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## Calorie restriction-induced insulin sensitivity is mediated by adipose mTORC2 and not required for lifespan extension

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### SUMMARY

Calorie restriction (CR) extends the healthspan and lifespan of diverse species. In mammals, a broadly conserved metabolic effect of CR is improved insulin sensitivity, which may mediate the beneficial effects of a CR diet. This model has been challenged by the identification of interventions that extend lifespan and healthspan, yet promote insulin resistance. These include rapamycin, which extends mouse lifespan yet induces insulin resistance by disrupting mTORC2 (mechanistic Target Of Rapamycin Complex 2). Here, we induce insulin resistance by genetically disrupting adipose mTORC2 via tissue-specific deletion of the mTORC2 component *Rictor* (AQ-RKO). Loss of adipose mTORC2 blunts the metabolic adaptation to CR, and prevents whole-body sensitization to insulin. Despite this, AQ-RKO mice subject to CR experience the same increase in fitness and lifespan on a CR diet as wild-type mice. We conclude that the CR-induced improvement in insulin sensitivity is dispensable for the effects of CR on fitness and longevity.

### INTRODUCTION

Calorie restriction (CR), a dietary regimen in which calories are reduced without causing malnutrition, extends the lifespan of many diverse species and is the gold standard for interventions that promote the health and longevity of mammals (Lamming and Anderson, 2014; Madeo et al., 2019). Importantly, CR not only extends longevity, it extends

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#### AUTHOR CONTRIBUTIONS

D.Y. and D.W.L. conceived the experiments. D.W.L. secured funding. D.Y., J.L.T., S.E.Y., B.R.M., M.H.W., D.S.S., N.E.C., E.L.B., J.A.B., F.A.S., and D.W.L. performed the experiments. D.Y., D.S.S., E.L.B. and D.W.L. analyzed the data. D.Y. and D.W.L. wrote the manuscript.

healthspan, decreasing the incidence of age-related diseases including cardiovascular disease, neurodegenerative disorders, cancer, and diabetes, and in addition reduces frailty (Balasubramanian et al., 2017; Kane et al., 2016). There has therefore been great interest in identifying the physiological and molecular mechanisms by which CR promotes health and longevity (Fontana and Partridge, 2015; Lopez-Lluch and Navas, 2016; Speakman et al., 2016).

In mammals fed a CR diet, one of the most striking and broadly conserved effects is improved sensitivity to insulin (Lamming and Anderson, 2014; Most et al., 2017). Many dietary and pharmaceutical interventions that extend mammalian lifespan and healthspan likewise promote insulin sensitivity, while conversely there is a well-known association of insulin resistance with diabetes and poor health. Given the central role of the insulin signaling pathway in the lifespan of worms, flies, and mammals (Kenyon, 2001; Lamming, 2014), improved insulin sensitivity has been proposed as an essential mechanism by which a CR diet extends mammalian lifespan (Lamming and Anderson, 2014). While the effects of CR are systemic, some of its most prominent effects are on adipose tissue; CR reduces adiposity in mammals, mobilizing fat stores in white adipose tissue (WAT) while also activating WAT lipogenesis, which is associated with improved systemic insulin sensitivity and metabolic health (Bruss et al., 2010; Chen et al., 2008; Herman et al., 2012).

Despite the strong correlative evidence that CR promotes health and longevity through improved insulin sensitivity, there is clear evidence that insulin sensitivity may not necessarily be essential for healthy aging. Several genetically modified mouse models in which insulin resistance has been induced in one or more tissues have extended lifespan (Bluher et al., 2003; Holzenberger et al., 2003; Selman et al., 2008), while mice treated with rapamycin, an inhibitor of the mTOR (mechanistic Target Of Rapamycin) protein kinase that extends lifespan, develop insulin resistance in multiple tissues (Lamming et al., 2012). Correctly interpreting these results is difficult; for example, the FIRKO (fat insulin receptor knockout) mice retain insulin sensitivity at the organismal level; and the FIRKO mouse study was conducted utilizing Cre recombinase under the control of an *AP2* promoter, which is now known not to be specific for adipose tissue (Bluher et al., 2003; Lee et al., 2013).

Over the last decade, a critical role for the mechanistic Target Of Rapamycin Complex 2 (mTORC2) in the control of organismal metabolism has become apparent. In contrast to the well-known mTOR Complex 1 (mTORC1), which functions as a key integrator of many different environmental and hormonal cues, mTORC2 functions primarily as an effector of PI3K signaling, contributing to the downstream activation of many kinases, including AKT, by insulin (Guertin et al., 2006; Sarbassov et al., 2005). Deletion of *Rictor*, which encodes an essential protein component of mTORC2, results in insulin resistance in tissues including liver, adipose tissue, and skeletal muscle (Cybulski et al., 2009; Hagiwara et al., 2012; Kleinert et al., 2017; Kumar et al., 2008; Kumar et al., 2010; Lamming et al., 2014a; Lamming et al., 2012; Yuan et al., 2012). The organismal consequences of inactivating adipose mTORC2 have been unclear, as distinct phenotypes have been observed in mice where *Rictor* was deleted using the *AP2-Cre* and a more recent study utilizing the adipose-specific *Adiponectin-Cre* (Cybulski et al., 2009; Kumar et al., 2010; Tang et al., 2016).

Published studies of the effects of adipose mTORC2 have also been limited to relatively young mice under 1 year of age.

While an important role for CR-induced insulin sensitivity in the health and survival benefits of CR has long been assumed, the contribution of improved insulin sensitivity to the benefits of CR has not been directly examined. Here, we have tested the role of CR-induced insulin sensitivity on the metabolic health, frailty, and longevity of mice by placing mice lacking adipose mTORC2 signaling (AQ-RKO: *Adiponectin-Cre; Rictor<sup>LoxP/LoxP</sup>*) and their wild-type littermates on either *ad libitum* or calorie restricted diets. Critically, the insulin sensitivity of AQ-RKO mice does not improve on a CR diet, enabling us to discern the role of CR-induced insulin sensitivity in CR-induced phenotypes. Although the white adipose tissue of AQ-RKO mice has a blunted metabolic response to CR, and female AQ-RKO mice fed an *ad libitum* diet have a slightly reduced lifespan, we find that AQ-RKO mice of both sexes fed a CR diet have increased fitness and extended lifespan. We conclude that the CR-induced increase in insulin sensitivity is dispensable for the effects of CR on fitness and longevity.

## RESULTS

### Deletion of adipose *Rictor* impairs insulin sensitivity

We generated adipose specific *Rictor* knockout mice by crossing *Rictor* floxed mice with mice expressing Cre recombinase under the control of the adiponectin promoter (AQ-RKO; *Adipoq-Cre; Rictor<sup>LoxP/LoxP</sup>*). As expected, we found that RICTOR protein expression was substantially reduced in the adipose tissues of AQ-RKO mice, including the inguinal white adipose tissue (iWAT) and epididymal white adipose tissue (eWAT), but was not altered in other organs including liver and skeletal muscle (Figure 1A). Consistent with this, phosphorylation of the mTORC2 substrate AKT S473 was likewise significantly reduced in iWAT and eWAT, but not in other tissues (Figure 1A).

Adipose mTORC2 has been proposed to negatively regulate whole body growth, as young *Ap2-Cre; Rictor<sup>LoxP/LoxP</sup>* mice have increased body weight and lean tissue mass with elevated IGF-1 (Cybulski et al., 2009). However, these phenotypes were not observed in mice in which *Rictor* was deleted using *Adiponectin-Cre* (Tang et al., 2016). In agreement with these latter results, we found that young AQ-RKO mice have approximately normal body weight (Figure 1B), with the mass of most AQ-RKO organs equivalent to that of their littermate controls (Figures 1C and 1D). However, the liver weight of female AQ-RKO mice was significantly greater than that of their littermate controls (Figure 1D); and correlated with this, we found that plasma levels of IGF-1 were elevated in female AQ-RKO mice, but not in males (Figure 1E).

We also examined the effect of adipose mTORC2 loss on glucose homeostasis. We found that AQ-RKO mice of both sexes were glucose tolerant (Figure 1F) but, as we anticipated given the established role of adipose tissue in insulin-stimulated glucose clearance (Rosen and Spiegelman, 2006), we observed that AQ-RKO mice were insulin resistant, with a more pronounced effect in male mice (Figure 1G).

### Adipose mTORC2 is required for CR to improve insulin sensitivity

We next tested our hypothesis that mice lacking adipose mTORC2 would be resistant to the metabolic benefits of CR by placing 10-week-old WT and AQ-RKO mice of both sexes on either *ad libitum* (AL) or CR diets. After 8 weeks, which is sufficient for CR to promote glucose tolerance and insulin sensitivity in C57BL/6J mice (Solon-Biet et al., 2015), we assessed the effects of CR on metabolic phenotypes. Both AQ-RKO mice and their WT littermates fed a CR diet had improved glucose tolerance compared to their AL fed counterparts (Figures 2A and 2B). However, the improvement of insulin sensitivity induced by CR feeding that we observed in both male and female WT mice was completely blocked by deletion of adipose *Rictor* (Figures 2C and 2D, Figures S1A and S1B). In accordance with these results, we observed increased phosphorylation of AKT T308 and S473 in the adipose tissue of WT CR-fed mice relative to their WT AL-fed littermates following either acute stimulation by insulin or prolonged stimulation by refeeding; while in the absence of adipose *Rictor*, CR had no effect on the phosphorylation of AKT in adipose tissue in response to these stimuli (Figures S1C–S1F and S2A–B). In contrast, we observed no effect of genotype on AKT phosphorylation in skeletal muscle (Figures S2C and S2D).

Consistent with the improved glucose tolerance of both genotypes of mice on a CR diet, and previous reports of fasting hyperinsulinemia in mice lacking adipose *Rictor* (Cybulski et al., 2009; Tang et al., 2016), we observed hyperinsulinemia following refeeding in AQ-RKO mice of both sexes fed either AL or CR; curiously, CR potentiated the hyperinsulinemia we observed in AL-fed mice (Figures 2E and 2F). Consistent with the above results, we found that CR improved pyruvate tolerance equally well in both WT and AQ-RKO mice, indicating effective suppression of hepatic gluconeogenesis by insulin (Figure 2G). In accordance with these results, we observed no effect of genotype on AKT phosphorylation in the liver (Figures S2E and S2F).

### Loss of adipose *Rictor* impairs the metabolic adaptation to a CR diet

The effects of CR on insulin sensitivity likely result in part from reduced adiposity. In addition, CR substantially alters whole-body lipid metabolism, in part by reprogramming WAT; CR upregulates lipogenesis in WAT upon refeeding (Bruss et al., 2010) which may contribute to the beneficial effects of CR on systemic insulin sensitivity. We observed that the loss of adipose mTORC2 did not substantially impact the effect of CR on adiposity; CR was equally effective at preventing the accretion of fat mass in both WT and AQ-RKO mice (Figures 3A and 3B). The body weight and lean mass of WT and AQ-RKO mice also responded similarly to CR (Figures 3C–3F). Thus, CR was equally effective at reducing adiposity in WT and AQ-RKO mice (Figures S3G and S3H).

