RagA, but not RagB, is essential for embryonic development and adult mice

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Abstract

The mechanistic target of rapamycin complex 1 (mTORC1) integrates cues from growth factors and nutrients to control metabolism. In contrast to the growth factor input, genetic disruption of nutrient-dependent activation of mTORC1 in mammals remains unexplored. We engineered mice lacking RagA and RagB genes, which encode the GTPases responsible for mTORC1 activation by nutrients. RagB has limited expression and its loss shows no effects on mammalian physiology. RagA deficiency leads to E10.5 embryonic death, loss of mTORC1 activity, and severe growth defects. Primary cells derived from these mice exhibit no regulation of mTORC1 by nutrients, and maintain high sensitivity to growth factors. Deletion of RagA in adult mice is lethal. Upon RagA loss, a myeloid population expands in peripheral tissues. RagA-specific deletion in liver increases cellular responses to growth factors. These results show the essentiality of nutrient sensing for mTORC1 activity in mice and its suppression of PI3K/Akt signalling.

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See Supplementary Experimental Procedures for details.

INTRODUCTION

The mechanistic target of rapamycin (mTOR) is a serine-threonine kinase that, as part of mTOR complex 1 (mTORC1), orchestrates cell growth and anabolism (Laplante and Sabatini, 2012). In mammals, mTORC1 activity is tightly controlled by growth factors, which trigger a cellular cascade that culminates in mTORC1 kinase activation. Nutrient abundance regulates mTORC1 through the activation of the Rag GTPases, which recruit mTORC1 to the lysosomal surface, an essential step for its kinase activation by growth factors. Hence, both inputs, growth factors and nutrients, must be present for maximal mTORC1 activity.

Germline deletion of the Raptor or mTOR genes in mice demonstrates the requirement for mTORC1 activity for mammalian development (Gangloff et al., 2004; Guertin et al., 2006; Murakami et al., 2004). In addition, acute deletion of Raptor in the tissues of adult mice showed that loss of mTORC1 activity in mammals has profound consequences (Polak and Hall, 2009). The physiological importance of growth factor- dependent regulation of mTORC1 in mammals is well established (Goorden et al., 2011). We have previously reported that constitutive RagA activity in mice leads to a neonatal energetic crisis and death (Efeyan et al., 2013), indicating that, unlike constitutive activation by growth factor signaling, constitutive activation of mTORC1 by the nutrient sensing pathway (i.e.: amino acids and glucose), is compatible with embryonic development, but has a detrimental role soon after birth. However, the requirement of the nutrient sensing machinery in mammals has not been determined (reviewed in (Efeyan and Sabatini, 2013; Laplante and Sabatini, 2012)).

Here, we show that abrogation of nutrient-dependent activation of mTORC1, by means of genetic loss of the Rag GTPases, leads to embryonic lethality. Conditional deletion of RagA (but not RagB) in adult mice strongly suppresses mTORC1 activity and leads to PI3K/Akt activation. Furthermore, acute deletion of RagA in adult mice is also lethal and leads to the expansion of a monocytic-like population, which colonizes multiple organs.

RESULTS

RagA is essential for mTORC1 activation and for embryonic development

We have previously generated mice conditionally expressing a constitutively active form of RagA (RagA^{GTP}), by means of a Lox-STOP-Lox strategy (Efeyan et al., 2013). In the absence of the Cre-recombinase, the RagA^{STOP} allele is not expressed and is functionally *null*, and we took advantage of this pre-recombined null allele to determine the consequences of the absence of the RagA gene in mice. While RagA$^{STOP/+}$ mice were viable and seemingly normal, RagA$^{STOP/STOP}$ mice succumbed embryonically. We also generated conventional RagA knock-out mice by means of generating a RagA *floxed* (RagA$^{fl/fl}$) allele; (Supplementary Figure 1a). We then crossed RagA$^{fl/+}$ mice with CMV-Cre transgenic mice, which delete RagA in the germline (referred to as RagA$^\Delta$ or '*deleted*' allele). Through intercrosses of RagA$^{STOP/+}$ mice or RagA$^{\Delta/+}$ mice, we observed that deletion of RagA (RagA$^{-/-}$) leads to embryonic lethality at ~E10.5 days (Supplementary Table 1). Embryos lacking the RagA gene show profound developmental aberrations,

including an open neural tube and severely decreased size, and a proportion was evidently reabsorbed by 10 days (Figure 1a and Supplementary Figure 1b). Consistent with a key role for RagA in the activation of mTORC1 *in vivo*, mTORC1 activity was severely reduced in total protein extracts of embryos lacking the RagA gene (Figure 1b, additional examples in Supplementary Figure 1c). It is unlikely that this reflects an overall decrease in cellular signaling secondary to reabsorption, as protein extracts were prepared from embryos with heartbeats, and furthermore, increased Akt activity, revealed by its phosphorylation at Thr308 or Ser473, was evident in RagA-deficient embryos (Figure 1b). Indeed, when two different RagA-null E10.5 embryos were obtained in the same litter, one alive and one reabsorbed, the former showed high Akt activity, whereas the latter showed decreased Akt activity, when both were compared with wild-type embryos (Supplementary Figure 1c). This result also shows the strong negative regulation that mTORC1 has on Akt activation, as shown previously (Harrington et al., 2004; Haruta et al., 2000; Um et al., 2004). RagB levels are very low or absent in most tissues of adult mice, and also in embryos. Interestingly, embryos lacking RagA frequently showed up-regulation of RagB protein (Figure 1b and Supplementary Figure 1c), suggesting the existence of a mechanism that tries to maintain the total level of the Rag GTPases.

We established primary mouse embryonic fibroblasts (MEFs) cultures from RagA$^{fl/+}$ and RagA$^{fl/fl}$ E13.5 embryos, and deleted the RagA gene by infecting the cells with adenovirus encoding Cre-recombinase. Acute loss of RagA led to partial loss of mTORC1 activation by amino acids or glucose, together with activation of Akt (Figure 1c and Supplementary Figure 1d and 1e). This signaling defect was accompanied by a moderate decrease in proliferation rate (Figure 1d). As non-deleted, RagA$^{fl/fl}$ cells outcompeted RagA$^{/}$ cells, attempts to establish cell lines lacking RagA gene were not successful with this approach.

