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REPTOR and REPTOR-BP regulate organismal metabolism and transcription downstream of TORC1

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SUMMARY

TORC1 regulates growth and metabolism in part by influencing transcriptional programs. We identify here REPTOR and REPTOR-BP as transcription factors downstream of TORC1, required for ~90% of the transcriptional induction that occurs upon TORC1 inhibition in *Drosophila*. Thus REPTOR and REPTOR-BP are major effectors of the transcriptional stress response induced upon TORC1 inhibition, analogous to the role of FOXO downstream of Akt. We find that when TORC1 is active, it phosphorylates REPTOR on Ser527 and Ser530, leading to REPTOR cytoplasmic retention. Upon TORC1 inhibition, REPTOR becomes dephosphorylated in a PP2A dependent manner, shuttles into the nucleus, joins its partner REPTOR-BP to bind target genes, and activates their transcription. *In vivo* functional analysis using knockout flies reveals that REPTOR and REPTOR-BP play critical roles in maintaining energy homeostasis and promoting animal survival upon nutrient restriction.

Keywords

Drosophila; metabolism; starvation; development; CREBL2; CREBRF

INTRODUCTION

Target of rapamycin complex 1 (TORC1) integrates information on energy and nutrient status in eukaryotic cells. Under high nutrient and energy conditions TORC1 drives translation, ribosome biogenesis, mitochondrial activity, lipid synthesis, nucleotide synthesis, and glycolysis (Dibble and Manning, 2013). TORC1 thereby couples activity of cellular anabolic and catabolic pathways to nutrient and energy supply (Sengupta et al., 2010b). TORC1 is frequently mis-regulated in diseases such as cancer, diabetes, obesity and neurodegeneration (Cornu et al., 2013; Menon and Manning, 2008; Zoncu et al., 2011).

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TORC1 regulates growth and metabolism by phosphorylating target proteins such as S6K and 4E-BP, involved in translational regulation (Hay and Sonenberg, 2004; Ma and Blenis, 2009; Morita et al., 2013; Thoreen et al., 2012). Phosphorylation of targets changes very rapidly upon altered TORC1 activity (Kang et al., 2013), allowing cells to adapt quickly to changing environmental conditions. In addition, TORC1 also has long-lasting impact on cellular behavior through the control of transcriptional programs (Duvel et al., 2010; Guertin et al., 2006). This occurs by directly or indirectly modulating activity of transcription factors such as SREBP, HIF1a, PGC-1a, TIF1a, PPARa, Atf4 (CREB2), TFEB and TFE3 (Csibi et al., 2013; Cunningham et al., 2007; Duvel et al., 2010; Horton et al., 2002; Hudson et al., 2002; Kim et al., 2012; Martina et al., 2014; Mayer et al., 2004; Peterson et al., 2011; Roczniak-Ferguson et al., 2012; Sengupta et al., 2010a).

The TORC1 signaling pathway is highly conserved through evolution (Kapahi et al., 2010), thereby enabling the use of model organisms such as *Drosophila* for discovery of novel pathway components (Saucedo et al., 2003; Stocker et al., 2003). Recent studies in *Drosophila* analyzed the impact of TORC1 signaling on cellular transcription (Guertin et al., 2006; Zinke et al., 2002). In *Drosophila* S2 cells, inhibition of TORC1 with rapamycin leads to numerous transcriptional changes (Guertin et al., 2006). Genes involved in anabolic processes such as ribosome biogenesis are strongly repressed upon TORC1 inhibition. We previously showed this occurs via down-regulation of myc activity (Teleman et al., 2008). A second class of genes is activated upon TORC1 inhibition. Although the function of these genes is less understood, they probably represent genes needed for cells to adapt to conditions yielding reduced TORC1 activity, such as low nutrient availability. We aimed to find the transcription factor responsible for mediating this up-regulation upon TORC1 inhibition. We report here the discovery of these factors, which surprisingly are required for mediating most of the transcriptional induction that takes place upon TORC1 inhibition, and play important roles in maintaining energy homeostasis *in vivo*.