We next examined the role of mTORC2 in the upregulation of lipogenesis by CR following refeeding. WT and AQ-RKO mice were placed in metabolic chambers, allowing us to assess substrate utilization by examining the respiratory exchange ratio (RER). RER is calculated using the ratio of O<sub>2</sub> consumed and CO<sub>2</sub> produced, and approaches 1.0 when carbohydrates are being primarily utilized for energy production and approaches 0.7 when lipids are the predominant energy sources (Hasek et al., 2010). An RER > 1.0 reflects the utilization of carbohydrates for active *de novo* lipogenesis (Bruss et al., 2010). Consistent with previous

reports that CR-fed mice engage in rapid lipogenesis following refeeding and sustain themselves the rest of the day via the utilization of these stored lipids (Bruss et al., 2010), we observed that RER rapidly rose above 1.0 in WT CR mice following refeeding, and approximately 6 hours later fell below 0.8 (Figures 4A and 4B). In contrast, AQ-RKO CR-fed mice of both sexes had a blunted lipogenic response to refeeding, and AQ-RKO CR male mice had an intermediate RER for the majority of the dark cycle, suggesting that these animals relied on a mix of carbohydrate and lipid stores (Figures 4A and 4B).

To verify our model that AQ-RKO mice fed a CR diet had a reduced ability to induce lipogenesis following refeeding, we sacrificed mice 3 hours after refeeding and examined lipogenic gene expression in inguinal WAT (iWAT). In agreement with our RER data, we observed that CR robustly induced the expression of many genes required for *de novo* lipogenesis, including ATP-citrate lyase (*Acly*), acetyl-CoA carboxylase (*Acc*), fatty acid synthase (*Fasn*), and elongation of very long chain fatty acids 6 (*Elovl6*), in WT mice (Figures 4C and 4D). However, CR had a substantially weaker impact on the expression of these lipogenic genes in AQ-RKO mice (Figures 4C and 4D).

*De novo* lipogenesis is regulated by several transcriptional factors including carbohydrate-responsive element-binding proteins (ChREBPs) (Uyeda and Repa, 2006) and sterol regulatory element-binding proteins (SREBPs) (Horton et al., 2002). In agreement with a recent report implicating adipose mTORC2 in the control of ChREBP- $\beta$ -dependent *de novo* lipogenesis (Tang et al., 2016), we observed that CR induced *Chrebp- $\beta$*  strongly in the iWAT of CR fed WT mice, but to a much lesser degree in mice lacking adipose mTORC2 (Figures 4C and 4D). Other transcriptional factors important for lipogenesis in adipose tissue such as *Chrebp-a* and *Srebp1-c* were also downregulated in AL-fed AQ-RKO mice; however, deletion of adipose *Rictor* attenuated the ability of CR to upregulate these transcriptional factors only in females (Figure S3A). While adipose tissue is the major site of lipogenesis in CR-fed mice (Bruss et al., 2010), the liver is also an important site for *de novo* lipogenesis. To assess possible indirect effects of adipose *Rictor* deletion on hepatic lipogenesis, we examined the expression of a panel of key lipogenic genes in this tissue. Although CR robustly increased the expression of almost of all these genes, we observed no effect of genotype on lipogenic gene expression on either AL or CR diets (Figure S3B).

CR activates thermogenesis as a mechanism to defend body temperature (Fabbiano et al., 2016). We found that WT males, but not WT females, fed a CR diet had increased expression of uncoupled protein 1 (*Ucp1*) mRNA as well as increased expression of UCP1 protein in the iWAT; however, neither male nor female AQ-RKO mice had increased expression of UCP1 at either the mRNA or protein level in response to CR (Figures S3C–S3F). The expression of two other key thermogenic genes, cell death-inducing DNA fragmentation factor alpha-like effector A (*Cidea*) and elongation of very long chain fatty acids protein 3 (*Elovl3*), were significantly upregulated by CR in the iWAT of WT mice of both sexes, but were not induced by CR in AQ-RKO mice (Figures S3C and S3D). Despite these significant differences at the molecular level, the morphology of iWAT from WT and AQ-RKO mice on either diet was very similar (Figure S4A). Furthermore, CR similarly remodeled the BAT of both WT and AQ-RKO mice (Figure S4B) at the histological level.

As in the iWAT, the expression of UCP1 was increased by CR in the BAT of WT male mice, but not in AQ-RKO mice (Figure S4C).

We reasoned that as adipose *Rictor* deletion impacts the effects of CR on both substrate utilization and adipose lipogenesis, AQ-RKO mice might have altered levels of circulating lipids following refeeding. We observed that loss of adipose *Rictor* increased circulating levels of triglycerides in both males and females, irrespective of diet, and CR significantly reduced the plasma triglycerides levels of both WT and AQ-RKO males (Figure 4E). Plasma free fatty acids were significantly decreased by CR in female WT mice but not in female AQ-RKO mice; in males, plasma free fatty acids were not significantly affected by genotype, but CR diet showed a strong trend to decrease plasma free fatty acids (Figure 4F). Furthermore, female AQ-RKO mice placed on a CR diet had significantly higher plasma cholesterol than WT CR-fed females (Figure S4D). Finally, we assessed lipid deposition in the liver and muscle by histology; we found that CR promoted a similar reduction in lipid content in the liver of both WT and AQ-RKO mice, and that no group had significant lipid deposition in the muscle (Figures S4E and S4F).

### Longitudinal assessment of the role of adipose mTORC2 in body composition and glucose homeostasis

The importance of adipose mTORC2 in the metabolic response to CR suggested to us that adipose mTORC2 might play a key role in regulating the many health benefits of long-term CR. To test this, we initiated a survival study in which 10-week-old WT and AQ-RKO mice were placed on either AL or CR diets; we then longitudinally assessed the effects of CR on weight and body composition, glycemic control, healthspan and lifespan.

As we anticipated, both WT and AQ-RKO mice of either sex fed a CR diet weighed substantially less than AL-fed mice throughout their life (Figures S5A and S5B), and were leaner with reduced levels of both fat and lean mass (Figures 5A–5D). However, we did observe significant differences in the body composition of AL-fed mice with age; in particular, male AQ-RKO AL-fed mice had significantly less fat mass than their WT littermates, particularly at 12 and 18 months of age (Figure 5A). Both male and female AL-fed AQ-RKO mice had greater lean mass throughout life than their AL-fed WT littermates, an effect that was statistically significant in females from 6–18 months of age (Figures 5C and 5D). The overall effect of *Rictor* loss was reduced adiposity over the course of the lifespan for AL-fed males, but not for AL-fed females (Figures 5E and 5F).

During the course of the study, we assessed glucose homeostasis as the mice aged. In agreement with our initial findings, and studies conducted by Tang and colleagues in young mice (Tang et al., 2016) we found that AQ-RKO mice fed an AL diet had normal glucose tolerance even at advanced ages; indeed, there was a trend towards improved glucose tolerance of 8 month old AQ-RKO male mice relative to their WT littermates (Figures S5C–S5F). This glucose tolerance was observed despite the lifelong insulin resistance of AQ-RKO mice of both sexes relative to their WT littermates (Figures 6A–6D). Consistent with these results and previous reports (Tang et al., 2016), we observed increased fasting hyperinsulinemia and hypersecretion of insulin in response to glucose stimulation in AL-fed AQ-RKO mice (Figures S5G and S5H).

As we observed in our short-term study, while WT mice placed on a CR diet showed significantly improved insulin sensitivity at 9 months of age relative to WT AL-fed mice, a CR diet did not improve the insulin sensitivity of AQ-RKO mice at this age (Figures 6A and 6B, Figures S6A and S6B). At 2 years of age, although CR improved the insulin sensitivity of AQ-RKO mice relative to AL-fed mice of the same genotype, CR-fed AQ-RKO mice remained significantly more insulin resistant than CR-fed WT mice (Figures 6C and 6D, Figures S6C and S6D). Critically, at no age were AQ-RKO mice fed a CR diet more insulin sensitive than age-matched WT AL-fed mice.

### Adipose mTORC2 does not mediate the effects of a CR diet on fitness and longevity

Mice and humans become increasingly frail with age, and starting at 18 months of age we utilized a recently validated mouse frailty index that permits the quantification of frailty by tracking the accumulation of deficits with age to assess the impact of CR on fitness in both genotypes (Kane et al., 2016; Rockwood et al., 2017; Whitehead et al., 2014). As we anticipated, we found that WT CR-fed male and female mice were significantly less frail than WT AL-fed mice of the same age and sex (Figures 7A and 7B). Surprisingly, we observed that the frailty of AQ-RKO AL-fed mice and WT AL-fed mice was similar, and that the fitness of both male and female AQ-RKO mice was improved by a CR diet to the same extent as CR improved the fitness of WT mice (Figures 7A and 7B).

Finally, we determined the effect of adipose *Rictor* deletion on longevity and the response to CR. In contrast to our previously reported observation of a sharp, male-specific decrease in the lifespan of mice lacking hepatic *Rictor* (Lamming et al., 2014b), here we find that loss of adipose mTORC2 signaling has no significant effect on male lifespan; instead, we find a 7% decrease in median female lifespan (Figures 7C–7E). As expected, a CR diet significantly extended the lifespan of both male and female WT mice. Surprisingly – but consistent with our frailty findings – we found that a CR diet robustly extends the lifespan of both male and female AQ-RKO mice; indeed, the lifespan of AQ-RKO mice fed a CR diet was indistinguishable from that of their WT littermates (Figures 7C–7E).

## DISCUSSION

Despite thorough investigation over the last century, the physiological and molecular mechanisms underlying the benefits of CR on healthspan and longevity have remained elusive. One of the most widely conserved effects of CR in mammals is improved insulin sensitivity (Lamming and Anderson, 2014). Given the well-known association of insulin resistance with obesity, diabetes, cardiovascular disease, Alzheimer's disease, and other diseases of aging, it is conceptually appealing to posit that insulin sensitivity mediates the beneficial effects of a CR diet (Evans and Goldfine, 2013; Fontana and Partridge, 2015; Williamson et al., 2012). Although this hypothesis has never been directly tested, the extended longevity of a mouse model of whole body insulin resistance (*Irs1*<sup>-/-</sup>, (Selman et al., 2008)) and other genetic or pharmaceutical mouse models in which insulin resistance has been induced in one or more tissues suggested to us that this hypothesis was incorrect or incomplete.

Here, we have directly tested the hypothesis that the beneficial effects of CR are mediated by improved insulin sensitivity using AQ-RKO mice. These mice, which in our hands have lifelong insulin resistance and fasting hyperinsulinemia when fed AL, remain glucose tolerant due to increased glucose-stimulated insulin secretion. The frailty and longevity of AQ-RKO mice of both sexes on an AL diet is similar to that of their WT littermates, with reduced lifespan only in female AQ-RKO mice. Finally, we have found that AQ-RKO mice have an impaired metabolic response to CR, resisting CR-induced improvements in insulin sensitivity. This combination of phenotypes has enabled us to test the theory that the beneficial effects of a CR diet are mediated by improved insulin sensitivity. We find that despite the impaired metabolic response of AQ-RKO mice to a CR diet, AQ-RKO mice benefit fully from the effects of a CR diet with respect to both fitness and longevity. We conclude that although CR improves systemic insulin sensitivity, this improvement is not required for the beneficial effects of a CR diet on healthspan and lifespan (Figure 7F).