Nutrient-insensitive mTORC1 activity in RagA-deficient cells in culture

To overcome out-competition of non-recombining RagA$^{fl/fl}$ MEFs in the adenovirus-Cre based approach, we attempted to obtain RagA$^{-/-}$ cells by culturing RagA E10.5 genetically deficient embryos. We readily obtained cultures from RagA-expressing embryos, but the proportion of RagA-deficient cell lines that survived initial *in vitro* conditions was vey low. In addition, longer times in culture were necessary for RagA- deficient cells to start proliferating. Nevertheless, we managed to obtain several independent cell lines. As in the embryos, RagA-deficient cells showed a moderate up- regulation of RagB (Figure 2a and Supplementary Figure 2a and 2b). In contrast to RagA-proficient cells, mTORC1 activity in RagA-deficient cells was insensitive to deprivation of either amino acids or glucose (as determined by phosphorylation of S6K1 and 4EBP1), but maintained regulation by growth factors (Figure 2b and 2c). This suggests that loss of RagA led to the emergence of a RagA-independent mechanism of maintaining mTORC1 activity that contrasts with the very low mTORC1 activity of Ragulator-deficient cells (Sancak et al., 2010). It is unlikely that another kinase, in RagA- deficient cells, is responsible for phosphorylating S6K1 and 4EBP1, as inhibition of mTORC1 with rapamycin ablated the phosphorylation of S6K1 (Supplementary Figure 2c). Furthermore, knockdown of the Rheb GTPase, responsible for stimulating the mTORC1 kinase activity and the endpoint of growth factor input, completely ablated mTORC1 activity in RagA$^{-/-}$ cells (Supplementary Figure 2d). RagA-deficient cells

also showed a marked increase in Akt activation, as determined by the phosphorylation of Thr308 and Ser473 on Akt, which likely helps to compensate for the decrease in mTORC1 caused by RagA loss (Figure 2b). In addition, increased levels of total IRS1 and a strong activation of the MAPK kinase pathway were also evident in these cells (Figure 2b), indicating that activation of growth factor signaling is not restricted to PI3k/Akt. We initially hypothesized that the increase in the abundance of RagB could rescue the loss of RagA in mTORC1 activation, allowing mTORC1 recruitment to the outer lysosomal surface, the site of mTORC1 activation. Inconsistent with this possibility, RagC, the obligate partner of RagA or RagB, did not co-localize with the lysosomal marker Lamp2 (Supplementary Figure 2e), suggesting that a mild elevation in RagB levels could not overcome loss of RagA. Addition of amino acids in RagA-proficient cells recruited mTORC1 to the outer lysosomal surface (Sancak et al., 2010; Sancak et al., 2008). In contrast, in cells lacking RagA, mTORC1 remained diffuse throughout the cytoplasm and did not co-localize with Lamp2 regardless of the presence or absence of amino acids (Figure 2d). To discard a qualitatively different lysosomal morphology that could mislead the interpretation of the results, we performed immunofluorescence staining for another lysosomal marker, Lamp1, in cells fixed in methanol. While methanol fixation precludes staining for mTORC1, it is optimal for preserving membrane structures and for the staining of membrane-bound proteins. We observed a similar lysosomal morphology in RagA-proficient and RagA-deficient cells stained for Lamp1 (Supplementary Figure 2f). This suggested that a RagA-independent mechanism for maintaining mTORC1 activity was at work. In order to explore the reversibility of this phenomenon, we reconstituted Rag activity in RagA$^{-/-}$ cells by stably expressing RagB from a lentiviral plasmid. In these cells, mTORC1 activity was readily inhibited by amino acid withdrawal, and re-addition of amino acids led to mTORC1 activation, thus correcting the insensitivity of RagA-deficient cells (Figure 2e). In agreement with this, forced expression of RagB allowed mTORC1 recruitment to the lysosome surface in the presence, but not in the absence of amino acids (Supplementary Figure 2g). In addition, expression of RagB in RagA$^{-/-}$ cells partially suppressed the activation of Akt (Supplementary Figure 2h). The similar reversion of the nutrient-dependent regulation of mTORC1 activity was observed when we transduced RagA (Supplementary Figure 2i), suggesting that overall Rag activity, rather than specific potential functions of RagA or RagB, was responsible for this rescue.

To analyze the consequences of the lack of regulation of mTORC1 activity by nutrients in RagA-deficient cells, we performed prolonged amino acid starvation and determined the extent to which autophagy, a cellular process of recycling cytoplasmic proteins and organelles that is regulated by mTORC1 (Mizushima et al., 2008), was affected in RagA-deficient cells. A 20-minute amino acid starvation was sufficient to inhibit mTORC1 activity in RagA-proficient cells. However, prolonged amino acid starvation (i.e., at least 2 hours) was necessary for triggering inhibition of mTORC1 activity in RagA-deficient cells (Supplementary Figure 2j). Interestingly, re-addition of amino acids for 10 minutes resulted in no mTORC1 activation in RagA-deficient cells, showing that the mechanism leading to mTORC1 activation in the absence of RagA does not rely on a fast signal transduction cascade (Supplementary Figure 2k). When we looked at the regulation of autophagy markers, we observed that phosphorylation of the mTORC1 target ULK1 was also affected

with slower kinetics in RagA-deficient cells (Supplementary Figure 2j). P62, which is degraded during the process of autophagy, decreased in RagA-proficient and RagA-deficient cells, suggesting that RagA-null are able to trigger autophagy upon prolonged amino acid starvation. In agreement with this, all cells, regardless of RagA status, showed a similar rate of death upon long nutrient withdrawal from the culture medium (data not shown).