RESULTS

REPTOR and REPTOR-BP are required for activation of a rapamycin inducible transcriptional reporter in S2 cells

To identify transcription factors responsible for up-regulating target genes upon TORC1 inhibition, we generated a luciferase reporter that recapitulates this regulation. We cloned promoter and/or intronic sequences from early-induced target genes (Guertin et al., 2006) into a luciferase reporter, and found a 1.4kb genomic fragment from the *unk* gene that was 2-fold induced when S2 cells are treated with rapamycin for 6 hours (Fig S1A). Truncations of this fragment identified a minimal 332bp region from *unk* intron 2, capable of inducing luciferase transcription 2.8 fold (Fig S1A). Further truncation of this fragment caused the rapamycin response to be progressively lost (Fig S1A). To make this reporter suitable for screening, we dimerized the enhancer (Fig 1A), yielding a reporter that is activated 10-fold after 6h rapamycin treatment (Fig 1A’). This reporter is induced in a dose-dependent manner by TORC1 inhibition with rapamycin or Torin1 (Fig S1B) (Liu et al., 2010; Thoreen et al., 2009), and is repressed by TORC1 hyperactivation (Fig S1C). Previous reports found that fork head (fkh) and Lipin (Lpin) mediate part of the transcriptional output of TORC1

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(Bulow et al., 2010; Peterson et al., 2011). Neither *fkh* nor *Lpin* knockdown significantly blunted induction of the unk reporter (Fig S1E and S1E') or of a panel of other genes upon rapamycin treatment (Fig S1D, with the exception of *ash2* and *4EBP*), suggesting additional transcriptional mediators remain to be discovered.

To find transcription factors that up-regulate the unk reporter upon TORC1 inhibition, we performed an RNAi screen in S2 cells. We depleted cells of all 1002 genes with predicted DNA binding activity individually (Dataset S1), and tested for unk reporter induction upon rapamycin treatment (Fig 1B). We thereby identified two uncharacterized genes, CG13624 and CG18619, to be required for rapamycin-mediated unk reporter activation (Fig 1B'). Knockdown of CG13624 or CG18619 also blunted induction of the endogenous *unk* gene (Fig 1C), without obvious effects on cell size or viability (not shown). Conversely, when over-expressed, CG13624 and CG18619 could activate the unk reporter (Fig 1D). Since proteome-wide protein-protein interaction screens suggested that CG13624 and CG18619 can bind each other (Guruharsha et al., 2011), we tested if they interact by co-immunoprecipitation. Indeed, myc-CG18619 co-immunoprecipitated CG13624-HA (Fig 1E), and the other way around (Fig S1F and S1F'). Furthermore, CG18619 was also able to homodimerize (Fig S1H). Based on these data, we hypothesized that CG13624 and CG18619 act as a transcriptional activator complex that is repressed by TORC1, and called CG13624 "REPTOR" (REPressed by TOR) and CG18619 "REPTOR-BP" (REPTOR-binding partner) (Fig S1I).

Using SMART (Letunic et al., 2012), we found that REPTOR and REPTOR-BP contain basic region leucine zippers (BRLZ, Fig S1I). This domain mediates both homo/hetero-dimerization and DNA binding through an adjacent basic region (Vinson et al., 1989). DNA binding specificity is determined by the homo- or heterodimer that is formed. To test if REPTOR and REPTOR-BP interact via their BRLZ domains, we performed serial N-terminal truncations of REPTOR, leaving the BRLZ domain intact, and tested if these fragments interact with REPTOR-BP. Indeed, all fragments of REPTOR, including a short one that consists of only the BRLZ domain (ΔN3), co-immunoprecipitated with REPTOR-BP (Fig S1G). In sum, REPTOR and REPTOR-BP form a complex required for up-regulation of *unk* and the unk-reporter upon TORC1 inhibition.