The question of what mediates the benefits of a CR diet on fitness and longevity remains open. While our results clearly demonstrate that improved insulin sensitivity is not required for CR to extend lifespan in C57BL/6J mice, numerous studies have found that interventions that promote insulin sensitivity do extend lifespan (e.g., (Garratt et al., 2017; Templeman et al., 2017)). While a CR diet protects against tumors and carcinogenesis (Kalaany and Sabatini, 2009), an effect that would likely extend the lifespan of tumor-prone C57BL/6J mice, a study conducted over a decade ago found that a CR diet robustly extended the lifespan of mice lacking NF-E2-related factor 2 (Nrf2), despite being unable to suppress tumorigenesis (Pearson et al., 2008). As we have outlined in Figure 7F, while insulin sensitivity and cancer protection are both induced by a CR diet, neither effect is solely responsible for the effects of CR on the fitness and longevity of rodents. Future research into the mediators of CR may benefit from taking a similar approach to our study and that conducted by Pearson, in which the healthspan and lifespan of mouse models with a reduced response to specific facets of CR is investigated. The model we have developed here may be useful for testing whether improved insulin sensitivity mediates the extended health and longevity of a number of pharmaceutical interventions and genetic mouse models (Garratt et al., 2017; Harrison et al., 2019; Strong et al., 2016; Templeman et al., 2017).

An important limitation of our results is that here we have primarily focused on systemic insulin sensitivity as measured by the acute clearance of glucose in response to an intraperitoneal injection of insulin, and we have not directly assessed many other aspects of insulin resistance or insulin action at different timescales. While we have not directly assessed tissue glucose uptake, the findings of Tang et al. (Tang et al., 2016), our insulin tolerance test results, and our molecular data showing that CR-fed mice have increased insulin/PI3K signaling in iWAT following either acute insulin stimulation or refeeding suggest that the insulin resistance of AQ-RKO mice fed either AL or CR diets is primarily mediated by reduced glucose uptake into adipose tissue. However, there may also be cell non-autonomous effects of adipose *Rictor* deletion mediated by the reduced lipogenesis in AQ-RKO mice fed a CR diet. ChREBP-mediated lipogenesis in adipose tissue promotes systemic insulin sensitivity (Herman et al., 2012; Vijayakumar et al., 2017; Yore et al., 2014); thus, the impaired lipogenic response of AQ-RKO mice to a CR diet may blunt the effects of CR on the insulin sensitivity of other tissues. While we did not conduct a



comprehensive examination of hormones and factors that might be altered in AQ-RKO mice and regulate insulin sensitivity, Tang and colleagues have reported that AQ-RKO mice have normal levels of TNF- $\alpha$ , leptin, and resistin, with changes in adiponectin that are insufficient to explain the insulin resistance of AQ-RKO mice (Tang et al., 2016). Although we did not observe an effect of adipose *Rictor* loss on insulin/PI3K signaling in skeletal muscle or liver tissue, our examination of these tissues was limited, and the insulin sensitivity of these or other non-adipose tissues could be improved by a CR diet, even in AQ-RKO mice, and could therefore potentially contribute to, or even drive, the beneficial effects of CR on fitness and lifespan. While distinguishing between these possibilities will require further detailed investigation, we consider the latter hypothesis unlikely; in agreement with other groups, we find that loss of adipose mTORC2 results in hyperinsulinemia (Cybulski et al., 2009; Tang et al., 2016), and as we report here, even on a CR diet AQ-RKO mice have relatively high levels of insulin. High insulin sensitivity in the context of hyperinsulinemia would result in increased signaling through the PI3K/AKT/mTORC1 signaling pathway, which would likely have deleterious rather than beneficial effects on health and longevity (Blagosklonny, 2012; Dumas and Lamming, 2019; Lamming, 2014).

Rapamycin treatment extends the lifespan of mice (reviewed in (Arriola Apelo and Lamming, 2016)). The effects of rapamycin on health and longevity were originally believed to result from inhibition of mTORC1, but we and other groups have demonstrated that rapamycin also inhibits mTORC2 *in vivo* in multiple tissues, including liver and WAT (Lamming et al., 2012; Schreiber et al., 2015). Inhibition of hepatic mTORC2 by rapamycin results in hepatic insulin resistance, and it is now thought that many side effects of rapamycin may be driven by mTORC2 inhibition (Kennedy and Lamming, 2016). However, this does not eliminate the possibility that inhibition of mTORC2 may also have some benefits with respect to longevity, and indeed mice heterozygous for the mTORC2 substrate *Akt1* have increased lifespan (Nojima et al., 2013). Thus, understanding both the positive and negative roles of mTORC2 in different tissues on health and longevity is of high importance as rapamycin and related compounds enter human clinical trials for diseases of aging (Kraig et al., 2018; Mannick et al., 2014; Mannick et al., 2018).

We have previously shown that diminishing mTORC2 activity specifically in the liver or in the whole body reduces male lifespan by at least 30% (Lamming et al., 2014b), while deletion of *Rictor* specifically in the hypothalamus reduces male lifespan by 10% and increases frailty in both sexes (Chellappa et al., 2019). Fitness was also reduced when *Rictor* was inducibly deleted in adult mice, which resulted in a progeroid phenotype. In contrast, we find that mTORC2 inhibition in adipose tissue has minimal effects on the fitness of either male or female mice, and specifically reduces median female lifespan by 7%, possibly as a consequence of elevated IGF-1 levels in *ad libitum* fed female mice. As loss of adipose mTORC2 signaling also significantly increases plasma triglycerides, these results suggest that inhibition of adipose mTORC2 signaling should be avoided when designing rapalog-based approaches to diseases of aging (Schreiber et al., 2019). Finally, we note that the induction of insulin resistance in adipose tissue did not result in the extended longevity observed in the FIRKO mouse (Bluhner et al., 2003). Possible explanations for the difference include the degree of insulin resistance induced and the different Cre driver utilized by Bluhner and colleagues. While we and others have hypothesized that insulin resistance may

promote longevity in part by decreasing mTORC1 signaling (Blagosklonny, 2012; Lamming, 2014), we did not observe altered mTORC1 signaling in mice lacking adipose *Rictor*.

In summary, we have tested the long-standing hypothesis that the beneficial effects of CR on health and longevity are the result of the CR-induced improvements in insulin sensitivity observed in both rodents and humans placed on a CR diet, using a mouse model lacking adipose mTORC2 activity. We have determined that adipose mTORC2 has an essential role in the regulation of body composition and the survival of female mice fed *ad libitum*. Further, adipose mTORC2 plays a role in the metabolic adaptation to CR, and is required for CR to improve insulin sensitivity. CR-induced improvements in insulin sensitivity are widely seen in mammals, and were proposed to mediate the beneficial effects of CR on health and longevity. Here, we disprove this hypothesis by showing that the CR-induced improvements in organismal insulin sensitivity are not required for the beneficial effects of CR on fitness and lifespan. We suggest that the utilization of mouse strains with impaired responses to different aspects of CR may be a useful approach to identifying the true physiological and molecular mechanisms by which CR promotes mammalian health and longevity.

## EXPERIMENTAL PROCEDURES

### Animals

All procedures were performed in conformance with institutional guidelines and were approved by the Institutional Animal Care and Use Committee of the William S. Middleton Memorial Veterans Hospital (Madison, WI, USA). Adipose specific *Rictor* knockout mice (AQ-RKO) were generated by crossing mice with a conditional allele of *Rictor* (Shiota et al., 2006) on a C57BL/6 background that we have previously described (Lamming et al., 2014b) to mice expressing Cre recombinase under the control of the mouse *Adipoq* promoter ((Eguchi et al., 2011), gift of Drs. Christine Patterson and Evan Rosen). Mice were kept on a daily 12 h light/dark cycle and fed Purina lab chow diet 5001 (Purina, St. Louis, MO, USA) until 10 weeks of age. At 10 weeks of age, mice either continued on an *ad libitum* (AL) regimen, or were placed on a CR regimen and fed using pre-weighed rodent diets tablets (Purina 1816539 and 1816541), with progressively increasing restriction over the course of 2 weeks, with a maximum level of restriction of 40%, as previously described (Lamming et al., 2014b; Yilmaz et al., 2012). Briefly, animals housed 1, 2, or 3 to cage were fed, respectively, 3, 5, or 6 grams of pre-weighed rodent diet tablets per day; the food intake of each cage was adjusted when the number of animals in the cage was altered, e.g. due to age-related mortality. CR animals were typically fed in the morning at approximately 8AM. For short-term studies, 10-week-old mice were placed on AL and CR regimens for 12 weeks before sacrifice. Mice were housed in a specific-pathogen-free (SPF) mouse facility under a 12:12 h light/dark cycle with free access to food and water, except for CR mice and where noted in the procedures below. Animals were group housed in static microisolator cages, except when temporarily housed in an Oxymax/CLAMS metabolic chamber system (Columbus Instruments, Columbus, OH, USA).

### **In vivo tests**

For glucose and pyruvate tolerance tests, food was withheld overnight from *ad libitum* fed animals for 16 hours, and mice were then injected intraperitoneally with glucose (1 g/kg) or pyruvate (2 g/kg) as described previously (Yu et al., 2018). The insulin tolerance test described in Figure 1 was performed by fasting mice at 5 pm and injecting 0.75 U/kg of insulin intraperitoneally around 9 am the next morning. As CR-fed WT mice are extremely insulin sensitive and this procedure leads to extremely low blood glucose values during an ITT, all other insulin tolerance tests (except for the ones performed in 25-month-old mice, Figures 6C, 6D, S6C, S6D) were performed by fasting mice at 6 am in the morning and then injecting 0.5 U/kg of insulin (0.5U/kg) intraperitoneally at 10 am. ITTs in 25 month-old mice were performed using 0.75 U/kg as the response of all groups of mice to 0.5 U/kg was limited. In all ITTs, WT CR-fed animals that experienced hypoglycemia and needed to be rescued by administration of glucose were not included in the presented results. A Bayer Contour blood glucose meter and test strips were used to measure blood glucose. For body composition analysis, lean and fat mass were measured using an EchoMRI 3-in-1 Body Composition Analyzer (EchoMRI™, Houston, TX, USA) according to the manufacturer's procedures. To assess RER, we utilized an Oxymax/CLAMS metabolic chamber system (Columbus Instruments, Columbus, OH, USA).