Deletion of the RagB gene in mice is innocuous

In order to determine the contribution of RagA versus RagB in the regulation of mTORC1 activity in mice, we generated RagB *floxed* mice by introducing LoxP sites flanking the endogenous exon 3 of RagB gene, encoded on the X chromosome (Supplementary Figure 3a). RagB^{floxed} mice were crossed with CMV-Cre transgenic mice to achieve complete deletion of RagB in the germline. Intercrosses of RagB$^{+/-}$ mice produced RagB$^{+/+}$, RagB$^{+/-}$ and RagB$^{-/-}$ (or male RagB$^{-/0}$) offspring with normal Mendelian ratios, suggesting that loss of RagB has no obvious consequences for embryonic development or for surviving the first weeks of life. Deletion of the RagB gene was confirmed in RagB$^{-/0}$ mice in whole brain extracts (Supplementary Figure 3b). Redundant, and hence, compensatory role of RagA in the context of RagB-null mice was very likely to occur, as RagA has a widespread pattern of expression in most tissues, and is more abundant than RagB in tissues where RagB is expressed to detectable levels, as in the brain (Supplementary Figure 3b). Overall mTORC1 activity was not regulated in whole mouse brain extracts upon fasting and re-feeding, and more importantly, it was also not affected by loss of RagB (Supplementary Figure 3c). In liver extracts, mTORC1 activity was equally regulated by fasting/re-feeding in RagB$^{+/0}$ and RagB$^{-/0}$ mice. It is possible that RagB plays specialized roles in the brain or in other organs, but nevertheless, the undetectable effects of its deletion are in sharp contrast to the deep signaling impairment and embryonic death seen in RagA-deficient mice.

Germline deletion of RagA and RagB

In order to ablate all RagA/B activity and determine if RagB was providing some level of compensation in the context of RagA loss, we generated RagA/B double knock- out mice (RagA/B DKO). At E10.5, the developmental time point when RagA knock-out embryos were found with profound defects but generally still alive, RagA/B DKO embryos were found in low proportions, and undergoing reabsorption (Supplementary Table 2). This suggests that in the absence of RagA, increased levels of RagB can sustain some mTORC1 activity in early stages of embryonic development, and also provides an explanation for the difference with the early death of mTOR-null or Raptor- null embryos (~E3.5 and ~and E6.5, respectively (Gangloff et al., 2004; Guertin et al., 2006; Murakami et al., 2004). Ragulator-null embryos, which ablate all Rag activity but not other inputs to mTORC1, also succumb before E10.5 (Teis et al., 2006), further suggesting a mild compensatory effect of RagB in RagA-deficient embryos.

Deletion of RagA and RagB in hepatocytes

We analyzed the effect of acute loss of RagA in mouse liver, as it is a key organ for coordinating fasting/feeding responses and contributes greatly to glucose homeostasis. We deleted RagA and RagB genes in mouse livers in which Cre recombinase is under the

expression of the albumin promoter (Alb-Cre (Postic et al., 1999). Deletion of RagA in liver led to robust suppression of mTORC1 activity upon overnight fasting and re-feeding, as seen by abrogated phosphorylation of mTORC1 targets in immunoblots (Figure 3a and Supplementary Figure 3d). Profound effects on mTORC1 activation was also observed when glucose or insulin were injected, showing the essentiality of RagA upstream of mTORC1 to allow its activation by the other main activator of mTORC1, growth factors. Similar results were observed when we assessed the phosphorylation of S6 by immunohistochemistry (Figure 3b). Isolated groups of cells in RagA^{fl/fl}; Alb-Cre[+] mice showed an increase in phospho-S6 upon re-feeding, which were clearly identified as hepatocytes at higher magnification (Supplementary Figure 3e), and may be escapers from Cre-mediated recombination. This explains, together with non-hepatocyte cells present in the liver, the residual levels of RagA in western blots. When the same fasting/re-feeding experiment was performed in old (>1 year) mice, regulation of mTORC1 activity was similar to that of wild-type mice (Supplementary Figure 3f), and to a large extent, so were levels of the RagA protein, suggesting the possibility that a proportion of cells that had escaped Cre-mediated DNA recombination increased with time.

Because RagA^{−/−} livers showed a mild increase in RagB protein (Figure 3a, and Supplementary Figure 3d), and in order to determine the impact of complete loss of Rag function, we generated RagA^{fl/fl}; RagB[fl/fl (or fl/0)]; AlbCre[+] mice (RagA/B DKO). In comparison to RagA KO livers, RagA/B DKO livers showed a further decrease in mTORC1 activity, again with hyperactivation of Akt (Supplementary Figure 3g). This suggests that, as in embryos, RagB partially compensates for RagA loss in the activation of mTORC1.

We searched for the consequences of reduced mTORC1 activity and increased PI3K/Akt activities in RagA^{−/−} livers on fasting/re-feeding responses. FoxO1/3 and GSK3, downstream phosphorylation targets of Akt involved in the regulation of glucose homeostasis, showed increased phosphorylation from fasted/re-fed RagA^{−/−} livers (Figure 3c). As expected from the increased inhibitory phosphorylation of the FoxO1/3 transcription factors, we observed transcriptional changes in genes regulated by fasting/re-feeding via the PI3K/Akt/FoxO axis, such as PEPCK, glucose-6-phosphatase and glucokinase (Zhang et al., 2006) (Figure 3d). This correlated with a mild reduction in *ad libitum* blood glucose levels (Supplementary Figure 3h) in young RagA^{fl/fl}; AlbCre[+] mice. While the mice were equally tolerant to glucose in glucose tolerance test (GTT), an increased reduction in glycaemia upon injection of insulin, in insulin tolerance test (ITT), was observed (Figure 3e), suggesting that loss of RagA leads to a better response to insulin *in vivo*. Together with the cell-autonomous effects on Akt activation in cultured RagA-null cells, these findings provide support to the efficiency of dieting (i.e., reduced nutrient intake) and pure mTORC1 inhibitors to control over-activation of mTORC1 and to ameliorate insulin resistance and metabolic syndrome.

