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Rapamycin-induced insulin resistance is mediated by mTORC2 loss and uncoupled from longevity

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Abstract

Rapamycin, an inhibitor of mechanistic target of rapamycin complex 1 (mTORC1), extends the lifespans of yeast, flies, and mice. Calorie restriction, which increases lifespan and insulin sensitivity, is proposed to function by inhibition of mTORC1, yet paradoxically, chronic administration of rapamycin substantially impairs glucose tolerance and insulin action. We demonstrate that rapamycin disrupted a second mTOR complex, mTORC2, *in vivo* and that mTORC2 was required for the insulin-mediated suppression of hepatic gluconeogenesis. Further, decreased mTORC1 signaling was sufficient to extend lifespan independently from changes in glucose homeostasis, as female mice heterozygous for both mTOR and mLST8 exhibited decreased mTORC1 activity and extended lifespan, but had normal glucose tolerance and insulin sensitivity. Thus, mTORC2 disruption is an important mediator of the effects of rapamycin *in vivo*.

Age-related diseases, including cancer, neurodegenerative disorders, cardiovascular disease, type II diabetes, and many others, are the major contributors to morbidity and mortality in Western society. The high frequency of these diseases in the elderly limits the benefit that can be obtained by targeting them individually (1). However, targeting the aging process directly may offer a way to delay the incidence of many age-related diseases simultaneously. To date, the only molecule that appears to influence the intrinsic rate of aging in mammals, as evidenced by a robust extension of maximum lifespan, is rapamycin, an inhibitor of

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mechanistic (previously referred to as mammalian) target of rapamycin complex 1 (mTORC1) (2, 3).

mTOR is a kinase that integrates inputs from many nutrients and growth factors. mTOR is found in two distinct protein complexes: mTORC1, which regulates numerous cellular processes related to growth and differentiation, and mTORC2, which has a regulatory role in the insulin signaling cascade. Genetic attenuation of mTORC1 signaling promotes longevity in diverse organisms, including *Saccharomyces cerevisiae*, *Caenorhabditis elegans*, and *Drosophila melanogaster*, and rapamycin-induced lifespan extension has been reported in *S. cerevisiae*, *D. melanogaster*, and mice (2-7). Moreover, deletion of the mTORC1 substrate S6 kinase 1 (S6K1) is sufficient to confer increased lifespan in female mice (8). These findings strongly implicate mTOR in the regulation of mammalian longevity.

Calorie restriction (CR), a reduction in caloric intake while maintaining adequate nutrition, improves health and extends lifespan in many organisms, including primates, through a mechanism that is not understood (9, 10). Yeast that lack the mTOR homolog *TOR1* have an extended replicative lifespan that is not further enhanced by CR, suggesting a common mechanism (4), but in flies, rapamycin increases lifespan beyond the maximum achieved by CR, and thus may act through additional mechanisms (11). Improved glucose tolerance and insulin sensitivity are hallmarks of CR in mammals, and a common feature of many models of increased longevity (12-16), although mice lacking insulin receptor substrate (IRS) proteins 1 or 2 provide a notable counterexample (17, 18). One possible mechanism for enhanced insulin sensitivity during CR is reduced negative feedback through a signaling loop mediated by mTORC1 and S6K1 (19). Indeed, deletion of S6K1 is sufficient to improve insulin sensitivity and extend life in mice (8), suggesting that rapamycin might act in a similar manner.

In contrast to the prediction that rapamycin would mimic the effects of CR or S6K1 deletion on glucose tolerance and insulin sensitivity, recent evidence suggests that chronic treatment with rapamycin impairs glucose homeostasis. Prolonged treatment with rapamycin leads to glucose intolerance in mice (20), as well as insulin resistance in rats (21, 22), and possibly humans (23, 24). We explored the mechanism by which rapamycin treatment impairs glucose homeostasis. Chronic rapamycin treatment, at approximately the same dose that was used to extend lifespan (~2 mg/kg/day), led to glucose intolerance and insulin resistance in both male and female C57BL/6 mice (fig. S1, A and B). In rats, rapamycin-induced glucose intolerance results, in part, from increased hepatic gluconeogenesis (22). We found that rapamycin-treated mice had increased expression of the gluconeogenic genes Phosphoenolpyruvate carboxykinase (PEPCK) and Glucose 6-Phosphatase (G6Pase) in their livers, and had substantially impaired tolerance to pyruvate, indicating a failure to suppress gluconeogenesis (fig. S1, C and D).