### **Tissue harvest**

Mice used for the short-term study were sacrificed in either in the fasted state, refed state or insulin-stimulated state after 12 weeks of feeding AL or CR. Mice sacrificed in the fasted state were fasted overnight starting at 5 pm and then sacrificed between 9 and 11 am the next morning. Mice sacrificed in the refed state were fasted at 5 pm for overnight and food was provided to both AL and CR mice at 8 am the next morning; the mice were then sacrificed approximately 3 hours later. Mice sacrificed in the insulin-stimulated state were fasted overnight for approximately 16 hours starting at 5 pm and the next morning were then injected I.P. with 0.75 U/kg of insulin 15 min prior to sacrifice.

### **Plasma hormone and lipid measurement**

Plasma IGF1 and insulin were quantified using a mouse IGF-1 ELISA kit (80574) and ultra-sensitive mouse insulin ELISA kit (90080), respectively, from Crystal Chem (Elk Grove Village, IL, USA). Plasma triglyceride and free fatty acid were assessed using triglyceride colorimetric assay kit (10010303) and free fatty acid fluorometric assay kit (700310) from Cayman Chemical (Cayman Chemical, Ann Arbor, MI, USA), respectively. Plasma cholesterol was assessed using the stanbio cholesterol liquicolor kit (1010225) from Stanbio Laboratory (Boerne, TX, USA).

### **Quantitative PCR**

RNA was extracted from liver or adipose tissue using Trizol (Sigma-Aldrich, St. Louis, MO, USA). Briefly, the concentration and purity of RNA were determined by absorbance at 260/280 nm using Nanodrop (Thermo Fisher, Waltham, MA, USA), and 1 µg of RNA was used to generate cDNA using Superscript III (Invitrogen, Carlsbad, CA, USA). Oligo dT primers and primers for real-time PCR were obtained from Integrated DNA Technologies

(Coralville, IA, USA); primer sequences are listed in Table S1. Reactions were run on an Applied Biosystems StepOne Plus machine (Applied Biosystems, Foster City, CA, USA) with Sybr Green PCR Master Mix (Invitrogen, Carlsbad, CA, USA) as described previously (Cummings et al., 2018).  $\beta$ -actin was used to normalize the results from gene-specific reactions.

### Immunoblotting

Tissue samples from liver and muscle were lysed in cold RIPA buffer supplemented with phosphatase inhibitor and protease inhibitor cocktail tablets (Thermo Fisher Scientific, Waltham, MA, USA) as previously described (Baar et al., 2016) using a FastPrep 24 (M.P. Biomedicals, Santa Ana, CA, USA) with bead-beating tubes (16466–042) from (VWR, Radnor, PA, USA) and zirconium ceramic oxide bulk beads (15340159) from (Thermo Fisher Scientific, Waltham, MA, USA). Protein lysates were then centrifuged at 13,300 rpm for 10 min and the supernatant was collected. For WAT lysates, protein lysates were then frozen overnight at  $-80^{\circ}\text{C}$ , thawed, and the aqueous non-lipids were collected using a needle and syringe; this supplemental step to remove lipids was repeated twice as previously described (Baar et al., 2016). Protein concentration was determined by Bradford (Pierce Biotechnology, Waltham, MA, USA). 20  $\mu\text{g}$  protein was separated by SDS–PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) on 8%, 10%, or 16% resolving gels (Thermo Fisher Scientific, Waltham, MA, USA) and transferred to PVDF membrane (EMD Millipore, Burlington, MA, USA). Rictor (2140), AKT (9272), HSP90 (4877), and pS473-AKT (4060) were purchased from Cell Signaling Technologies. Imaging was performed using a GE ImageQuant LAS 4000 imaging station (GE Healthcare, Chicago, IL, USA). Quantification was performed by densitometry using NIH ImageJ software.

### Lifespan and necropsy

All mice for the lifespan study were bred by crossing mice homozygous for a conditional allele of *Rictor* (*Rictor<sup>L/L</sup>*) with mice homozygous for a conditional allele of *Rictor* and hemizygous for *Adipoq-Cre* (*Adipoq-Cre; Rictor<sup>L/L</sup>*). Mice were co-housed (grouped by sex and dietary regimen) in SPF housing. Mice were inspected daily and were euthanized for humane reasons if moribund, if the mice developed other specified problems (e.g. excessive tumor burden), or upon the recommendation of the facility veterinarian. Mice found dead were noted at each daily inspection and saved in a refrigerator for necropsy, during which the presence or absence of cancer was recorded. Mice that died during the adaptation to a CR diet, or as a result of the conduct of their cagemates, were excluded from the lifespan analysis. Mice that died within a one week period following the conduct of an *in vivo* procedure, as a result of husbandry errors, or due to anal or uterine prolapse prior to 20 months of age were included and censored as of the date of death. A complete list of mice for the lifespan study is in Table S2.

**Frailty.**—Frailty was assessed longitudinally in a subset of mice using a 26-item frailty index based on the procedures defined by (Whitehead et al., 2014). The items scored included alopecia, loss of fur color, dermatitis, loss of whiskers, coat condition, tumors, distended abdomen, kyphosis, tail stiffening, gait disorders, tremor, body condition score, vestibular disturbance, cataracts, corneal opacity, eye discharge/swelling, microphthalmia,

vision loss, menace reflex, nasal discharge, malocclusions, rectal prolapse, vaginal/uterine/penile prolapse, diarrhea, breathing rate/depth, and piloerection.

### Statistics

Statistical analyses were conducted using Prism 8 (GraphPad Software, La Jolla, CA, USA), except for stratified log-rank survival analyses which were conducted in R (version 3.5.0) using the ‘survival’ package (version 2.38) (T, 2015). Statistical analyses were performed using two-way ANOVA, with or without repeated-measures as appropriate, and followed by a Holm-Sidak or Tukey-Kramer post-test as specified in the figure legends.

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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#### DECLARATION OF INTERESTS

D.W.L. has received funding from, and is a scientific advisory board member of, Aeonian Pharmaceuticals, which seeks to develop novel, selective mTOR inhibitors for the treatment of various diseases.

### REFERENCES

- Arriola Apelo SI, and Lamming DW (2016). Rapamycin: An InhibiTOR of Aging Emerges From the Soil of Easter Island. *J Gerontol A Biol Sci Med Sci* 71, 841–849. [PubMed: 27208895]
- Baar EL, Carbajal KA, Ong IM, and Lamming DW (2016). Sex- and tissue-specific changes in mTOR signaling with age in C57BL/6J mice. *Aging Cell* 15, 155–166. [PubMed: 26695882]
- Balasubramanian P, Howell PR, and Anderson RM (2017). Aging and Caloric Restriction Research: A Biological Perspective With Translational Potential. *EBioMedicine* 21, 37–44. [PubMed: 28648985]
- Blagosklonny MV (2012). Once again on rapamycin-induced insulin resistance and longevity: despite of or owing to. *Aging (Albany NY)* 4, 350–358. [PubMed: 22683661]

