E: Food intake (n = 9).

F: Serum creatine kinase activity (n = 6–8). Values shown are mean ± SEM. *P < 0.05 compared with TN, #P < 0.05 compared with 4°C, %P < 0.05 compared with rapamycin.
Figure 7—Adipocyte-specific ablation of mTORC1 blocks the ability of β-adrenergic signaling to induce thermogenic genes. A: Expression of Raptor mRNA and protein in the iWAT of Raptorfl/fl and Raptorfl/flAdipoCre mice (n = 5–6). B: Ucp1 expression in iWAT and eWAT of Raptorfl/fl and Raptorfl/flAdipoCre mice 24 h after CL injection (n = 7–11). C and D: Expression of beige-fat-associated genes, Elovl3, Cidea, and Pgc-1α, in iWAT (C) and eWAT (D). E: Basal serum FFAs and responses to CL injection in Raptorfl/flAdipoCre mice (n = 5–7). F: Representative sections of iWAT stained with hematoxylin-eosin. Values shown are mean ± SEM. *P < 0.05 compared with Raptorfl/fl, #P < 0.05 compared with CL, %P < 0.05 compared with Raptorfl/flAdipoCre.
Adipocyte-specific deletion of Raptor confirmed that mTORC1 is required for beiging in response to CL yet raised two additional questions. Puzzlingly, deletion of Raptor, but not treatment with rapamycin, causes a mild beiging response under unstimulated conditions. Since the Raptor deletion occurs at adipocyte maturation, it is possible that this reflects a compensatory response initiated in early life or a chronic process that would become apparent only after months of rapamycin treatment. Acute deletion of Raptor using a tamoxifen-inducible system would be an important step toward resolving these possibilities. The second unexpected result is the reversal of CL’s effects such that some transcripts, including Ucp1, are decreased by CL in the absence of Raptor. While this may appear to be related to the high basal expression of these transcripts in Raptor knockout mice, closer examination reveals the same trend in respiratory capacity of iWAT and in the expression of Cidea and Pgc-1α from rapamycin-treated mice, which do not have elevated baselines (Fig. 1). Teasing apart these complex relationships will be an important goal for future work.

In line with the inhibition of β-adrenergic signaling, rapamycin blunted FFA release in CL-injected mice. Consistently, Fang et al. (11) observed lower plasma glycerol and FFA after rapamycin treatment. However, isoproterenol-induced lipolysis has been reported to increase with rapamycin in 3T3-L1 adipocytes or in fat pads treated with rapamycin ex vivo (36). This difference is unlikely to reflect mTORC2 inhibition by chronic treatment in our mice, since ablation of mTORC2 in adipose has also been reported to enhance lipolysis (37). One complicating factor is insulin resistance in the presence of rapamycin, since insulin signaling dampens lipolysis via activation of phosphodiesterase 3B (38). Consistent with impaired suppression of lipolysis by insulin, we have observed increased FFA in rapamycin-treated animals after a fasting/refeeding regimen (data not shown), despite the attenuated response to CL. Importantly, stimulation of lipolysis by CL appears to be completely prevented in adipocyte-specific Raptor knockout mice despite normal or elevated FFA under basal conditions, again suggesting that mTORC1 disruption impairs β-adrenergic signaling specifically.

Prior studies have suggested relationships between β-adrenergic signaling and/or cAMP and the mTOR complexes. For example, cAMP promotes mTORC1 activity in thyroid cells (39) and β-cells (40), whereas it disrupts both mTORC1 and mTORC2 in MEFs (41) and, via the downstream effects on lipolysis, in white adipocytes (42). In brown adipocytes, β-adrenergic signaling promotes translocation of GLUT1 to the plasma membrane in an mTORC2-dependent, but cAMP-independent, manner, whereas in muscle cells, β-adrenergic signaling activates mTORC2 via PKA. Therefore, there are likely multiple interactions between β-adrenergic signaling and the mTOR complexes that change based on cell type, stimulus, and environment.

We speculate that rapamycin’s suppressive effects may have been less robust in the cold owing to the lower body temperature (i.e., stronger stimulus) in rapamycin-treated animals and that cold activates many redundant signaling pathways besides those linked to β3-adrenergic receptor activation. A number of hormones, including irisin, fibroblast growth factor 21, and Meteorin-like 1, are inducible by cold and may have some effects on beiging that are independent from β3-adrenergic signaling (43–45). Moreover, a direct response of adipocytes to changes in ambient temperature was demonstrated to be independent of the classical cAMP/PKA signaling pathway downstream of β-adrenergic receptors (46). That the block in CL responses was much more robust indicates that rapamycin may have specific suppressive effects on the β3-adrenergic receptor signaling pathway. Importantly, rapamycin-treated mice did not exhibit improved glucose tolerance after cold exposure and were cold intolerant, failing to maintain body weight or temperature at 4°C. Since typical housing conditions (22°C) represent a mild cold stress for mice, the failure of cold adaptation mechanisms may play an important role in the metabolic consequences of rapamycin.

It is interesting to consider whether the attenuation of beige fat alone is sufficient to explain the cold intolerance of rapamycin-treated mice. Rapamycin blocked the ability of CL, a stimulator of beige fat formation, to enhance respiratory capacity in iWAT, and relatively specific loss of beige fat has been shown to have detrimental effects on metabolism (16). However, the respiratory capacity of BAT was 5- to 10-fold greater in our hands and was only mildly impaired by rapamycin, suggesting that significant capacity for BAT-mediated nonshivering thermogenesis might still remain in cold-challenged, rapamycin-treated animals. In the future, it will be important to test whether these trends in adipose tissue respiratory capacity are maintained in animals that have been exposed to cold. Interestingly, we did find that rapamycin treatment diminished the shivering response as well as cold-induced hyperphagia (Fig. 5E and F). The latter likely contributes to weight loss and the reduced ability to maintain fasting glucose, and we suspect that the cold intolerance of rapamycin-treated animals is likely the net effect of reduced nonshivering thermogenesis and impaired compensatory mechanisms.

Overall, our studies reveal an inhibitory effect of rapamycin on the β-adrenergic signaling cascade that recruits beige adipocytes in WAT, which may contribute to the detrimental effects of rapamycin on metabolism. In contrast to the prevailing view, we demonstrate that mTORC1 plays a positive role in the induction of beige adipocytes by β-adrenergic signaling, acting downstream of PKA and on expression of Adbb3. While this article was in press, an independent report was published that confirms our central finding of a positive role for mTORC1 signaling in the beiging of white fat (47). Future experiments will further delineate the specific signaling mechanisms.
involved, which may open up new therapeutic avenues to promote beiging of WAT and avoid the detrimental side effects of rapamycin in humans.

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