We used a hyperinsulinemic-euglycemic clamp to measure insulin sensitivity in rapamycin-treated mice (Fig 1, A to F). Briefly, mice received a constant infusion of insulin, and radio-labeled glucose was co-infused as needed to prevent hypoglycemia. Calculating whole-body glucose uptake and hepatic glucose production, based on the fraction of labeled glucose in the circulation, revealed marked hepatic insulin resistance in rapamycin-treated mice (Fig. 1, C and D). In contrast, uptake of labeled glucose into white adipose and skeletal muscle was unaffected under clamp conditions (Fig. 1E and F). In rapamycin-treated animals, plasma insulin was unchanged during fasting, and higher after re-feeding (Fig. 1G), suggesting an adaptive response to insulin resistance, rather than a defect in insulin production by pancreatic beta cells. These results indicate that hepatic insulin resistance is a major contributor to the impairment of glucose homeostasis by rapamycin, and that white adipose tissue and skeletal muscle take up glucose normally in response to continuous insulin

stimulation, despite inhibition of mTORC1 signaling to S6K1 in all three tissues (Fig. 1H and fig. S2 A and B).

We specifically disrupted hepatic mTORC1 signaling by expressing Cre under the control of the liver-specific albumin promoter in mice carrying a conditional allele of the mTORC1 subunit Raptor (fig. S2C). Alb-Cre *Raptor*^{loxP/loxP} mice, despite lacking hepatic mTORC1 activity, had normal glucose tolerance and remained responsive to rapamycin treatment (Fig. 1I and J). These results indicate that disruption of hepatic mTORC1 signaling cannot account for the effects of rapamycin on glucose homeostasis. Although rapamycin is generally considered to be a specific inhibitor of mTORC1, extended treatment of cells with rapamycin can also physically disrupt mTORC2, independently from changes in protein expression in certain cell lines (25). We therefore examined mTORC2 signaling in livers, white adipose tissue, and muscles from rapamycin-treated mice. Mice were fasted overnight, and then stimulated by re-feeding or by intraperitoneal injection of insulin. Phosphorylation of mTORC2 substrates, including the protein kinases PKC α S657 and Akt S473, as well as the SGK substrate NDRG1 T346 (Fig. 2, A to D) was attenuated in all three tissues. To confirm that these effects resulted from mTORC2 disruption, we immunoprecipitated mTOR from the liver, white adipose tissue, and skeletal muscle of mice treated with rapamycin, and visualized components of mTORC1 and mTORC2 by protein immunoblotting (Fig. 2, E to G). Chronic rapamycin treatment disrupted the association of mTOR with both Raptor (mTORC1) and Rictor (mTORC2) in all three tissues. Therefore, direct disruption of mTORC2 is a second molecular mechanism that may contribute to the effects of rapamycin *in vivo*. Although rapamycin inhibited the phosphorylation of hepatic PKC α and the activity of SGK (as evidenced by decreased phosphorylation of the SGK substrate NDRG1 (26)) in animals that were fasted and re-fed for 45 minutes, the phosphorylation of hepatic Akt S473 was inhibited by rapamycin only in animals stimulated with insulin (49.7% of wild-type level, $p = 0.013$) (Fig. 2, A and B) (25). This may indicate that a small amount of residual mTORC2 is sufficient for basal, but not maximal signaling to Akt.

To examine the effect of hepatic mTORC2 on glucose homeostasis, we generated mice that carried a Cre-excisable allele of the mTORC2 subunit *Rictor* and expressed Cre under the control of the albumin promoter (fig. S3A). Alb-Cre *Rictor*^{loxP/loxP} mice displayed pronounced glucose intolerance (Fig. 3A), but had a mild defect in an insulin tolerance test (fig. S3B), which likely reflected normal glucose uptake into skeletal muscle and adipose tissue. As with rapamycin-treated mice, Alb-Cre *Rictor*^{loxP/loxP} mice had impaired ability to suppress hepatic gluconeogenesis in a pyruvate tolerance test (Fig. 3B), had increased hepatic expression of G6Pase (fig. S4A), and did not show any overt defect in insulin secretion (fig. S4B). Thus, disruption of mTORC2 in the liver is sufficient to impair hepatic insulin sensitivity, and is likely a major contributor to rapamycin-induced glucose intolerance.