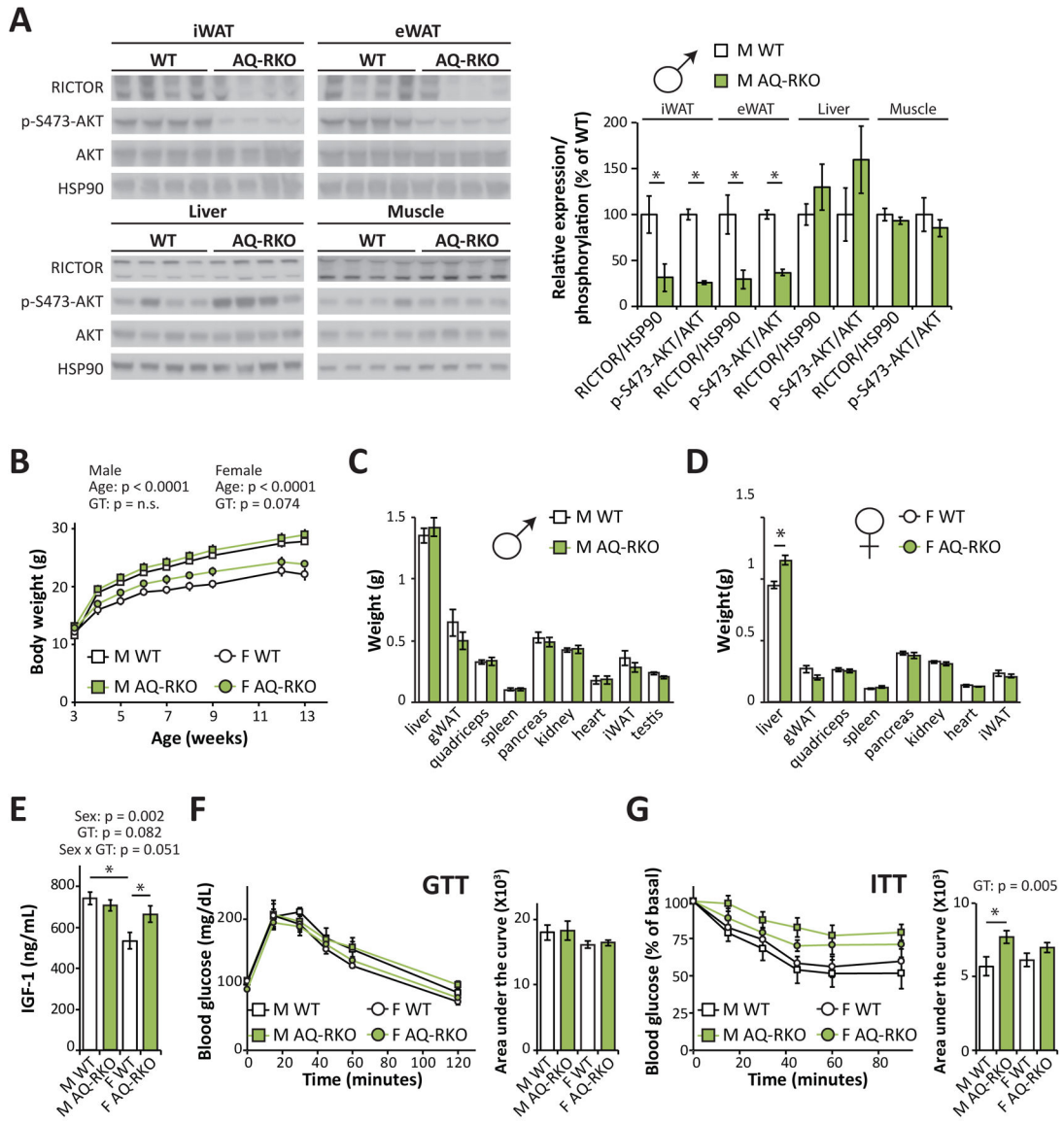
- Bluhner M, Kahn BB, and Kahn CR (2003). Extended longevity in mice lacking the insulin receptor in adipose tissue. *Science* 299, 572–574. [PubMed: 12543978]
- Bruss MD, Khambatta CF, Ruby MA, Aggarwal I, and Hellerstein MK (2010). Calorie restriction increases fatty acid synthesis and whole body fat oxidation rates. *Am J Physiol Endocrinol Metab* 298, E108–116. [PubMed: 19887594]
- Chellappa K, Brinkman JA, Mukherjee S, Morrison M, Alotaibi MI, Carbajal KA, Alhadeff AL, Perron IJ, Yao R, Purdy CS, et al. (2019). Hypothalamic mTORC2 is essential for metabolic health and longevity. *Aging Cell*, e13014. [PubMed: 31373126]
- Chen D, Bruno J, Easlson E, Lin SJ, Cheng HL, Alt FW, and Guarente L (2008). Tissue-specific regulation of SIRT1 by calorie restriction. *Genes Dev* 22, 1753–1757. [PubMed: 18550784]
- Cummings NE, Williams EM, Kasza I, Konon EN, Schaid MD, Schmidt BA, Poudel C, Sherman DS, Yu D, Arriola Apelo SI, et al. (2018). Restoration of metabolic health by decreased consumption of branched-chain amino acids. *The Journal of physiology* 596, 623–645. [PubMed: 29266268]
- Cybulski N, Polak P, Auwerx J, Ruegg MA, and Hall MN (2009). mTOR complex 2 in adipose tissue negatively controls whole-body growth. *Proc Natl Acad Sci U S A* 106, 9902–9907. [PubMed: 19497867]
- Dumas SN, and Lamming DW (2019). Next generation strategies for geroprotection via mTORC1 inhibition. *J Gerontol A Biol Sci Med Sci*.
- Eguchi J, Wang X, Yu S, Kershaw EE, Chiu PC, Dushay J, Estall JL, Klein U, Maratos-Flier E, and Rosen ED (2011). Transcriptional control of adipose lipid handling by IRF4. *Cell Metab* 13, 249–259. [PubMed: 21356515]
- Evans JL, and Goldfine ID (2013). Aging and insulin resistance: just say iNOS. *Diabetes* 62, 346–348. [PubMed: 23349542]
- Fabbiano S, Suarez-Zamorano N, Rigo D, Veyrat-Durebex C, Dokic AS, Colin DJ, and Trajkovski M (2016). Caloric Restriction Leads to Browning of White Adipose Tissue through Type 2 Immune Signaling. *Cell Metab* 24, 434–446. [PubMed: 27568549]
- Fontana L, and Partridge L (2015). Promoting health and longevity through diet: from model organisms to humans. *Cell* 161, 106–118. [PubMed: 25815989]
- Garratt M, Bower B, Garcia GG, and Miller RA (2017). Sex differences in lifespan extension with acarbose and 17-alpha estradiol: gonadal hormones underlie male-specific improvements in glucose tolerance and mTORC2 signaling. *Aging Cell* 16, 1256–1266. [PubMed: 28834262]
- Guertin DA, Stevens DM, Thoreen CC, Burds AA, Kalaany NY, Moffat J, Brown M, Fitzgerald KJ, and Sabatini DM (2006). Ablation in mice of the mTORC components raptor, rictor, or mLST8 reveals that mTORC2 is required for signaling to Akt-FOXO and PKCalpha, but not S6K1. *Dev Cell* 11, 859–871. [PubMed: 17141160]
- Hagiwara A, Cornu M, Cybulski N, Polak P, Betz C, Trapani F, Terracciano L, Heim MH, Ruegg MA, and Hall MN (2012). Hepatic mTORC2 activates glycolysis and lipogenesis through Akt, glucokinase, and SREBP1c. *Cell Metab* 15, 725–738. [PubMed: 22521878]
- Harrison DE, Strong R, Alavez S, Astle CM, DiGiovanni J, Fernandez E, Flurkey K, Garratt M, Gelfond JAL, Javors MA, et al. (2019). Acarbose improves health and lifespan in aging HET3 mice. *Aging Cell* 18, e12898. [PubMed: 30688027]
- Hasek BE, Stewart LK, Henagan TM, Boudreau A, Lenard NR, Black C, Shin J, Huypens P, Malloy VL, Plaisance EP, et al. (2010). Dietary methionine restriction enhances metabolic flexibility and increases uncoupled respiration in both fed and fasted states. *Am J Physiol Regul Integr Comp Physiol* 299, R728–739. [PubMed: 20538896]
- Herman MA, Peroni OD, Villoria J, Schon MR, Abumrad NA, Bluhner M, Klein S, and Kahn BB (2012). A novel ChREBP isoform in adipose tissue regulates systemic glucose metabolism. *Nature* 484, 333–338. [PubMed: 22466288]
- Holzenberger M, Dupont J, Ducos B, Leneuve P, Geloën A, Even PC, Cervera P, and Le Bouc Y (2003). IGF-1 receptor regulates lifespan and resistance to oxidative stress in mice. *Nature* 421, 182–187. [PubMed: 12483226]
- Horton JD, Goldstein JL, and Brown MS (2002). SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. *The Journal of clinical investigation* 109, 1125–1131. [PubMed: 11994399]

- Kalaany NY, and Sabatini DM (2009). Tumours with PI3K activation are resistant to dietary restriction. *Nature* 458, 725–731. [PubMed: 19279572]
- Kane AE, Hilmer SN, Boyer D, Gavin K, Nines D, Howlett SE, de Cabo R, and Mitchell SJ (2016). Impact of Longevity Interventions on a Validated Mouse Clinical Frailty Index. *J Gerontol A Biol Sci Med Sci* 71, 333–339. [PubMed: 25711530]
- Kennedy BK, and Lamming DW (2016). The Mechanistic Target of Rapamycin: The Grand ConducTOR of Metabolism and Aging. *Cell Metab* 23, 990–1003. [PubMed: 27304501]
- Kenyon C (2001). A conserved regulatory system for aging. *Cell* 105, 165–168. [PubMed: 11336665]
- Kleinert M, Parker BL, Fritzen AM, Knudsen JR, Jensen TE, Kjobsted R, Sylow L, Ruegg M, James DE, and Richter EA (2017). Mammalian target of rapamycin complex 2 regulates muscle glucose uptake during exercise in mice. *The Journal of physiology* 595, 4845–4855. [PubMed: 28464351]
- Kraig E, Linehan LA, Liang H, Romo TQ, Liu Q, Wu Y, Benavides AD, Curiel TJ, Javors MA, Musi N, et al. (2018). A randomized control trial to establish the feasibility and safety of rapamycin treatment in an older human cohort: Immunological, physical performance, and cognitive effects. *Exp Gerontol* 105, 53–69. [PubMed: 29408453]
- Kumar A, Harris TE, Keller SR, Choi KM, Magnuson MA, and Lawrence JC Jr. (2008). Muscle-specific deletion of rictor impairs insulin-stimulated glucose transport and enhances Basal glycogen synthase activity. *Mol Cell Biol* 28, 61–70. [PubMed: 17967879]
- Kumar A, Lawrence JC Jr., Jung DY, Ko HJ, Keller SR, Kim JK, Magnuson MA, and Harris TE (2010). Fat cell-specific ablation of rictor in mice impairs insulin-regulated fat cell and whole-body glucose and lipid metabolism. *Diabetes* 59, 1397–1406. [PubMed: 20332342]
- Lamming DW (2014). Diminished mTOR signaling: a common mode of action for endocrine longevity factors. *SpringerPlus* 3, 735. [PubMed: 25674466]
- Lamming DW, and Anderson RM (2014). *Metabolic Effects of Caloric Restriction In eLS* (Chichester: John Wiley & Sons, Ltd).
- Lamming DW, Demirkan G, Boylan JM, Mihaylova MM, Peng T, Ferreira J, Neretti N, Salomon A, Sabatini DM, and Gruppuso PA (2014a). Hepatic signaling by the mechanistic target of rapamycin complex 2 (mTORC2). *FASEB J* 28, 300–315. [PubMed: 24072782]
- Lamming DW, Mihaylova MM, Katajisto P, Baar EL, Yilmaz OH, Hutchins A, Gultekin Y, Gaither R, and Sabatini DM (2014b). Depletion of Rictor, an essential protein component of mTORC2, decreases male lifespan. *Aging Cell* 13, 911–917. [PubMed: 25059582]
- Lamming DW, Ye L, Katajisto P, Goncalves MD, Saitoh M, Stevens DM, Davis JG, Salmon AB, Richardson A, Ahima RS, et al. (2012). Rapamycin-induced insulin resistance is mediated by mTORC2 loss and uncoupled from longevity. *Science* 335, 1638–1643. [PubMed: 22461615]
- Lee KY, Russell SJ, Ussar S, Boucher J, Vernochet C, Mori MA, Smyth G, Rourk M, Cederquist C, Rosen ED, et al. (2013). Lessons on conditional gene targeting in mouse adipose tissue. *Diabetes* 62, 864–874. [PubMed: 23321074]
- Lopez-Lluch G, and Navas P (2016). Calorie restriction as an intervention in ageing. *The Journal of physiology* 594, 2043–2060. [PubMed: 26607973]
- Madeo F, Carmona-Gutierrez D, Hofer SJ, and Kroemer G (2019). Caloric Restriction Mimetics against Age-Associated Disease: Targets, Mechanisms, and Therapeutic Potential. *Cell Metab* 29, 592–610. [PubMed: 30840912]
- Mannick JB, Del Giudice G, Lattanzi M, Valiante NM, Praestgaard J, Huang B, Lonetto MA, Maecker HT, Kovarik J, Carson S, et al. (2014). mTOR inhibition improves immune function in the elderly. *Sci Transl Med* 6, 268ra179.
- Mannick JB, Morris M, Hockey HP, Roma G, Beibel M, Kulmatycki K, Watkins M, Shavlakadze T, Zhou W, Quinn D, et al. (2018). TORC1 inhibition enhances immune function and reduces infections in the elderly. *Sci Transl Med* 10.
- Most J, Tosti V, Redman LM, and Fontana L (2017). Calorie restriction in humans: An update. *Ageing Res Rev* 39, 36–45. [PubMed: 27544442]
- Nojima A, Yamashita M, Yoshida Y, Shimizu I, Ichimiya H, Kamimura N, Kobayashi Y, Ohta S, Ishii N, and Minamino T (2013). Haploinsufficiency of akt1 prolongs the lifespan of mice. *PLoS One* 8, e69178. [PubMed: 23935948]