Almost all genes that are transcriptionally induced by rapamycin are REPTOR and REPTOR-BP dependent

In addition to *unk*, other genes such as *ash2* and *stai* are induced by rapamycin in S2 cells (Guertin et al., 2006). Upregulation of *ash2* and *stai* was also REPTOR and REPTOR-BP dependent (Fig 2A), suggesting a more general role for REPTOR and REPTOR-BP in regulating transcription downstream of TORC1. To test this, we performed genome wide expression analysis on cells treated with dsRNA against REPTOR, REPTOR-BP or GFP (as a control) and induced 2 hours +/− rapamycin. In control knockdown cells, 202 genes were induced and 231 genes were downregulated by rapamycin ("GFP", Fig 2B). Strikingly, in cells depleted of REPTOR or REPTOR-BP only 30 and 8 genes were induced, respectively (Fig 2B). Hence ~90% of all genes repressed by TORC1 in S2 cells are downstream of REPTOR and REPTOR-BP. This can be visualized by plotting fold-change upon rapamycin

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treatment for all genes that are significantly ($p<0.05$) induced in control cells (x-axes Fig 2C) versus knockdown cells (y-axes Fig 2C), with equal induction in both conditions on the diagonal. Almost all genes lie off the diagonal, indicating they are induced in control S2 cells but not in REPTOR knockdown cells. In contrast, genes repressed by rapamycin treatment are equally well repressed in the presence or absence of REPTOR and REPTOR-BP (Fig 2D). Hence REPTOR and REPTOR-BP are specifically required for the class of genes induced upon TORC1 inhibition. These microarray data were confirmed by randomly picking strongly and weakly induced genes and measuring their mRNA levels by qRT-PCR (Fig S2A).

189 genes require both REPTOR and REPTOR-BP for their induction in response to TORC1 inhibition, which we define here as 'REPTOR target genes' (Fig S2B, Dataset S2). When subjected to GO-term-enrichment analysis, REPTOR target genes were not strongly enriched for a specific biological process, based on current GO-term annotations, but were generally involved in 'metamorphosis' and 'development' ($p<0.002$, Fig S2C). REPTOR target genes include genes from the insulin/IGF and TOR signaling pathway itself (*4E-BP*, *chico*, *Rheb*), genes involved in autophagy (*Atg8a*, *Atg9*, *Atg2*), metabolic enzymes (*alpha-Est3*, *Glycogenin*), and mitochondrial regulators (*Pink1*, *Marf*) (Fig S2D), processes linked to TORC1 signaling in previous studies (Cunningham et al., 2007; Kim et al., 2011; Morita et al., 2013; Scott et al., 2004). In sum, REPTOR and REPTOR-BP appear to be the main transcription factors responsible for mediating ~90% of the transcriptional repression downstream of TORC1 in S2 cells.

TORC1 controls the subcellular localization of REPTOR to control target gene expression in S2 cells

The data in Fig 1 suggest REPTOR and REPTOR-BP are repressed under normal culture conditions and become active when TORC1 is inhibited. To study the underlying mechanism, we tested if TORC1 activity abrogates binding between REPTOR and REPTOR-BP. However REPTOR-HA binds myc-REPTOR-BP under both high and low TORC1 conditions (Fig 1E and S1F), ruling out this hypothesis. Of note, the conditions used for this coIP experiment completely rupture the cells, allowing cytoplasmic and nuclear proteins to mix in the lysate. Therefore, it only assays the binding affinity between REPTOR and REPTOR-BP, but does not take into account the subcellular partitioning of REPTOR and REPTOR-BP in intact cells. To address this, we studied the subcellular localization of REPTOR and REPTOR-BP. REPTOR-BP is constitutively nuclear regardless of TORC1 activity (in red, Fig 3A). In contrast, REPTOR was enriched in the cytoplasm under normal culture conditions (not shown) or in the presence of insulin (top panels, Fig 3A and S3A), and it translocated into the nucleus upon TORC1 inhibition with rapamycin or Torin1 for 30 minutes (Fig 3A lower panels, S3A, and S3C-C'). To confirm these findings with endogenous REPTOR, we fractionated S2 cells into nuclear and cytoplasmic fractions, and found that endogenous REPTOR (Fig S3B) also accumulated in the nuclear fraction upon rapamycin treatment (Fig 3B, lanes 1 to 4). These data suggest TORC1 represses REPTOR by inhibiting its nuclear accumulation, in a manner analogous to Akt and FOXO.