Rapamycin disrupts mTORC2 in multiple tissues (Fig. 2, E to G). Deletion of *Rictor* in muscle impairs phosphorylation of Akt S473, although the effect on glucose homeostasis has not been described (27). Deletion of *Rictor* in adipose tissue leads to weight gain, hyperinsulinemia and insulin resistance, with discordant findings reported as to the net effect on glucose tolerance (28, 29). To better understand the consequences of whole-body mTORC2 disruption, we created a strain of mice in which *Rictor* could be conditionally deleted in adult animals with a tamoxifen-inducible Cre (CreERT2) allele expressed under the control of the Ubiquitin C promoter.

Consistent with the idea that rapamycin-induced hepatic insulin resistance is primarily mediated by mTORC2 disruption, there was no further effect on glucose homeostasis when

Ubiquitin C-CreERT2 *Rictor*^{loxP/loxP} were treated for two weeks with rapamycin (Fig. 3C). Interestingly, Alb-Cre *Rictor*^{loxP/loxP} mice treated with rapamycin were hyperglycemic after 6 hours of fasting (fig. S6A) or re-feeding (fig. S4B). This difference may reflect alterations in metabolism as a result of lifelong deficiency in hepatic Rictor. Nevertheless, rapamycin had no further effect on gluconeogenic gene expression, glucose tolerance, or pyruvate tolerance in Alb-Cre *Rictor*^{loxP/loxP} mice (fig. S4A and fig. S6, B to E).

During a hyperinsulinemic-euglycemic clamp (Fig. 3, D to I), tamoxifen-treated Ubiquitin C-CreERT2 *Rictor*^{loxP/loxP} mice had a decreased glucose infusion rate as compared to tamoxifen-treated *Rictor*^{loxP/loxP} controls (Fig. 3D). As with the rapamycin-treated mice, the predominant defect in these animals under clamp conditions was hepatic insulin resistance, resulting in increased glucose production (Fig. 3, F and G). While the overall rates of glucose removal from the blood and uptake into skeletal muscle were not significantly affected, glucose uptake into white adipose tissue was increased (Fig. 3, H and I). Thus, Rictor appears to have a role in the regulation of glucose homeostasis in white adipose tissue, although chronic rapamycin treatment is either not sufficient to elicit this phenotype (Fig. 1E), or the effect is offset by other rapamycin targets, such as mTORC1. Notably, *Rictor* was not completely excised in skeletal muscle, meaning that a role for mTORC2 in this tissue cannot be excluded at present. Thus, both rapamycin treatment and *Rictor* deletion induce clear hepatic insulin resistance, whereas the precise role of mTORC2 in regulating glucose uptake into other tissues remains to be fully defined.

Because the negative effects of rapamycin on glucose tolerance and hepatic insulin sensitivity appear to be mediated by mTORC2 disruption, whereas lifespan extension has generally been presumed to result from mTORC1 inhibition, it may be possible to uncouple the beneficial and detrimental effects of mTOR inhibition. We therefore bred mice carrying only a single copy of either *mtor*, *Raptor*, or *mlst8* or double mutant *mtor*^{+/-} *Raptor*^{+/-} and *mtor*^{+/-} *mlst8*^{+/-} mice, and followed them as they aged.

There was no increase in lifespan in either female (Fig. 4A) or male (Fig. 4B) *mtor*^{+/-}, *Raptor*^{+/-}, *mlst8*^{+/-} or *mtor*^{+/-} *Raptor*^{+/-} mice (see also table S1). However, female *mtor*^{+/-} *mlst8*^{+/-} mice were long-lived, with a 14.4% increase in mean lifespan relative to wild type (Fig. 4C) (Dunnett's test, P = 0.046; Cox P = 0.027)(table S1). The longevity of male *mtor*^{+/-} *mlst8*^{+/-} mice was unaffected (Fig. 4D). Female *mtor*^{+/-} *mlst8*^{+/-} mice were not calorie restricted through reduced food intake or increased energy expenditure, and had normal body weights and levels of activity (fig. S7). Consistent with the phenotypic effects, *mtor*^{+/-} *mlst8*^{+/-} mice exhibited an approximately 30-60% reduction in the abundance of hepatic mTOR, Raptor, mLST8, and Rictor, whereas the expression of mTOR complex subunits was less affected in *Raptor*^{+/-} and *mtor*^{+/-} *Raptor*^{+/-} heterozygotes (fig. S8).