- Pearson KJ, Lewis KN, Price NL, Chang JW, Perez E, Cascajo MV, Tamashiro KL, Poosala S, Csiszar A, Ungvari Z, et al. (2008). Nrf2 mediates cancer protection but not longevity induced by caloric restriction. *Proc Natl Acad Sci U S A* 105, 2325–2330. [PubMed: 18287083]
- Rockwood K, Blodgett JM, Theou O, Sun MH, Feridooni HA, Mitnitski A, Rose RA, Godin J, Gregson E, and Howlett SE (2017). A Frailty Index Based On Deficit Accumulation Quantifies Mortality Risk in Humans and in Mice. *Scientific reports* 7, 43068. [PubMed: 28220898]
- Rosen ED, and Spiegelman BM (2006). Adipocytes as regulators of energy balance and glucose homeostasis. *Nature* 444, 847–853. [PubMed: 17167472]
- Sarbassov DD, Guertin DA, Ali SM, and Sabatini DM (2005). Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science* 307, 1098–1101. [PubMed: 15718470]
- Schreiber KH, Arriola Apelo SI, Yu D, Brinkman JA, Velarde MC, Syed FA, Liao CY, Baar EL, Carbajal KA, Sherman DS, et al. (2019). A novel rapamycin analog is highly selective for mTORC1 in vivo. *Nature communications* 10, 3194.
- Schreiber KH, Ortiz D, Academia EC, Anies AC, Liao CY, and Kennedy BK (2015). Rapamycin-mediated mTORC2 inhibition is determined by the relative expression of FK506-binding proteins. *Aging Cell* 14, 265–273. [PubMed: 25652038]
- Selman C, Lingard S, Choudhury AI, Batterham RL, Claret M, Clements M, Ramadan F, Okkenhaug K, Schuster E, Blanc E, et al. (2008). Evidence for lifespan extension and delayed age-related biomarkers in insulin receptor substrate 1 null mice. *FASEB J* 22, 807–818. [PubMed: 17928362]
- Shiota C, Woo JT, Lindner J, Shelton KD, and Magnuson MA (2006). Multiallelic disruption of the rictor gene in mice reveals that mTOR complex 2 is essential for fetal growth and viability. *Dev Cell* 11, 583–589. [PubMed: 16962829]
- Solon-Biet SM, Mitchell SJ, Coogan SC, Cogger VC, Gokarn R, McMahon AC, Raubenheimer D, de Cabo R, Simpson SJ, and Le Couteur DG (2015). Dietary Protein to Carbohydrate Ratio and Caloric Restriction: Comparing Metabolic Outcomes in Mice. *Cell reports* 11, 1529–1534. [PubMed: 26027933]
- Speakman JR, Mitchell SE, and Mazidi M (2016). Calories or protein? The effect of dietary restriction on lifespan in rodents is explained by calories alone. *Exp Gerontol* 86, 28–38. [PubMed: 27006163]
- Strong R, Miller RA, Antebi A, Astle CM, Bogue M, Denzel MS, Fernandez E, Flurkey K, Hamilton KL, Lamming DW, et al. (2016). Longer lifespan in male mice treated with a weakly estrogenic agonist, an antioxidant, an alpha-glucosidase inhibitor or a Nrf2-inducer. *Aging Cell* 15, 872–884. [PubMed: 27312235]
- T T. (2015). A Package for Survival Analysis in S.
- Tang Y, Wallace M, Sanchez-Gurmaches J, Hsiao WY, Li H, Lee PL, Vernia S, Metallo CM, and Guertin DA (2016). Adipose tissue mTORC2 regulates ChREBP-driven de novo lipogenesis and hepatic glucose metabolism. *Nature communications* 7, 11365.
- Templeman NM, Flibotte S, Chik JHL, Sinha S, Lim GE, Foster LJ, Nislow C, and Johnson JD (2017). Reduced Circulating Insulin Enhances Insulin Sensitivity in Old Mice and Extends Lifespan. *Cell reports* 20, 451–463. [PubMed: 28700945]
- Uyeda K, and Repa JJ (2006). Carbohydrate response element binding protein, ChREBP, a transcription factor coupling hepatic glucose utilization and lipid synthesis. *Cell Metab* 4, 107–110. [PubMed: 16890538]
- Vijayakumar A, Aryal P, Wen J, Syed I, Vazirani RP, Moraes-Vieira PM, Camporez JP, Gallop MR, Perry RJ, Peroni OD, et al. (2017). Absence of Carbohydrate Response Element Binding Protein in Adipocytes Causes Systemic Insulin Resistance and Impairs Glucose Transport. *Cell reports* 21, 1021–1035. [PubMed: 29069585]
- Whitehead JC, Hildebrand BA, Sun M, Rockwood MR, Rose RA, Rockwood K, and Howlett SE (2014). A clinical frailty index in aging mice: comparisons with frailty index data in humans. *J Gerontol A Biol Sci Med Sci* 69, 621–632. [PubMed: 24051346]
- Williamson R, McNeilly A, and Sutherland C (2012). Insulin resistance in the brain: an old-age or new-age problem? *Biochem Pharmacol* 84, 737–745. [PubMed: 22634336]

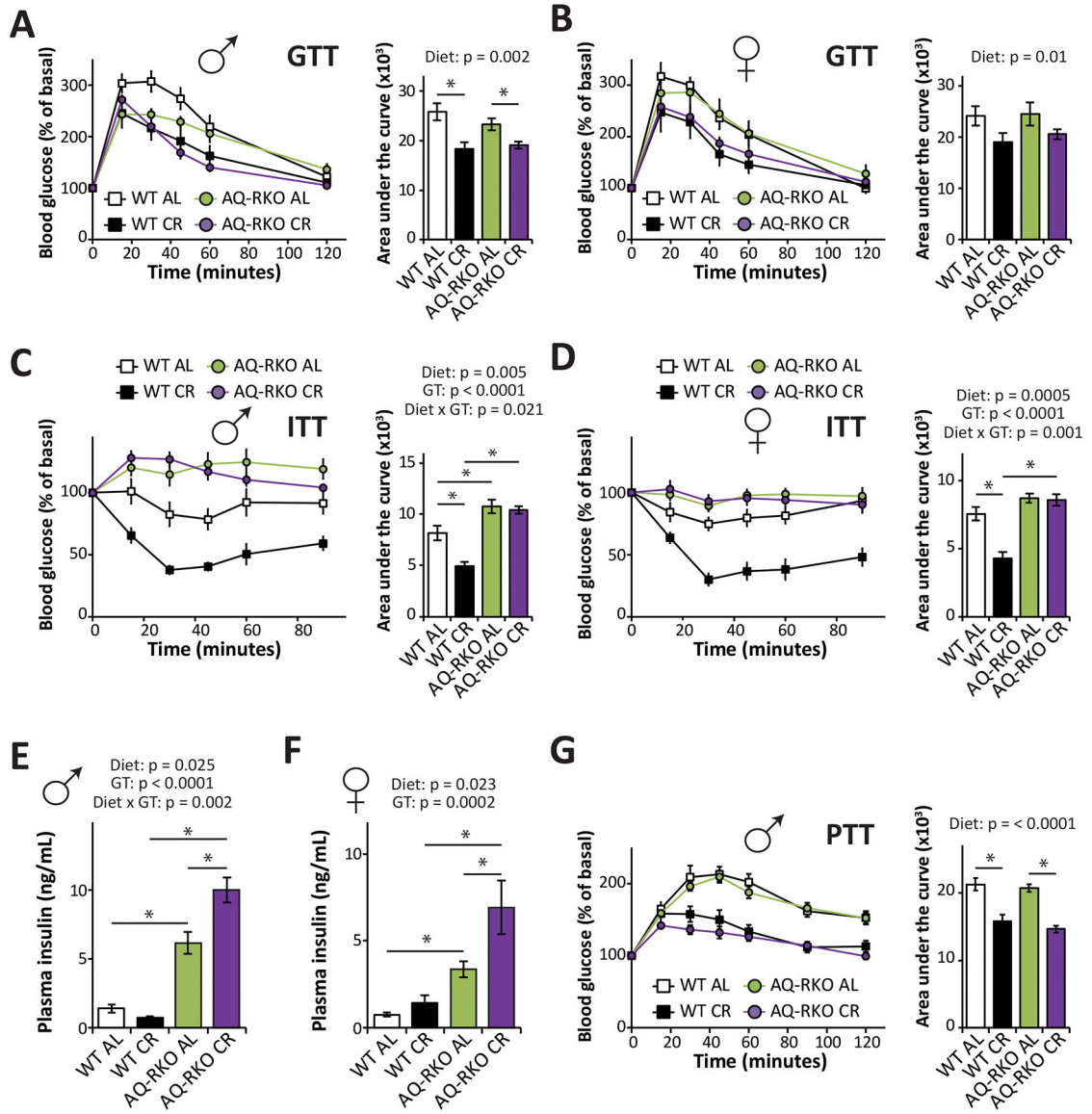


- Yilmaz OH, Katajisto P, Lamming DW, Gultekin Y, Bauer-Rowe KE, Sengupta S, Birsoy K, Dursun A, Yilmaz VO, Selig M, et al. (2012). mTORC1 in the Paneth cell niche couples intestinal stem-cell function to calorie intake. *Nature* 486, 490–495. [PubMed: 22722868]
- Yore MM, Syed I, Moraes-Vieira PM, Zhang T, Herman MA, Homan EA, Patel RT, Lee J, Chen S, Peroni OD, et al. (2014). Discovery of a class of endogenous mammalian lipids with anti-diabetic and anti-inflammatory effects. *Cell* 159, 318–332. [PubMed: 25303528]
- Yu D, Yang SE, Miller BR, Wisinski JA, Sherman DS, Brinkman JA, Tomasiewicz JL, Cummings NE, Kimple ME, Cryns VL, et al. (2018). Short-term methionine deprivation improves metabolic health via sexually dimorphic, mTORC1-independent mechanisms. *FASEB J* 32, 3471–3482. [PubMed: 29401631]
- Yuan M, Pino E, Wu L, Kacergis M, and Soukas AA (2012). Identification of Akt-independent regulation of hepatic lipogenesis by mammalian target of rapamycin (mTOR) complex 2. *J Biol Chem* 287, 29579–29588. [PubMed: 22773877]