Deletion of RagA in adult mice is lethal

Because deletion of RagA in the germline led to embryonic lethality, precluding the analysis of adult RagA-deficient mice, we took advantage of a tamoxifen-inducible Cre-recombinase driven by a ubiquitous promoter (UBC-CRE-ERT2, also denoted herein as UBC-CreER)

(Ruzankina et al., 2007), to achieve full-body acute deletion of RagA in adult mice. Injection of mice with tamoxifen caused excision of RagA and decrease in mTORC1 activity in several tissues (Supplementary Figure 4a and b). RagA$^{fl/fl}$; UBC-CreER$^+$ (RagA-iKO) mice transiently lost ~20% of body weight within 1 week (Figure 4a). More than 50% of RagA-iKO mice succumbed within 2–3 weeks after the start of tamoxifen injections (Figure 4b). The time of death suggested atrophy of the small intestine, which has also been reported for full-body Raptor$^{/}$ mice (Hoshii et al., 2012). Indeed, the small intestine in RagA-iKO mice showed frequent apoptotic figures (Figure 4c).

In addition, RagA-iKO showed a prominent accumulation of cells that resembled monocytes in spleen and bone marrow (Figure 4d). Immunostaining using antibodies against monocyte surface markers confirmed the increase proportion of monocytes in bone marrow and spleen of RagA-iKO compared to their wild-type counterparts (Figure 4e). We then assessed the proliferative status of this lineage by *in vivo* bromodeoxiuridine (BrdU) incorporation. A population of CD11b^{low} Gr-1$^+$ CD115$^-$ cells from bone marrow of RagA-iKO incorporated nearly twice as much BrdU as RagA- sufficient mice (Supplementary Figure 4c). The rapid expansion of monocytes was accompanied by a profound reduction in B lymphocytes and/or their progenitors (Supplementary Figure 4d), as shown previously for Raptor-deleted mice (Hoshii et al., 2012; Kalaitzidis et al., 2012).

The rapid death (within 2–3 weeks) of RagA-iKO mice presumably due to intestinal atrophy was partially penetrant. In the ~40% of mice that survived acute deletion of the RagA gene, the expansion of myeloid cells that could be already detected 2 weeks after tamoxifen, continued to spread in peripheral tissues. It was evident in liver, spleen, and meninges (Figure 4f), and was so severe that mice had to be sacrificed. This disease was histopathologically reminiscent of histiocytic sarcoma, the murine equivalent of monocytic leukemia, and did not stem from escapers from the action of the Cre recombinase, as the deletion of the RagA gene was confirmed by PCR (Supplementary Figure 4e). Furthermore, spleen and purified splenocytes, as well as liver from treated RagA-iKO mice, showed barely detectable levels of RagA protein and low mTORC1 activity (Supplementary Figure 4f). In addition, cultured macrophages obtained from RagA-iKO mice 2 weeks after starting tamoxifen treatment showed reduced levels of RagA, reduced mTORC1 activation, and increased Akt activity (Supplementary Figure 4g).

We also performed bone marrow reconstitution experiments to analyze this myeloid expansion without the detrimental effects of RagA deletion in other tissues, as the small intestine. Irradiated wild-type hosts were reconstituted with bone marrow cells from either RagA$^{fl/fl}$; UBC-CreER$^+$ or RagA$^{fl/fl}$; UBC-CreER$^-$ mice, and treated with tamoxifen after engraftment. Tamoxifen injections in bone marrow chimeras did not lead to the acute (within 2–3 weeks) death observed in full-body RagA$^{fl/fl}$; UBC-CreER$^+$ mice. However, RagA$^{fl/fl}$; UBC-CreER$^+$ chimeras recapitulated the splenomegaly (Supplementary Figure 4h) and the monocyte expansion in bone marrow and peripheral tissues (Figure 4g) that was observed in full-body RagA-iKO mice, and that limited their survival.

DISCUSSION

Absence of RagB in embryos and mice did not show any consequences, had no obvious phenotypes, and were fertile. This is consistent with the limited expression of the RagB gene in most tissues and with the higher expression of RagA throughout the body. In contrast, RagA-null embryos succumbed within ~10–11 days of development, with profound defects and a severe reduction in mTORC1 activity. On the one hand, RagA-deficient embryos demonstrate the requirement of the nutrient dependent input upstream of mTORC1 for its activity. The similar onset of lethality with Rheb-deficient embryos (Goorden et al., 2011) indicates that both major regulators of mTORC1 are essential for life. On the other hand, the difference in the onset of death between RagA- null and mTOR-null (or Raptor-null) embryos can be explained by residual mTORC1 activity in the absence of RagA, which may be due to increased levels of RagB. Finally, the relatively high levels of RagA in all tissues can explain the lack of phenotypes associated with RagB deletion.

The inability to acutely induce deletion of RagA in MEFs prompted us to establish cell lines from E10.5 embryos genetically deficient for RagA. To our surprise, instead of having residual mTORC1 activity, these lines grew with comparable levels of mTORC1 activity that did not respond to acute changes in nutrients, but were able to regulate mTORC1 via slow, perhaps transcriptionally regulated mechanisms. Interestingly, its was recently shown that Rheb-null cells also retain significant levels of mTORC1 activity in culture (Groenewoud et al., 2013). In RagA-null cells, mTORC1 did not localize at the lysosomal surface, indicating that cells underwent an adaptation that enabled high mTORC1 activity in a Rag-independent manner and that involved an up-regulation of the growth factor input.

The effects of the liver-specific ablation of Rag function on mTORC1 activity were severe and comparable to Raptor loss (Sengupta et al., 2010). Deletion of RagA led to activation of PI3K/Akt, and downstream inhibition of the FoxO1/3 transcription factors. Because the FoxO transcriptional program increases *de novo* glucose production, glycaemia in *ad libitum* hepatocyte-specific RagA-null mice was mildly lower. This result has implications in the context of metabolic syndrome therapeutic approaches, as nutrient overload has a strong correlation with the onset of type 2 diabetes (Wang et al., 2011). The data suggest that Rag-dependent regulation of mTORC1 may play a significant role in the correction of insulin resistance and glucose homeostasis observed when nutrient intake is lowered. Interestingly, the biguanide metformin, used to control glucose output in the liver, has been reported to inhibit, among other pathways, the Rag GTPases (Kalender et al., 2010). The data presented herein are consistent with at least part of the beneficial effects of metformin being related to its effects on the regulation of Rag-dependent mTORC1 activation.