Consistent with the increased lifespan of female *mtor*^{+/-} *mlst8*^{+/-} mice, hepatic mTORC1 signaling, as assessed by S6K1 phosphorylation, was decreased by approximately 50% (fig. S9A and Fig. 4E). Although both mTOR and mLST8 are subunits that are shared between the two mTOR complexes, there was no significant change in mTORC2 signaling as assessed by Akt, PKC α , or NDRG1 phosphorylation. Phosphorylation of 4E-BP1 was not changed in the livers of female *mtor*^{+/-} *mlst8*^{+/-} mice, however, we observed decreased phosphorylation of 4E-BP1 in mouse tail fibroblasts *in vitro* (fig. S10, A and B), suggesting that both S6K and 4E-BP1 signaling may be affected. Hepatic S6K1 T389 phosphorylation was also lower in male *mtor*^{+/-} *mlst8*^{+/-} mice than in controls (fig. S10C, P < 0.08).

Female *mtor*^{+/-} *mlst8*^{+/-} mice had normal glucose tolerance (fig. S9B), insulin sensitivity (fig. S11A), and fasting insulin levels (fig. S11B), further indicating that that mTORC2 signaling was intact. Moreover, female *mtor*^{+/-} *mlst8*^{+/-} mice had decreased expression of

PEPCK and G6Pase, suggesting that control of hepatic glucose production may even have been improved (Fig. 4H). Although absolute amounts of both mTORC1 and mTORC2 decreased in the livers of female *mtor*^{+/-} *mlst8*^{+/-} mice, the ratio of Raptor to mTOR declined more than the ratio of Rictor to mTOR (Fig. 4, I and J). Thus, mTORC2 appears to compete more effectively for a limiting amount of the mTOR catalytic subunit.

Glucose tolerance (area under the curve) was not significantly affected in young animals from any of the strains heterozygous for mTORC1 components, although the glucose tolerance of male *Raptor*^{+/-} mice improved with age (fig. S11, C and D). The increased lifespan and normal glucose tolerance of female *mtor*^{+/-} *mlst8*^{+/-} mice, and the unchanged lifespan despite increased glucose tolerance in male *Raptor*^{+/-} mice are both indicative that the effects of rapamycin on longevity and glucose homeostasis can be uncoupled.