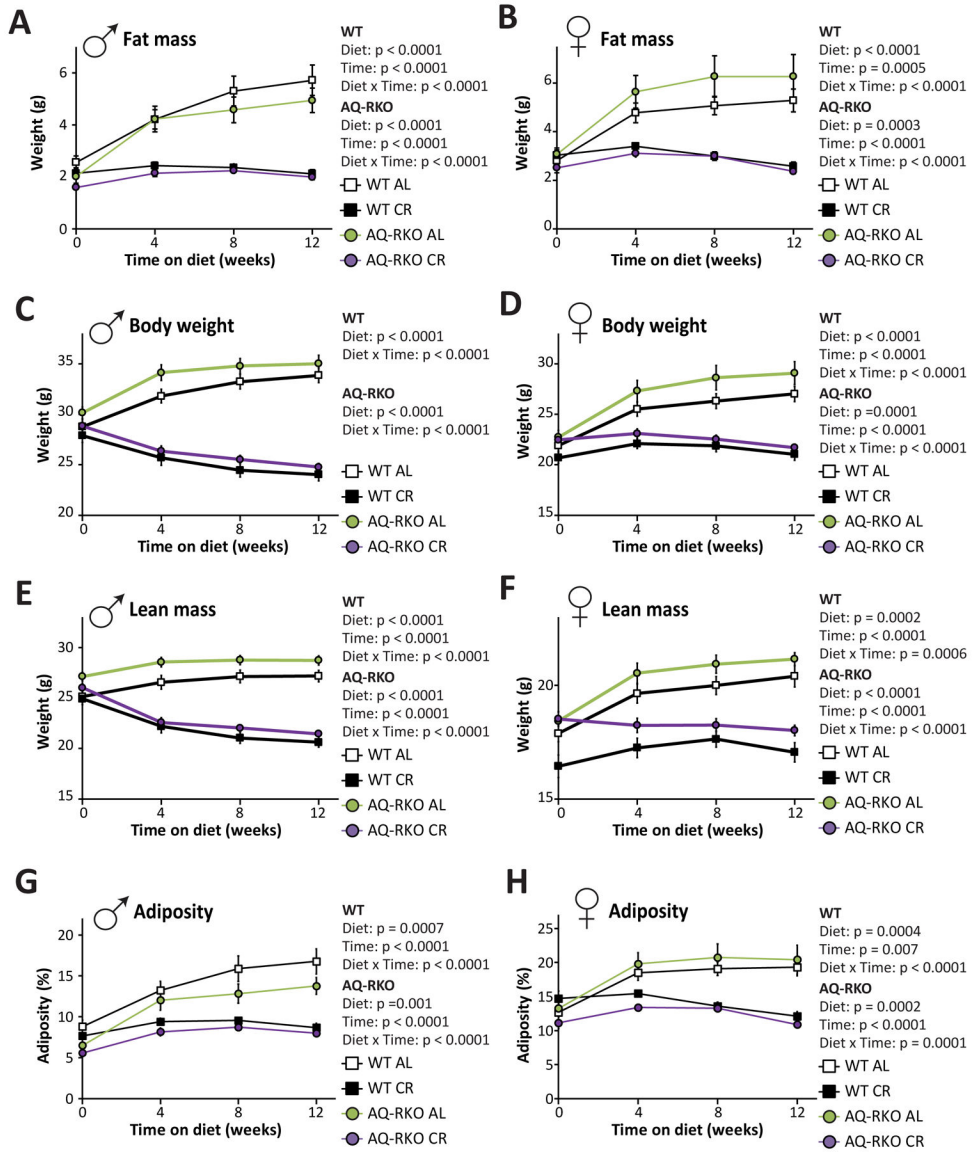


**Figure 1. Mice lacking adipose mTORC2 are glucose tolerant and insulin resistant.** (A) Western blot analysis and quantification of RICTOR expression and mTORC2 activity in the iWAT, eWAT, liver, and muscle of AQ-RKO mice and their wild-type (WT) littermates (n=4/group, \* =  $p < 0.05$ , t-test). (B) Weights of male and female WT and AQ-RKO mice (n = 8–12/group (males), n=4/group (female); statistics for the overall effects of age, genotype (GT), and the interactions represent the p-value from a repeated measures two-way ANOVA conducted separately for each sex). (C and D) Tissue weights of 20-week-old male (C) and female (D) WT and AQ-RKO mice (n=5–9/group, \* =  $p < 0.05$ , t-test). (E) Plasma IGF-1 was determined in male and female WT and AQ-RKO mice (n=5/group, statistics for the overall effects of sex, genotype (GT), and the interaction represent the p-value from a two-way ANOVA; \* =  $p < 0.05$ , from a Holm-Sidak’s post-test examining the effect of parameters identified as significant in the two-way ANOVA). (F and G) Glucose (F) and (G) insulin tolerance was determined in 10–12 week old mice (n=6–9/group; for AUC,

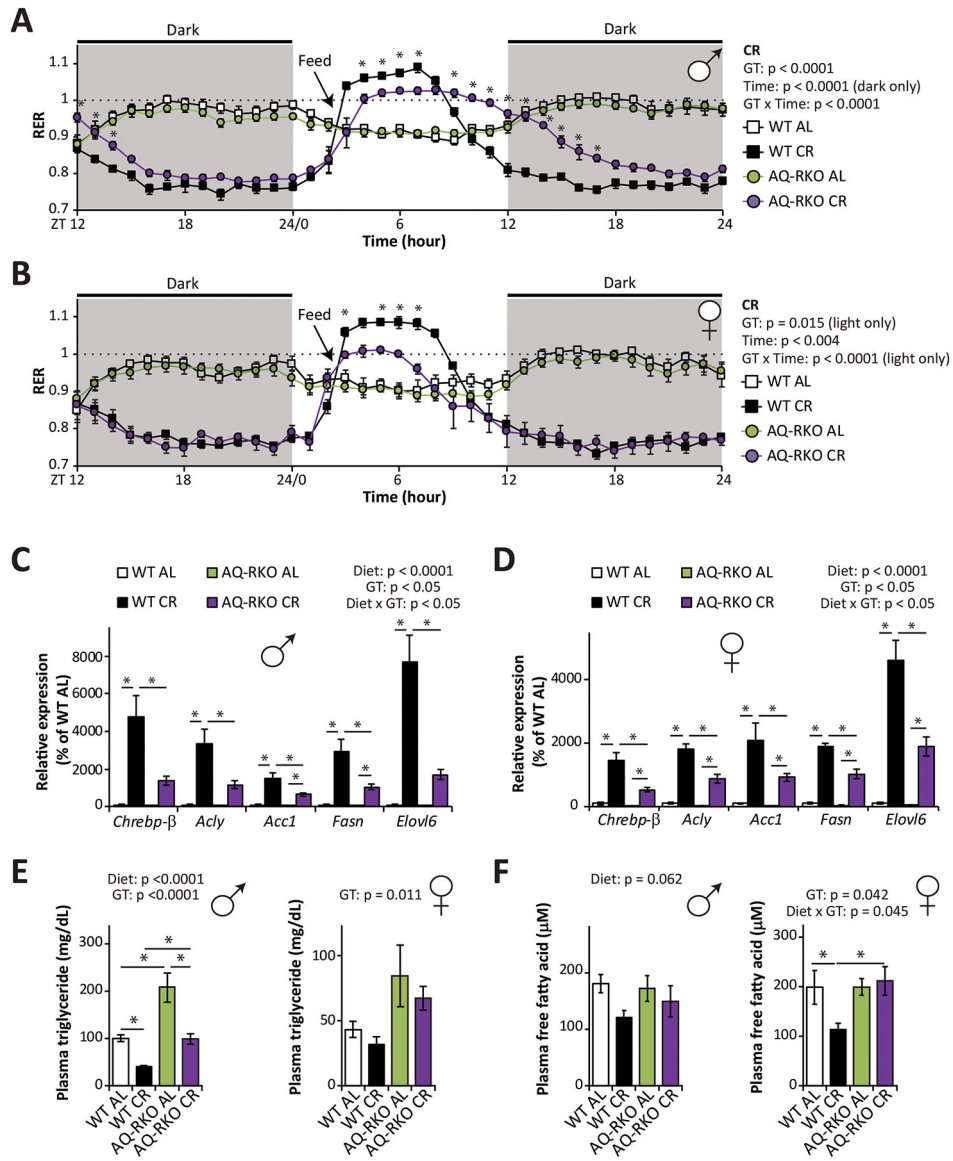
statistics for the overall effects of sex, genotype (GT), and the interaction represent the p-value from a two-way ANOVA, \* =  $p < 0.05$ , from a Holm-Sidak's post-test examining the effect of parameters identified as significant in the two-way ANOVA). Error bars represent SEM.



**Figure 2. Adipose mTORC2 is required for the CR-induced increase in insulin sensitivity.** (A and B) Glucose tolerance test of male (A) and female (B) WT and AQ-RKO mice after 9 weeks on indicated dietary regimens (n=5–14 mice/group). (C and D) Insulin tolerance test of male (C) and female (D) WT and AQ-RKO mice after 10 weeks on indicated dietary regimens (n=9–19 mice/group). (E and F) Plasma insulin in male (E) and female (F) mice after an overnight fast followed by refeeding for 3 hours (n=5–8/group). (G) Pyruvate tolerance test of male WT and AQ-RKO mice after 11 weeks on indicated dietary regimens (n=14–19/group). (A–G) Statistics for the overall effects of diet, genotype (GT), and the interaction represent the p-value from a two-way ANOVA; \* = p < 0.05, from a Holm-Sidak’s post-test examining the effect of parameters identified as significant in the two-way ANOVA. Error bars represent SEM.



**Figure 3. Loss of adipose mTORC2 does not affect the response of body composition to CR.** Fat mass, weight, lean mass, and adiposity of male (A, C, E, and G) and female (B, D, F, and H) WT and Aq-RKO mice fed either AL or CR diets starting at 10 weeks of age (n=12–16/group; Statistics for the overall effects of diet, genotype (GT), and the interaction represent the p-value from a repeated measures two-way ANOVA conducted separately for each genotype). Error bars represent SEM.



**Figure 4. Loss of adipose mTORC2 attenuates CR-mediated reprogramming of adipose lipid metabolism.**

(A and B) Respiratory exchange ratio (RER) of male (A) and female (B) WT and AQ-RKO mice after 7-week AL or CR regimens ( $n = 4-12$ /group; statistics for the overall effects of genotype (GT), time and the interaction represent the p-value from a repeated measures two-way ANOVA conducted on WT CR and AQ-RKO CR mice; \* =  $p < 0.05$ , from a Holm-Sidak's post-test; dark (shaded) and light phases were analyzed separately). (C and D) Expression of *Chrebp-β* and related lipogenic genes in the iWAT of male (C) and female (D) WT and AQ-RKO mice after 12 weeks on the indicated diets ( $n = 6$ /group; statistics for the overall effects of diet, genotype (GT), and the interaction represent the p-value from a two-way ANOVA for each gene; \* =  $p < 0.05$ , from Holm-Sidak's post-tests conducted for the effect of diet and genotype). (E and F) Plasma triglycerides (E) and free fatty acids (F) in WT and AQ-RKO mice after 12 weeks on the indicated diets ( $n = 5-8$ /group; statistics for the overall effects of diet, genotype (GT), and the interaction represent the p-value from a two-