Our work shows that deletion of the RagA gene in adult mice leads to many changes similar to those resulting from the loss of the key mTORC1 component Raptor. This is highlighted by the rapid death following tamoxifen injection in adult RagA^{fl/fl}; UBC-CreER[+] mice. Deletion of RagA leads to a rapid expansion of a similar population that is found in meninges, liver, and other tissues, and that limits survival. PTEN deletion, which leads to Akt activation, can also lead to different types of myeloproliferative disease (Tesio et al.,

2013; Yilmaz et al., 2006), again linking reduced mTORC1 activity with increased PI3K/Akt activity.

The solid body of evidence pointing towards mTORC1 as a key regulator of aging is supported by the anti-aging and anti-tumoral effects of rapamycin, a specific albeit incomplete mTORC1 inhibitor. The realization that rapamycin can also block mTORC2 (Lamming et al., 2012; Sarbassov et al., 2006), as mTOR-catalytic inhibitors do (Laplante and Sabatini, 2012), has spurred interest in the development of pure (and complete) mTORC1 inhibitors, that spare mTORC2 as an anti-aging strategy. The results presented herein provide support for beneficial effects of such compounds on insulin sensitivity.

EXPERIMENTAL PROCEDURES

Generation of RagA and B knock out mice

All animal studies and procedures were approved by the MIT Institutional Animal Care and Use Committee. RagA locus was targeted by introducing LoxP sites and frt- neomycin-frt cassette flanking RagA exon (map of the targeting allele is shown in Supplementary Figure 1a). Similarly, RagB locus was targeted by introducing LoxP sites flanking exon 3 (map of the RagB targeting allele is shown in Supplementary Figure 3a). RagA^{STOP} allele was previously described (Efeyan et al., 2013).

Treatments of mice

Fasting was performed from 6 pm to 9 am and then mice were injected with 30 % w/v glucose in PBS 100 μl per 30 g mouse, allow to feed *ad libitum*, or injected i.p. with 11.25 μl of insulin (HumulinR, Lilly) dissolved in 5 ml of PBS at a dose of 100 μl per 30 g mouse.

Treatments of MEFs

Sub-confluent cells were rinsed twice and incubated in RPMI without amino acids and/or glucose, and supplemented with 10% dialyzed FBS, as described (Sancak et al., 2008). Stimulation with glucose (5 mM) or amino acids (concentration as in RPMI) was performed for 10 min. For serum withdrawal, cells were rinsed twice in serum-free DMEM and incubated in serum-free DMEM for the indicated times; 100 nM was used for insulin stimulation.

Statistical analyses

For Kaplan-Meier survival curves, comparisons were made with the Log-rank Mantel- Cox method. For qtPCR analyses, measurements of glycemia, and other comparison between pairs, non-parametric t-tests were performed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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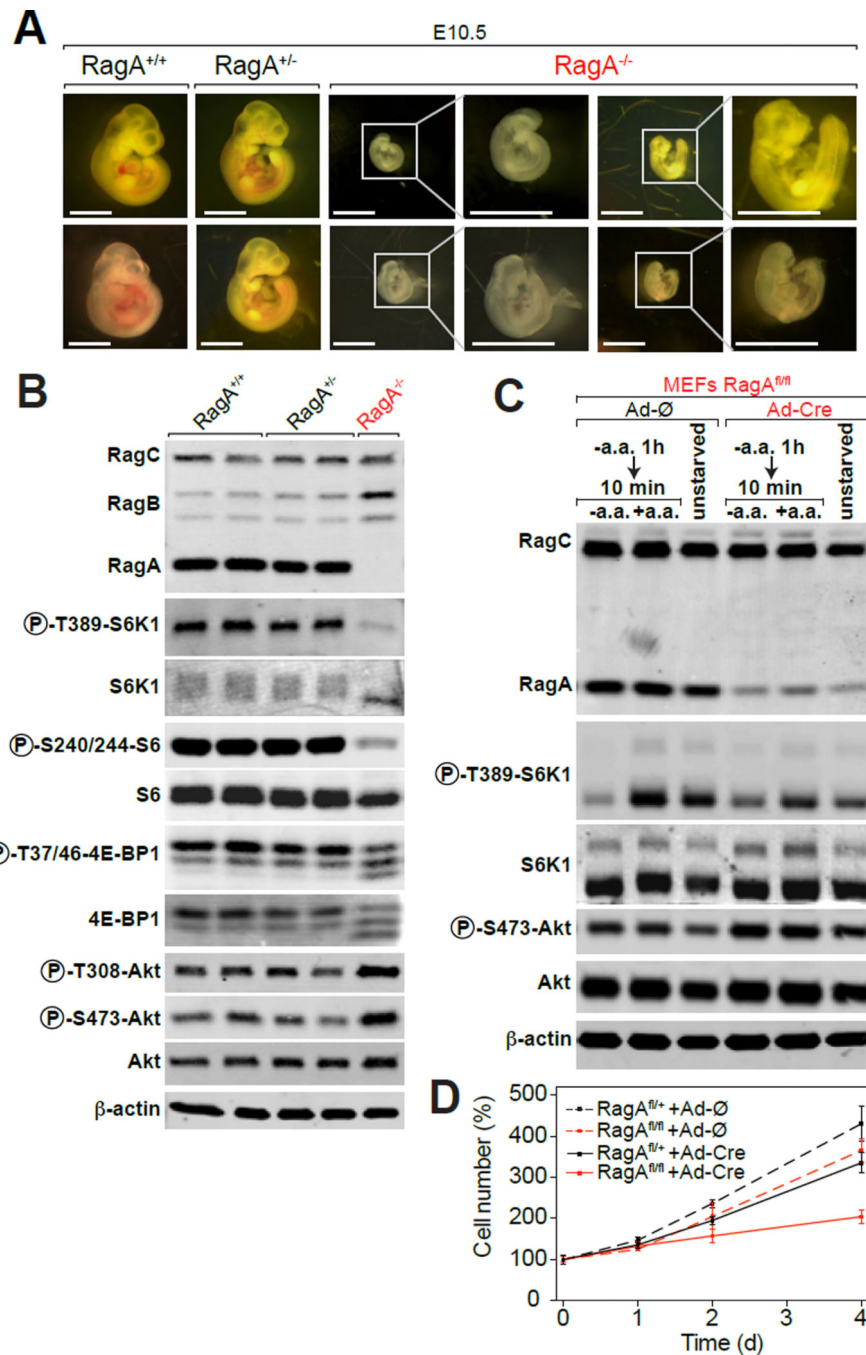
Figure 1. Embryonic lethality of RagA KO mice