Our results suggest that rapamycin extends lifespan at least in part via inhibition of mTORC1, and despite impairing glucose homeostasis via disruption of mTORC2 signaling. Female *mtor*^{+/-} *mlst8*^{+/-} mice, which had selectively impaired mTORC1 signaling, had increased longevity without overt changes in insulin signaling. Our findings emphasize that mTORC2 disruption profoundly affects metabolism, and may be relevant to the pathogenesis of type 2 diabetes and the metabolic syndrome. *S6K1*^{-/-} mice, which, like *mtor*^{+/-} *mlst8*^{+/-} mice, exhibit female-specific lifespan extension, are resistant to diet-induced weight gain and insulin resistance (8). However, *mtor*^{+/-} *mlst8*^{+/-} mice did not display these phenotypes (fig. S12), demonstrating that factors related to energy balance can also be uncoupled from longevity. Specific disruption of mTORC2 extends lifespan in worms fed a nutrient-rich diet (30). If this effect is conserved in mammals, disruption of mTORC2 may contribute to the pro-longevity effect of rapamycin. Nevertheless, our present findings suggest that specific inhibitors of mTORC1 might provide many of the benefits of rapamycin on health and longevity, while avoiding side effects that currently limit its utility.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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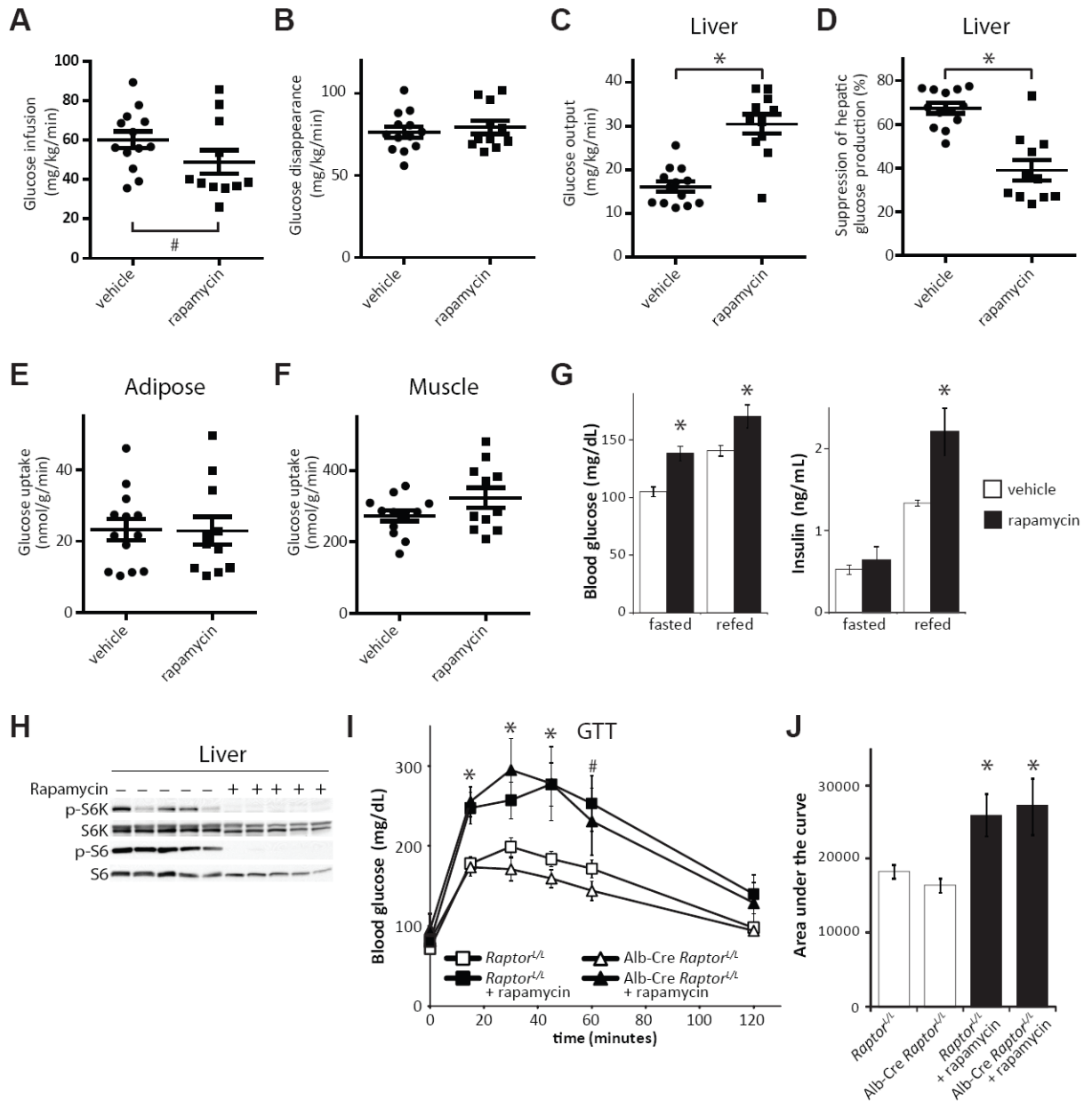


Figure 1. Rapamycin-induced insulin resistance is independent of hepatic mTORC1. A-F) Glucose infusion rate (A), rate of disappearance of glucose from the circulation (B), hepatic gluconeogenesis (C) and insulin responsiveness (D), as well as glucose uptake by white adipose tissue (E) and skeletal muscle (F) were determined during a hyperinsulinemic-euglycemic clamp in mice treated with 2 mg/kg/day rapamycin or vehicle control for two weeks. (Each dot represents a single animal, n = 13 vehicle-treated mice, 11 rapamycin-treated mice, * = $P < 1 \times 10^{-5}$, Student's t-test; # = $P < 0.045$ by Brown-Mood k-sample median test.) G) Serum glucose and insulin concentration in rapamycin-treated mice during fasting and after re-feeding for 4 hours (* = $P < 0.02$). H) Phosphorylation of the mTORC1 substrate S6K1 T389 and subsequent phosphorylation of S6 in the livers of rapamycin-treated mice. I,J) Glucose tolerance with or without rapamycin treatment in mice lacking

hepatic *Raptor* (* = $P < 0.05$, # = $P < 0.06$ for rapamycin treated groups vs. untreated). All bars indicate mean and SEM.