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S6K mediates a large number of outputs of TORC1 (Ben-Sahra et al., 2013; Wang et al., 2001; Zoncu et al., 2011). To test if TORC1 is acting on REPTOR directly or indirectly via S6K, we first asked if REPTOR binds directly to TORC1. Indeed, REPTOR can co-immunoprecipitate raptor, a component of TORC1 (Fig 3C and 3C') (Hara et al., 2002), suggesting the regulation might be direct. We then asked if TORC1 keeps REPTOR inactive via S6K, but this was not the case; S6K knockdown did not induce the unk reporter in the absence of rapamycin, even when S6K protein amounts were knocked down to undetectable levels (Figs 3D-D'). Thus, TORC1 appears to regulate REPTOR directly and independently of S6K.

Since TORC1 is a protein kinase, the most simple explanation would be that it phosphorylates REPTOR, thereby causing its cytoplasmic retention. To identify possible phosphorylation sites, we delineated the region of REPTOR protein required to regulate its subcellular localization. Successive N-terminal truncations of REPTOR showed that the first 500 amino acids are dispensable for regulating its subcellular localization, whereas further truncations led to a protein that was constitutively nuclear (Fig S3D), suggesting that the region around a.a. 520 contains regulatory information. To pinpoint the phosphorylated residues in REPTOR, we mutated 24 serines and threonines to alanine to mimic dephosphorylation, and searched for mutations rendering REPTOR constitutively nuclear. For instance, a mutant form of REPTOR simultaneously containing 16 alanine mutations (T116A/S457A/S487A/S504A/S505A/T508A/T528A/T542A/S587A/S595A/S596A/S639A/T665A/T689A/S762A/S763A) still shuttled correctly in response to rapamycin treatment (not shown). In contrast, the combined mutation of two serines (S527A and S530A) in the regulatory region identified by the truncation analysis (Fig S3D) rendered REPTOR constitutively nuclear even in the presence of TORC1 activity (Figs 3E-E'). Targeted mass spectrometry analysis of REPTOR-HA immunoprecipitated from S2 cells confirmed that these two serines are phosphorylated when TORC1 is active ("Ins", Fig 3F) and de-phosphorylated when TORC1 is inhibited with rapamycin for 30 minutes ("Ins +Rapa" Fig 3F). By probing immunoprecipitated REPTOR with a phospho-specific antibody recognizing REPTOR doubly-phosphorylated on S527 and S530, we confirmed these sites are phosphorylated only when TORC1 is active (Fig 3G).

Cytoplasmic retention of phospho-proteins can be mediated in part by binding to 14-3-3 proteins. An unbiased screen for REPTOR binding partners, whereby REPTOR co-immunoprecipitating proteins were analyzed by mass spectrometry, identified 14-3-3 proteins as one of the strongest interacting partners (Fig S3E, Dataset S3). Interestingly, this interaction was drastically reduced in rapamycin treated cells (Fig S3E). In agreement with the data presented above, we also detected strong interactions with endogenous REPTOR-BP and TOR kinase in this analysis (Fig S3E). To validate the 14-3-3 results, we raised antibodies against *Drosophila* 14-3-3 proteins (Fig S3F) and found that endogenous 14-3-3ε and 14-3-3ζ bind REPTOR-HA in a TORC1 dependent manner (Fig 3H and S3G).

Upon rapamycin treatment, REPTOR becomes quickly de-phosphorylated (Fig 3G), indicating active dephosphorylation by a phosphatase. Amongst the REPTOR-interacting proteins identified by mass spectrometry were subunits of protein phosphatase 1 (PP1) and 2A (PP2A) (Fig S3H). PP2A caught our attention since PP2A dephosphorylates components

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of the IIS-TOR pathway (Hahn et al., 2010). To test requirement of PP2A for REPTOR dephosphorylation, we inhibited PP2A with okadaic acid (OA) and found this completely abrogated nuclear accumulation of REPTOR (Figs 3I and 3B, lanes 5-8). OA also causes an upshift of REPTOR protein (Fig 3B) in agreement with REPTOR being phosphorylated on many additional sites that are not insulin or rapamycin responsive (Fig S1I). To ensure specificity, okadaic acid was used at a concentration of 50nM, the minimum required to block de-phosphorylation of S6K on T398 upon TORC1 inhibition (Fig S3I) (Hahn et al., 2010).

In sum, these data suggest that when TORC1 is active, REPTOR is phosphorylated and kept in the cytoplasm in part by interaction with 14-3-3 proteins. Upon TORC1 inhibition, REPTOR becomes dephosphorylated, in part via PP2A, and shuttles into the nucleus.