(**A**) Representative pictures of E10.5 embryos of the indicated RagA genotype (see also Supplementary Figure 1). (**B**) Whole- embryo protein extracts from RagA$^{+/+}$, RagA$^{+/-}$ and RagA$^{-/-}$ littermates were analyzed by immunoblotting for the indicated proteins. (**C**) RagA$^{fl/fl}$ MEFs were infected with control (Ad-⊘) or adenovirus encoding the Cre recombinase (Ad-Cre), starved for amino acids for 1 h and, where indicated, re-stimulated for 10 min. Total protein extracts were then analyzed by immunoblotting for the indicated

proteins. (**D**) Total cell count of cells infected as in (C) was monitored for 4 days. Data are mean ± SD; n=3.
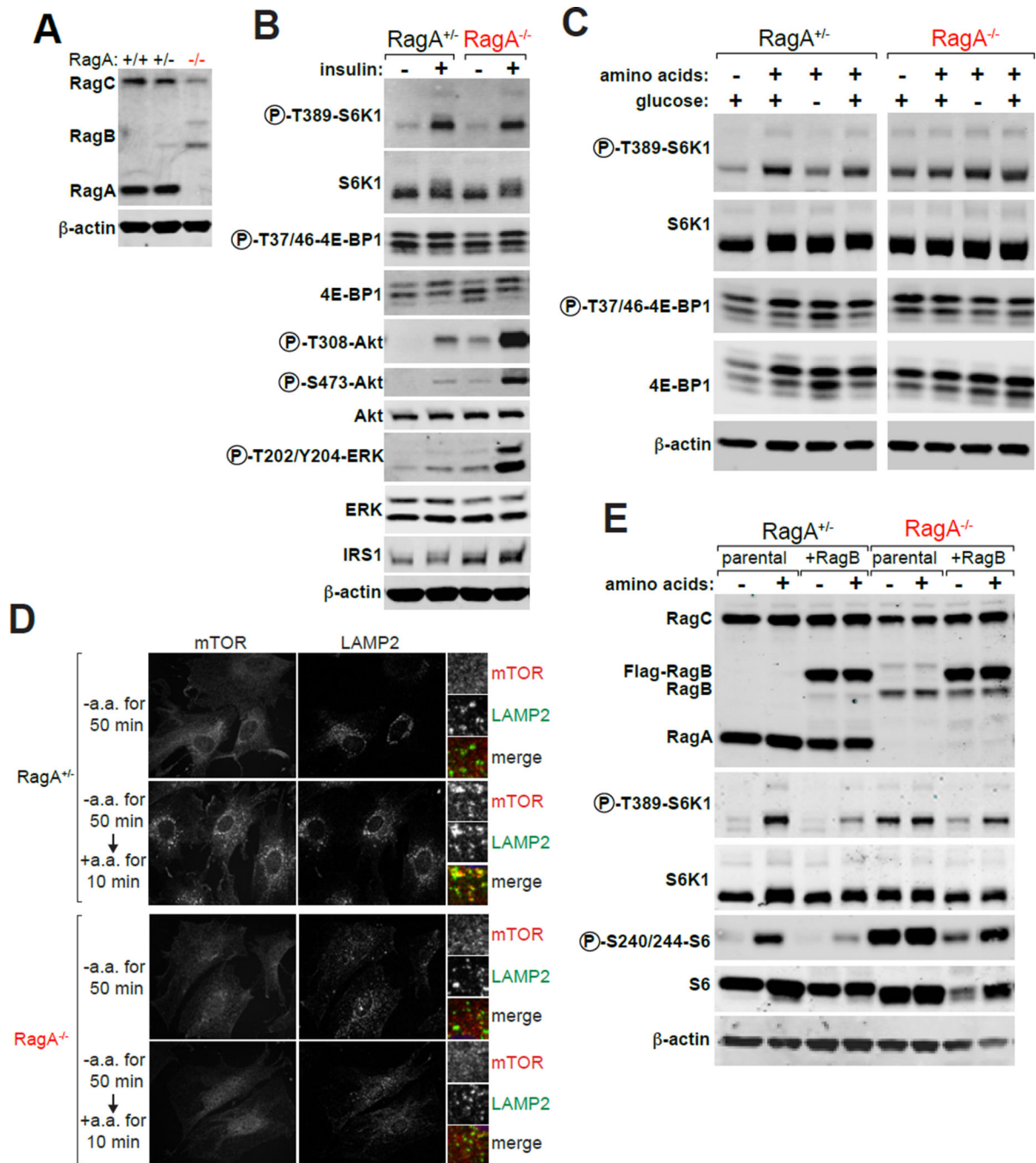
Figure 2. RagA-null cells exhibit nutrient-independent mTORC1 activity
(**A**) Absence of RagA protein in surviving RagA$^{-/-}$ cell lines. (**B**) RagA$^{-/-}$ cells maintain high sensitivity to growth factor deprivation, as determined by immunoblotting for mTORC1 and Akt activation markers. (**C**) RagA$^{-/-}$ cells exhibit constitutive mTORC1 activity regardless of nutrient (amino acid or glucose) deprivation, as determined by immunoblotting for mTORC1 activation markers from whole cell extract after amino acid or glucose starvation of cells followed by re-stimulation. (**D**) mTOR localization by immunofluorescence. In RagA$^{+/-}$ cells, amino acid deprivation causes mTOR to localize to