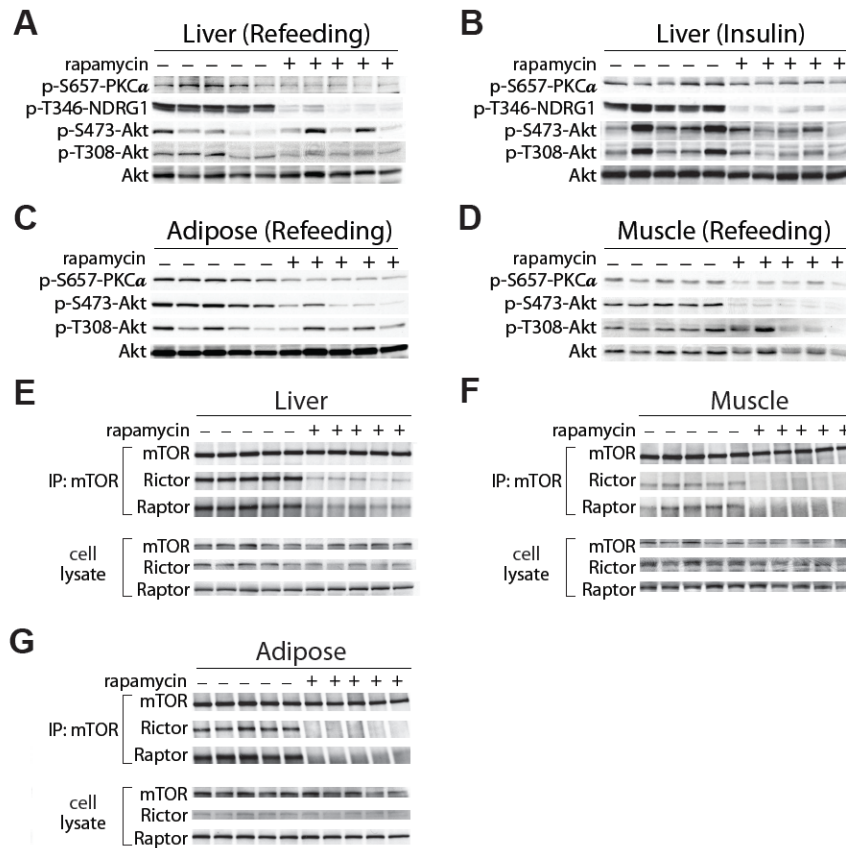


Figure 2. Disruption of mTORC2 *in vivo* after chronic rapamycin treatment. A, B) Effects of rapamycin on phosphorylation of PKC α , Akt, and the SGK substrate NDRG1 in liver in response to re-feeding (A) or insulin (B) after an overnight fast. C, D) Effects of rapamycin on phosphorylation of PKC α and Akt in response to re-feeding in white adipose tissue (C) and muscle (D). E-G) Effects of rapamycin on the integrity of mTORC1 and mTORC2. mTOR was immunoprecipitated from liver (E), skeletal muscle (F), and white adipose tissue (G), followed by immunoblotting for Raptor and Rictor (subunits of mTORC1 and mTORC2, respectively).