REPTOR has transactivation activity and binds target genes together with REPTOR-BP

To understand how REPTOR and REPTOR-BP activate transcription, we fused REPTOR or REPTOR-BP to the DNA binding domain (DBD) of GAL4 and assayed their ability to activate transcription of a luciferase reporter bearing GAL4 binding sites (UAS) (Fig 4A). This assay revealed that REPTOR has strong transactivation activity whereas REPTOR-BP does not (Fig 4A'). The transactivation activity of REPTOR did not require REPTOR-BP (Fig 4A").

Of the two proteins in the REPTOR/REPTOR-BP complex, our data indicate that REPTOR is the protein regulated by TORC1 phosphorylation, that shuttles in and out of the nucleus, and that has transactivation capacity. This left us with no clear function for its binding partner REPTOR-BP, despite REPTOR-BP being required for activation of target genes (Figs 1B' and 2). We suspected REPTOR-BP might help accumulate REPTOR in the nucleus upon TORC1 inhibition, but this was not the case (Fig S4B-B'). Alternatively, REPTOR-BP could help recruit REPTOR to target genomic DNA – a function performed artificially by the GAL4 DBD in the transactivation assay (Fig 4AA"). To test this, we performed chromatin immunoprecipitation (ChIP) experiments on REPTOR-myc from S2 cells. Upon rapamycin treatment REPTOR binds strongly to DNA within the 2nd intron of *unk*, the region from which the unk reporter is derived (Fig 4B-B'), confirming that *unk* is a direct transcriptional target of REPTOR, as well as to the promoter regions of all target genes that we tested such as 4E-BP, CG16721, REPTOR-BP, CG6770 and CG11658 (Figs 4B" and S4A). In contrast, REPTOR did not bind a control intergenic region downstream of *unk* (Fig 4B-B', "unk IGS"). ChIP of the binding partner REPTOR-BP revealed that it binds unk intron 2 as well as the intergenic region downstream of unk (Fig 4C). Although binding of REPTOR-BP increases upon rapamycin treatment, it also binds chromatin in the absence of rapamycin (Fig 4C). To test if REPTOR-BP helps REPTOR bind target genes, we performed a REPTOR ChIP from cells treated with REPTOR-BP dsRNA. When REPTOR-BP is depleted, REPTOR is unable to efficiently bind target genes upon rapamycin treatment (Fig 4D) despite it entering the nucleus (Fig S4B). Thus, REPTOR-BP resides constitutively in the nucleus and helps REPTOR bind target DNA.

To study REPTOR-BP binding genome-wide, we performed a REPTOR-BP ChIP and sequenced the bound DNA. This revealed that REPTOR-BP binds ~2300 regions genome-

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wide in the presence of rapamycin (Fig S4D, Dataset S5), including the promoter regions of target genes such as CG6770 and PEPCK (Figs S4E-E'). Almost all these regions are also bound by REPTOR-BP in the absence of rapamycin (Figs S4D, E-E', Dataset S5), suggesting that REPTOR-BP is always present at these locations.

To identify a possible binding motif for REPTOR-BP or the REPTOR/REPTOR-BP dimer, we performed a motif search on the REPTOR-BP bound regions using MEME-ChIP software (Machanick and Bailey, 2011). Interestingly, this identified the FOXO binding motif GTAAACAA (Teleman et al., 2008) as the most enriched motif (Fig S4F), suggesting that REPTOR/REPTOR-BP and FOXO bind the same enhancer regions. In addition, other motifs were significantly enriched (Fig S4F). We then scanned the 332nt unk reporter region and found 8 nucleotides at the 3′ end of the reporter required for rapamycin responsiveness (Fig S4G). We mutated each of these 8 nucleotides to all other possible nucleotides, thereby obtaining a matrix of possible sequences that support rapamycin induction (Figs S4G). Interestingly, this sequence has similarities to the motifs identified in the REPTOR-BP ChIP (Fig S4F). Unfortunately we were not able to test if these are REPTOR or REPTOR-BP binding sequences as we were not able to obtain soluble recombinant proteins for gel-shift assays.