a diffuse puncta throughout the cytoplasm. Re-addition of amino acids leads to mTOR shuttling to the lysosomal surface, co-localizing with the lysosomal protein Lamp2. In contrast, RagA$^{-/-}$ cells show mTOR constitutively diffuse throughout the cytoplasm, regardless of amino acid levels. (**E**) Constitutive mTORC1 activity regardless of amino acid levels can be reversed by reconstituting Rag activity. RagA$^{-/-}$ cells were infected with a lentivirus encoding RagB^{wt} protein, after starvation and re-stimulation with amino acids whole cell protein extracts were immunoblotted for the indicated proteins (see also Supplementary Figure 2).
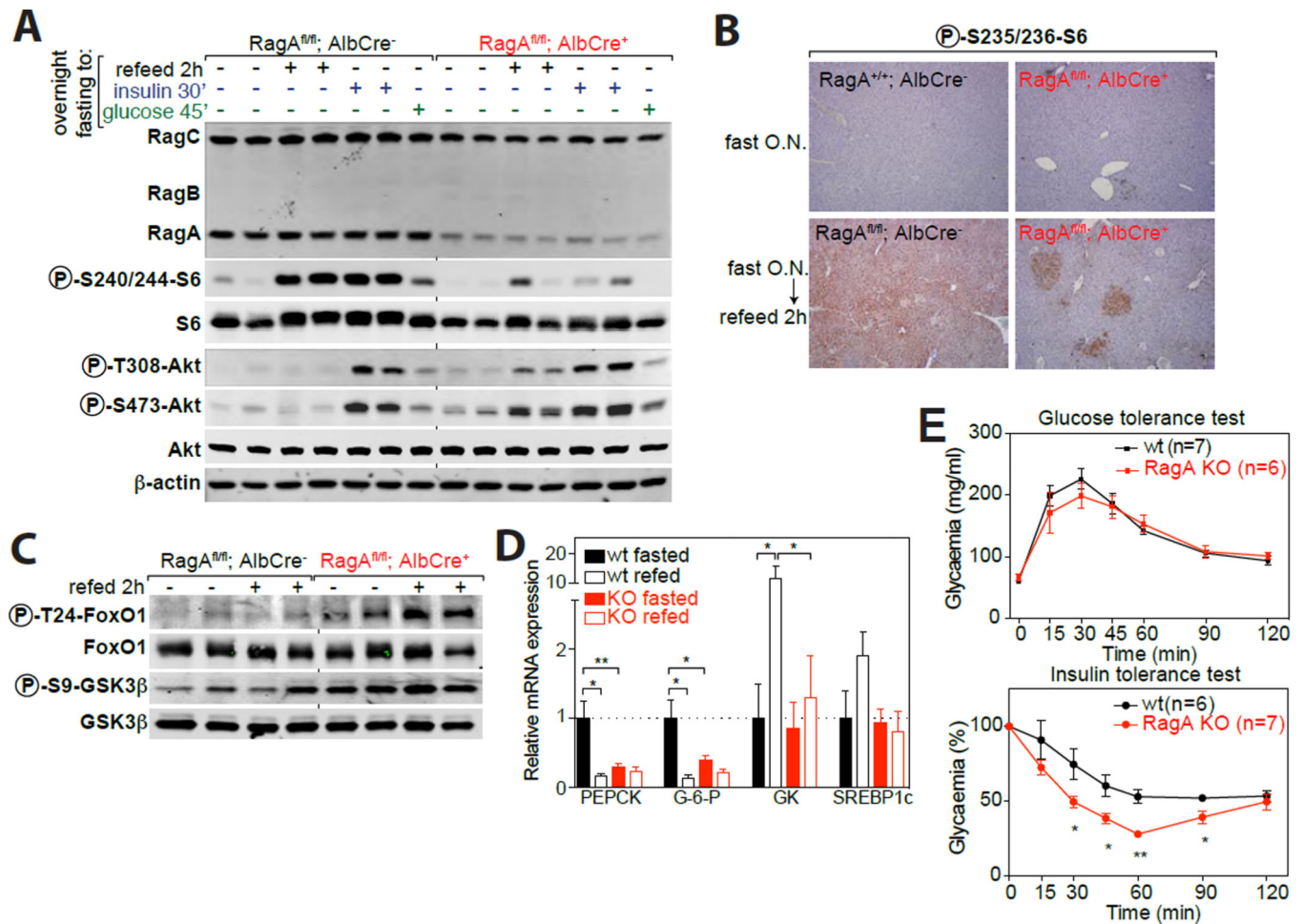
Figure 3. Defective mTORC1 activation in RagA-deficient livers
(**A**) Mice of the indicated genotypes were starved overnight and then either re-fed, injected with glucose or insulin for the indicated times. Whole protein extracts from liver were immunoblotted for indicated proteins. (**B**) Samples as in (A) were analyzed by immunohistochemistry with anti-phospho-S235/236 S6 (see also Supplementary Figure 3). (**C**) Liver samples as in (A) were immunoblotted for Akt phosphorylation targets. (**D**) RNA was extracted from control and RagA$^{fl/fl}$; Alb-Cre$^+$ mice and mRNA expression of the indicated genes determined by qPCR (mean ± SEM, n=5 (wt fasted), 3 (wt re-fed), 7 (KO fasted), 7 (KO refed). (**E**) Glucose tolerance and insulin tolerance tests performed in wt and RagA liver KO mice (mean ± SEM). *: p<0.05; **: p<0.01.
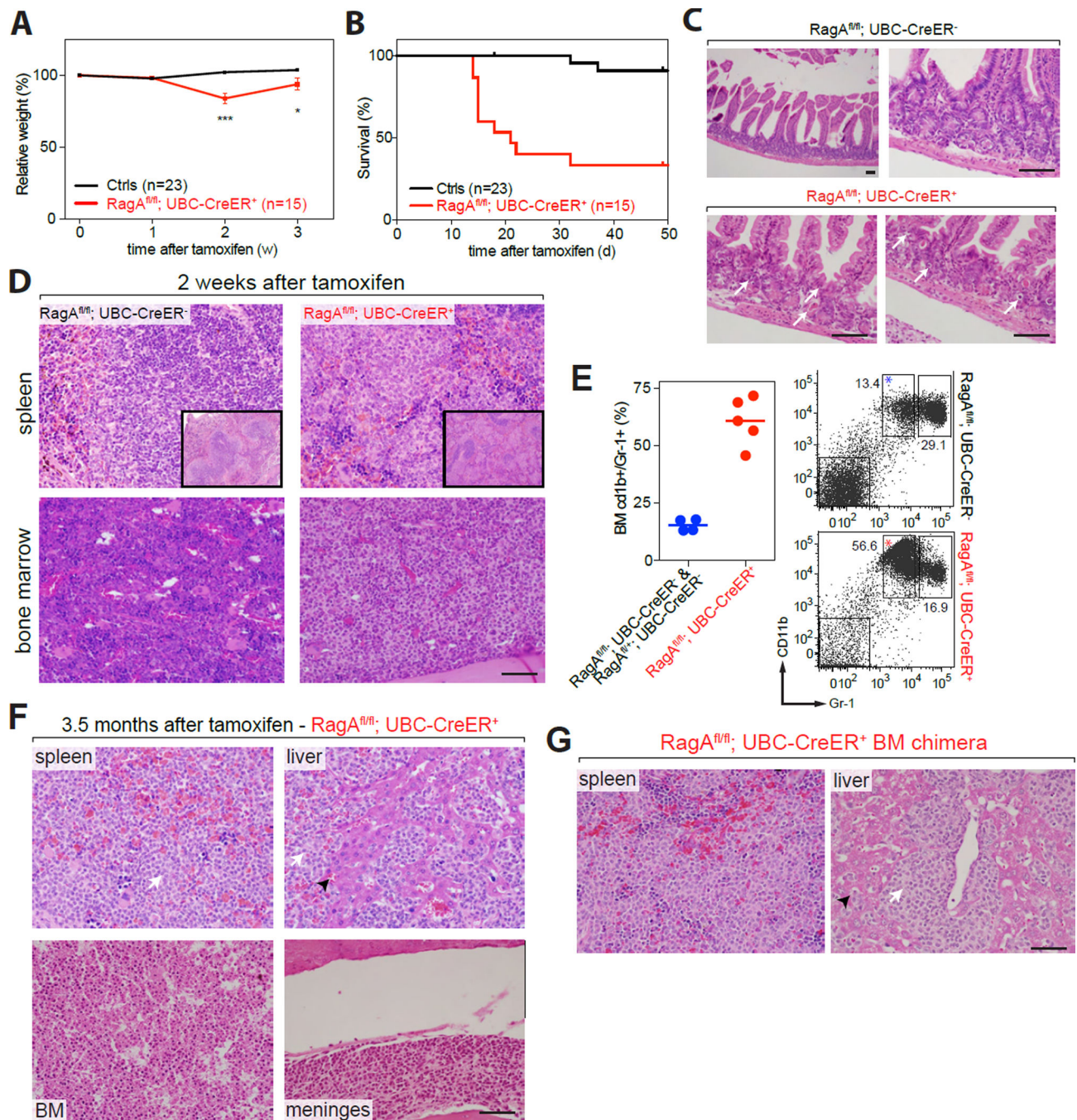
Figure 4. Acute deletion of RagA in adult mice