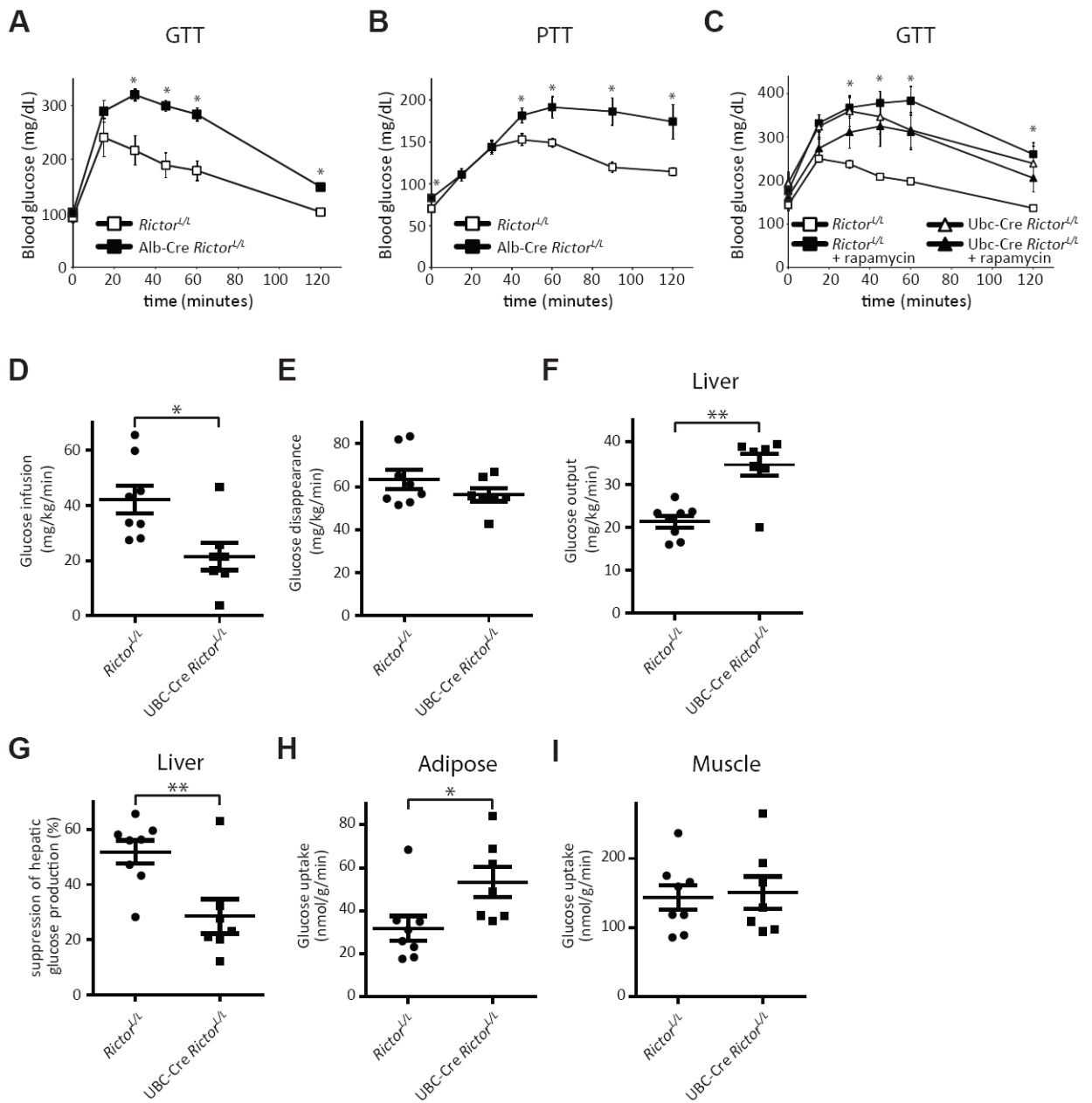


Figure 3. Regulation of glucose homeostasis by mTORC2. A) Glucose tolerance of Alb-Cre *Rictor^{LoxP/LoxP}* mice (* = $P < 0.002$). B) Pyruvate tolerance of Alb-Cre *Rictor^{LoxP/LoxP}* mice (* = $P < 0.03$). C) Effect of rapamycin on glucose tolerance of mice with whole-body deletion of Rictor fasted for 6 hr (* = $P < 0.008$ for all groups vs. wt). D-I) Glucose infusion rate (D), rate of disappearance of glucose from the circulation (E), hepatic gluconeogenesis (F) and insulin responsiveness (G), and glucose uptake by white adipose tissue (H) and skeletal muscle (I) were determined during a hyperinsulinemic-euglycemic clamp in tamoxifen-treated UbiquitinC-CreERT2 *Rictor^{LoxP/LoxP}* mice fasted for 6 hr (n = 8 *Rictor^{LoxP/LoxP}*, 7 UBC-Cre *Rictor^{LoxP/LoxP}*, ** = $P < 0.008$; * = $P < 0.05$). All bars indicate mean and SEM.