Since progressive truncation of the 332nt unk reporter from the 5′ end leads to progressive loss in enhancer activity (Fig S1A), we wondered if epigenetic mechanisms might be at play, which spread over the chromatin. Indeed, ChIP analysis revealed that histone 3 lysine 9 acetylation (H3K9ac), a mark associated with promoter and enhancer activation (Ernst et al., 2011), increases specifically in the unk reporter region after rapamycin treatment (Fig 4E and 4E'). This increase is not due to an overall increase in transcription of the area, as a slightly more upstream region (unk exon 2) did not show such an increase (Fig 4E'). As a control, H3K4me3, also associated with promoter and enhancer activation (Ernst et al., 2011), did not increase (Fig S4C). Hence, REPTOR might be inducing transcription in part via recruitment of epigenetic factors, although further work will be required to study this in detail.

REPTOR and REPTOR-BP regulate organismal metabolism

We asked if REPTOR activity is also regulated by TORC1 in the animal. We generated transgenic flies carrying the unk luciferase reporter (Fig 1A) and equivalent control flies carrying a reporter lacking the unk region. Both unk and control reporters were integrated into the fly genome at exactly the same position by phiC31-mediated site-directed integration, yielding two fly strains that are genetically identical, except for the presence or absence of the 332bp unk enhancer. This controls for all transcriptional effects besides the 332bp unk enhancer. As in S2 cells, the unk reporter, but not the control reporter, is activated by feeding rapamycin to larvae or adults (Figs 5A, S5A and S5B). To generate a tool for assaying inhibition of TORC1 *in vivo*, we generated flies carrying a GFP/lacZ version of the unk reporter. Inhibition of TORC1 in a stripe down the middle of the wing imaginal disc, by expressing PRAS40 (Pallares-Cartes et al., 2012) with patched-GAL4, led to a marked increase in reporter expression (Fig S5C), confirming that this reporter can monitor TORC1 inhibition *in vivo*.

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We generated knockout flies lacking all of the REPTOR ORF (Fig S5D) or most of the REPTOR-BP ORF (Fig S5D'). In REPTOR knockouts, induction of the unk reporter was completely abolished (Fig 5A), indicating that the unk reporter is a good readout for REPTOR, as well as TORC1, activity *in vivo*. Consistent with the activation being direct, we could detect robust induction of reporter expression at the mRNA level already 30 and 120 minutes after rapamycin feeding (Fig S5A). REPTOR activity was also induced in larvae and adult flies upon nutrient withdrawal (Figs 5A' and S5E), indicating that REPTOR, via TORC1, is sensitive to physiological perturbations in nutrient status. Mated females responded strongly to 24h nutrient deprivation, whereas virgin females did not, perhaps due to the increased metabolic demands of egg laying in mated females (Fig S5E). Luciferase assays on tissues isolated from fed or starved animals showed induction of the unk reporter in all tissues (Fig S5F) in agreement with REPTOR and REPTOR-BP being expressed ubiquitously in the animal (Fig S5G-H). Overexpression of dominantly-active REPTOR ("REPTOR[AA]", Fig 3E-G) induces target gene expression in wing imaginal discs more strongly than wild type REPTOR (Fig S5I-I'), indicating that these two sites regulate REPTOR activity also *in vivo*.

We asked if REPTOR and REPTOR-BP mediate a large fraction of the transcriptional response to TORC1 inhibition also in flies. Microarray expression analysis revealed that 407 genes are induced and 377 are repressed at least 2-fold (with $p<0.01$) upon 6 hour rapamycin feeding of control larvae, and that ~90% of the induced (380) and ~80% of the repressed genes do so in a REPTOR dependent manner (Fig S2E, Dataset S4). The overlap of regulated genes in larvae versus S2 cells is not 100% (Fig S2F) likely because larvae are a mixture of many different tissue types. These results were confirmed by qRT-PCR on a panel of genes from a separate biological replicate, with rapamycin-mediated induction of these genes being completely dependent on REPTOR and REPTOR-BP *in vivo* (Figs 5B-B'). GO analysis on REPTOR target genes induced by rapamycin in larvae revealed an enrichment for stress response genes and genes involved in metabolic processes (e.g. Glutamine synthetase 1, Glutamine synthetase 2, alpha/beta hydrolase 2, alpha-Esterase-1, CG31683) (Fig S2G). Repressed genes were enriched for protein transport and folding, with 39% of them also involved in metabolic processes (Fig S2G').