(**A**) Control (RagA$^{fl/+}$; UBC-CreER$^-$, RagA$^{fl/+}$; UBC-CreER$^+$, RagA$^{fl/fl}$; UBC-CreER$^-$) and RagA$^{fl/fl}$; UBC-CreER$^+$ mice were injected with tamoxifen for Cre activation and weight was monitored every week. (**B**) Kaplan-Meier survival curves after tamoxifen injection of Control (RagA$^{fl/+}$; UBC-CreER$^-$, RagA$^{fl/+}$; UBC-CreER$^+$, RagA$^{fl/fl}$; UBC-CreER$^-$) versus RagA$^{fl/fl}$; UBC-CreER$^+$ mice. (**C**) Representative H&E sections of small intestine from RagA$^{fl/fl}$; UBC-CreER$^-$ mice and RagA$^{fl/fl}$; UBC-CreER$^+$ mice 2 weeks after start of tamoxifen injections. Arrowhead indicates apoptotic figures (**D**) Representative H&E

sections spleen and bone marrow from RagA$^{fl/fl}$; UBC-CreER$^-$ mice and RagA$^{fl/fl}$; UBC-CreER$^+$ mice 2 weeks after start of tamoxifen. (**E**) Bone marrow cells were harvested, immune-stained and analyzed by flow cytometry. Dot plots show the cells gated on Lin$^-$ (CD3/CD19/Ter119/NK1.1) and are representative of one mouse out of 4–5. Gr-1 and CD11b staining was used to the to identify monocytes. Graph shows the quantification of the proportion of resident (or patrolling) BM monocytes (determined by CD11b$^+$ Gr-1^{low}). CD11b$^+$ Gr-1^{high} corresponds to inflammatory monocytes. (**F**) Representative H&E sections of indicated tissues from RagA$^{fl/fl}$; UBC-CreER$^-$ mice and RagA$^{fl/fl}$; UBC- CreER$^+$ mice 3.5 months after tamoxifen injections. Black arrowhead indicates normal tissue; white arrowhead indicates monocytic population. (**G**) Representative H&E sections of liver and spleen from wild-type mice reconstituted with bone marrow from RagA$^{fl/fl}$; UBC-CreER$^-$ mice and RagA$^{fl/fl}$; UBC-CreER$^+$ mice, injected with tamoxifen 6 weeks after bone marrow reconstitution (see also Supplementary Figure 4). Black arrowhead indicates normal tissue; white arrowhead indicates monocytic population. Bar indicates 40 μm.