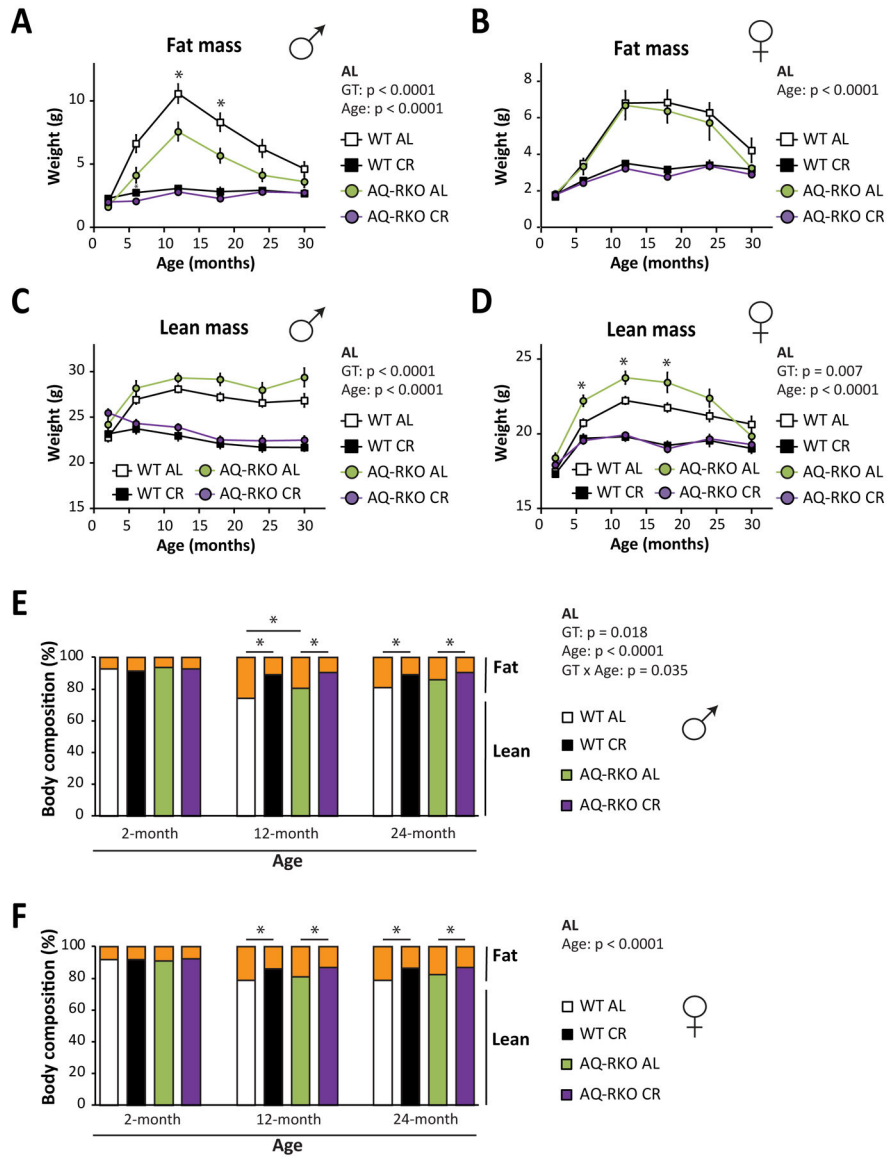
way ANOVA conducted separately for each sex; \* =  $p < 0.05$ , from Holm-Sidak's post-tests conducted for the effect of diet and genotype). Error bars represent SEM.

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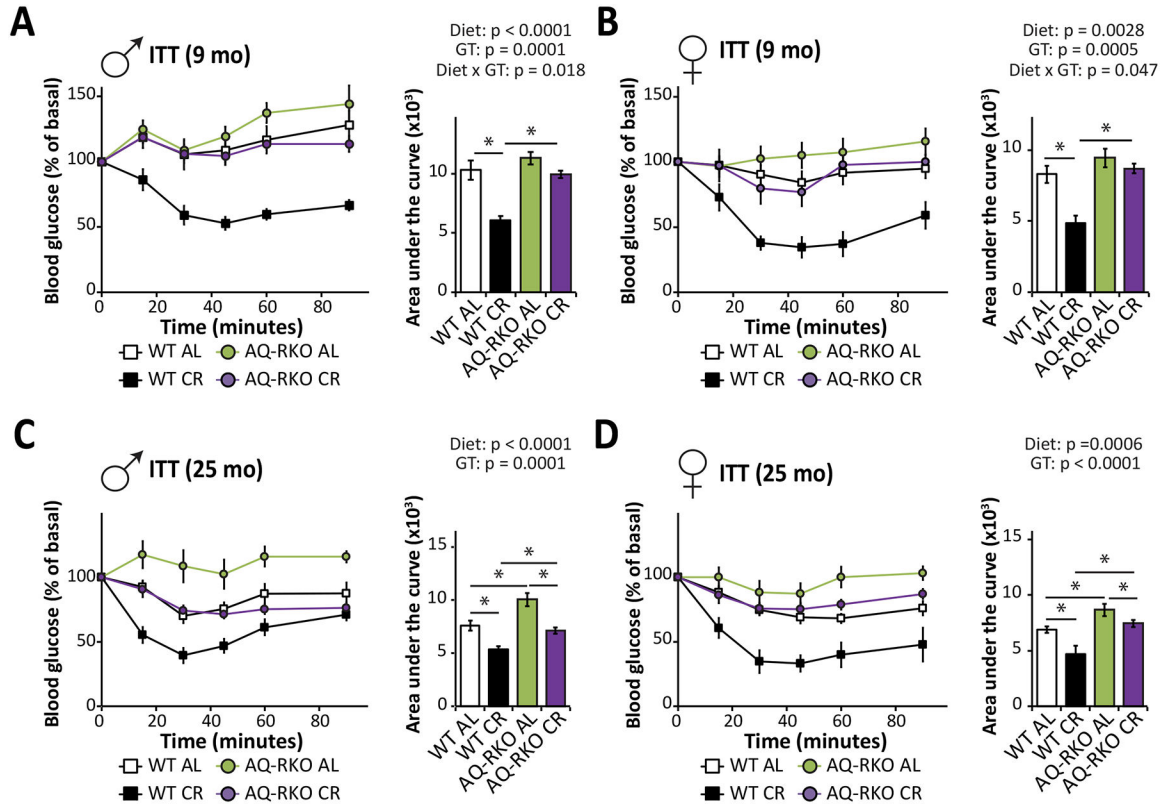
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**Figure 5. Longitudinal assessment of body composition of WT and AQ-RKO mice fed AL or CR diet.**

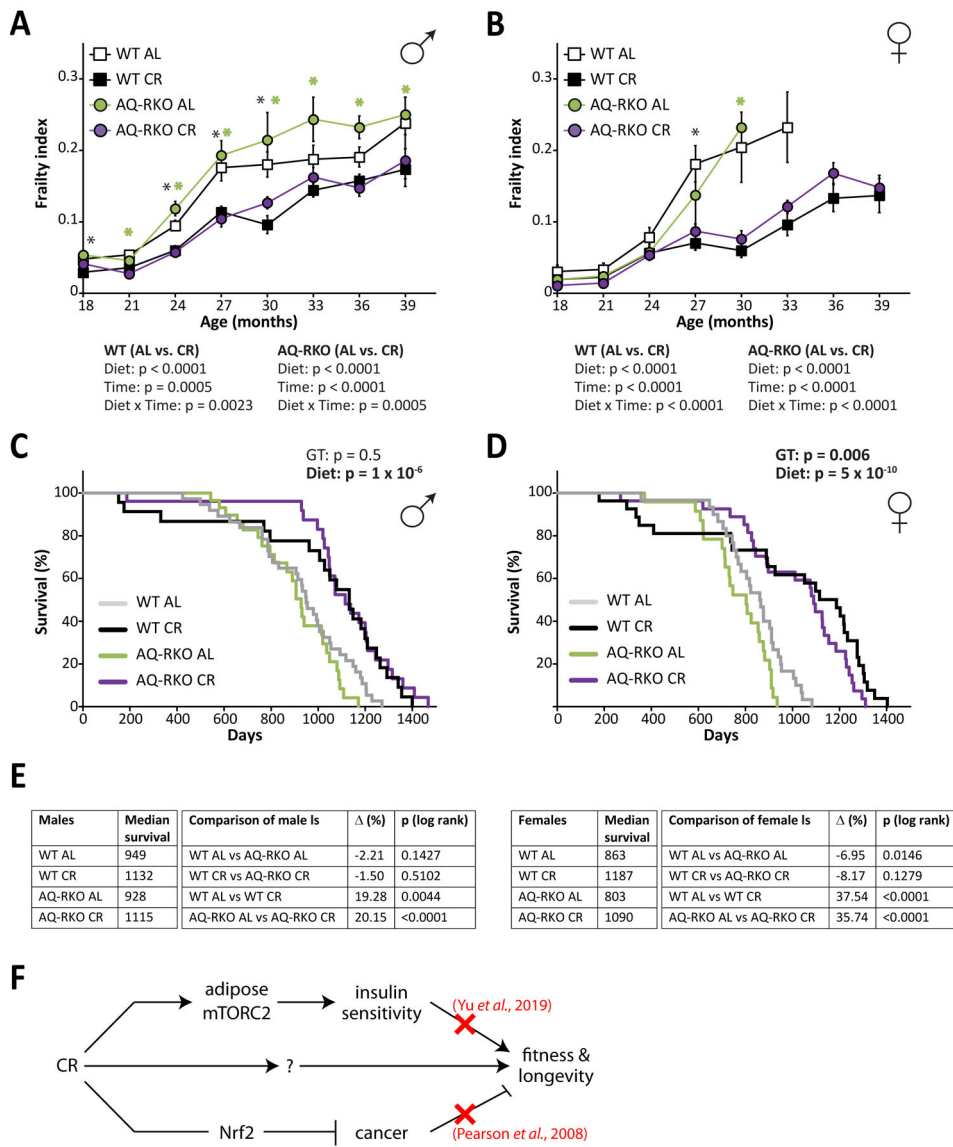
(A-F) Longitudinal body composition analysis of WT and AQ-RKO mice. (A and B) Fat mass of male (A) and female (B) mice. (C and D) Lean mass of male (C) and female (D) mice. (A-D) Numbers vary month-by-month; maximum n = 24–39/group. (E and F) Percentage of body fat mass and lean mass of male (E) and female (F) mice at 2, 12 and 24 months of age (n = 13–28 mice/group). Statistics for the overall effects of genotype (GT), age, and the interaction represent the p-value from a repeated measures two-way ANOVA conducted for AL fed mice; \* = p < 0.05, from Holm-Sidak’s post-test comparing WT AL and AQ-RKO AL fed mice at each age. Error bars represent SEM.





**Figure 6. Deletion of adipose *Rictor* blocks calorie restriction from improving organismal insulin sensitivity.**

Insulin tolerance tests were performed on (A and B) 9-month-old male (A) and female (B) WT and AQ-RKO mice as well as (C and D) 25 month-old male (C) and female (D) WT and AQ-RKO mice. n = 6–13/group; statistics for the overall effects of diet, genotype (GT), and the interaction represent the p-value from a two-way ANOVA for each measurement; \* = p < 0.05, Tukey post-test. Error bars represent SEM.



**Figure 7. Adipose mTORC2 is dispensable for the effects of CR on healthspan and lifespan.** (A and B) Frailty was assessed longitudinally in male (A) and female (B) WT and AQ-RKO mice starting at 18 months of age (numbers vary month by month; maximum n =20–37/ group). Statistics for the overall effects of diet, age, and the interaction represent the p-value from a mixed-effects model (Restricted Maximum Likelihood (REML)) conducted separately for each genotype; \* (black) = p < 0.05, from Holm-Sidak’s post-test comparing WT AL and WT CR-fed mice at each age, \* (green) = p < 0.05, from Holm-Sidak’s post-test comparing AQ-RKO AL and AQ-RKO CR-fed mice at each age). (C and D) Kaplan-Meier plot showing the lifespan of male (C) and female (D) WT and AQ-RKO mice. For each sex, the two-tailed stratified log-rank p-value for the effect of genotype (GT) and diet is indicated. (E) Median lifespans for the survival curves plotted in C and D, and the individual log-rank p-values for the indicated pairwise comparisons. (F) CR effects on fitness and

longevity are independent from its effects on insulin sensitivity and cancer. Error bars represent SEM.

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