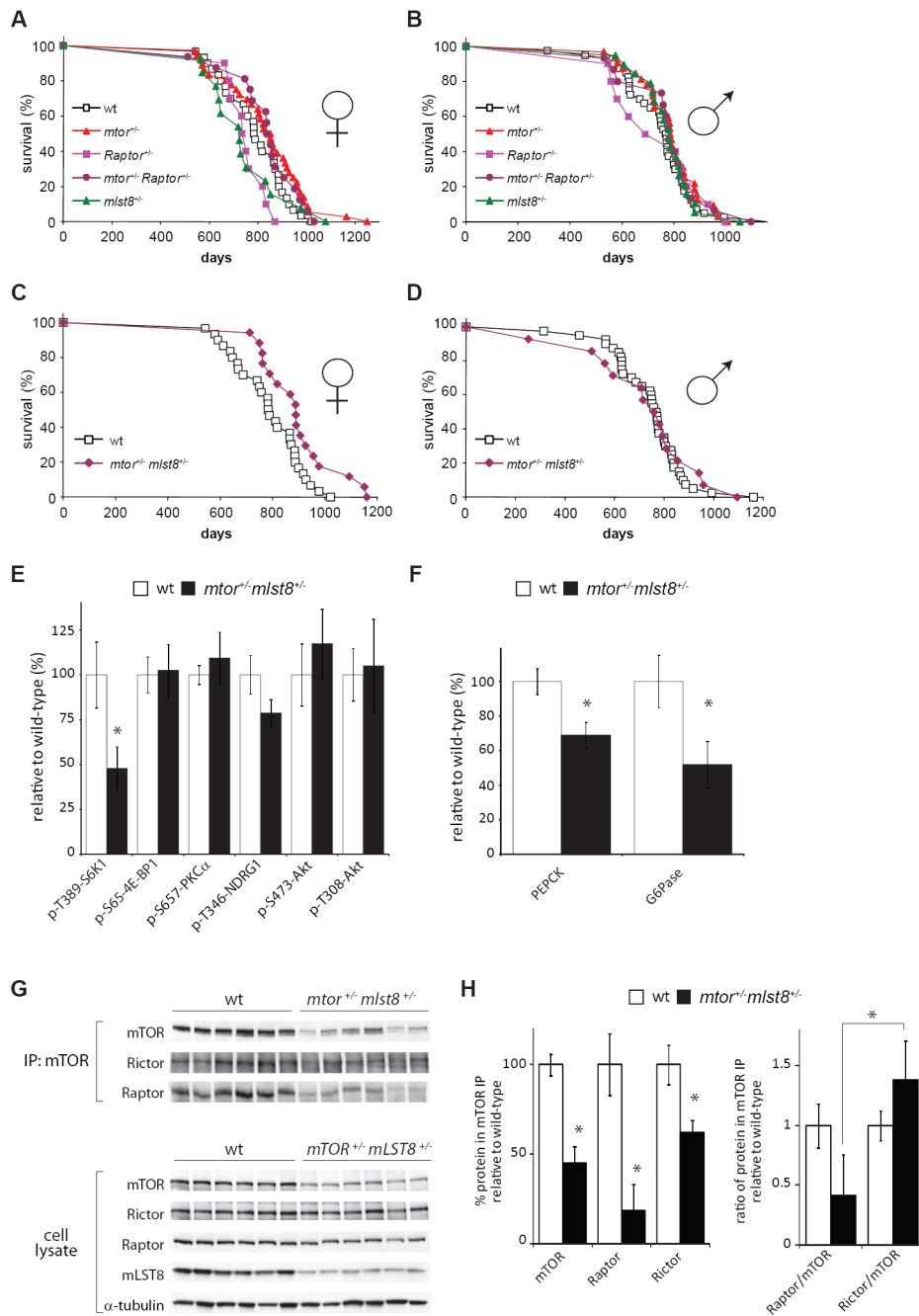


Figure 4. Depletion of mTOR and mLST8 uncouples longevity from decreased glucose tolerance. A, B) Kaplan-Meier plots showing lifespans of female (A) and male (B) mice heterozygous for components of the mTOR signaling pathway. C, D) Lifespans of female (C) and male (D) *mtor*^{+/-} *mLst8*^{+/-} mice. Wild-type curves are repeated for comparison. E) Quantification of phosphorylated proteins in female wild-type and *mtor*^{+/-} *mLst8*^{+/-} livers after an overnight fast and 45 minutes of refeeding (n = 13 wild-type vs. 13 *mtor*^{+/-} *mLst8*^{+/-} female mice, * = P < 0.03). F) Q-PCR of mRNA levels for PEPCK and G6Pase in the livers of young female wild type and *mtor*^{+/-} *mLst8*^{+/-} mice (* = P < 0.03). G, H) Immunoprecipitation of mTOR

complexes reveals preferential loss of Raptor association in *mtor*^{+/-} *mlst8*^{+/-} female mice (* = P < 0.02). All bars indicate mean and SEM.