Drosophila larvae eat continuously and grow very rapidly. Once they reach the correct final size, they stop eating and pupate to become adults. Several data indicate that REPTOR activity is low during the nutrient-rich, rapid-growth stages of larval development, when TORC1 activity is high, and that REPTOR activity increases at the end of larval development as animals stop eating and growing: (1) Activity of the unk reporter increases in animals as they progress from larval to pupal stages (Fig S6A). (2) Knocking out REPTOR or REPTOR-BP has little effect on basal (- rapamycin) levels of target genes in larvae (Figs 5B-B' and S6B), suggesting that REPTOR activity is low unless activated by nutrient withdrawal or rapamycin (Figs 5B-B'). In contrast, knocking out REPTOR in adults leads to a clear reduction in basal levels of target genes (Fig S6B'). Correspondingly, $REPTOR^{KO}$ and $REPTOR\text{-}BP^{KO}$ animals do not show strong phenotypes during larval stages under well-fed conditions. They do not have reduced levels of stored nutrients such as glycogen or triacylglycerides (TAG) (wandering 3rd instar larvae "wL3", Figs S6C-C'), and they are not delayed in development (Fig S6D), indicating their rate of growth is normal.

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Consequently, the size of knockout animals is not reduced (Fig S6E), since the mass accumulation occurs during larval stages. In contrast, as REPTOR^{KO} and REPTOR-BP^{KO} animals enter pupation and become adults, they display very strong metabolic phenotypes. REPTOR^{KO} and REPTOR-BP^{KO} pupae and adults have strongly reduced TAG and glycogen levels (Figs 5C-D' and S6C-C'). This leads to extreme sensitivity to starvation; REPTOR^{KO} and REPTOR-BP^{KO} flies die within 18 hours of starvation, whereas control animals survive up to 2.5 days without food (Figs 5E-E'). These phenotypes were rescued by re-introducing a leaky UAS-REPTOR-BP transgene (Fig S6H) into the REPTOR-BP^{KO} background (Fig S5F-G), and phenocopied by knocking down REPTOR with an RNAi construct *in vivo* (Fig S5I). In sum, REPTOR and REPTOR-BP play important roles in the adult in regulating metabolic homeostasis and in enabling flies to survive nutrient deprivation.

Since REPTOR/REPTOR-BP are inactive in larvae due to high TORC1 activity during animal growth, this raised the possibility that REPTOR/REPTOR-BP might be physiologically important also in larvae if TORC1 activity is reduced. To achieve this, we grew larvae on food diluted to 25% the usual concentration using PBS/agarose. Under these conditions, control animals grow slowly and yield small adults, but are viable (Fig 5F). In contrast, under these nutrient conditions REPTOR^{KO} animals display 50% lethality (Fig 5F). Thus REPTOR^{KO} animals are sensitive to nutrient stress both during development and in adulthood (Figs 5E-F). In contrast, REPTOR^{KO} were not hypersensitive to oxidative stress (Fig 5F).

Since REPTOR and REPTOR-BP, like most transcription factors, regulate expression of many genes, the REPTOR/REPTOR-BP knockout phenotype likely results from the combined effect of misregulation of many genes. Microarray analysis of fed REPTOR^{KO} adult flies found 122 genes that are down-regulated (Dataset S6) amongst which are genes involved in gluconeogenesis and TAG biosynthesis (Figs S7C-D), and 86 genes that are up-regulated (Dataset S6), amongst which are genes involved in lipid or glycogen breakdown (Figs S7C-D). Combined, these transcriptional changes likely contribute to the leanness of REPTOR knockouts. REPTOR target genes can also be considered stress response genes, induced when TORC1 is inactivated to cope with the stress. REPTOR^{KO} adults have reduced expression of one such gene, 4E-BP (Fig S7C), which acts as a metabolic brake in tissues such as muscle upon starvation (Teleman et al., 2005). Consistent with this, muscle-specific knockdown of REPTOR partially phenocopies the leanness of knockout animals (Fig S7A-A'). In sum REPTOR/REPTOR-BP likely regulate organismal metabolism by acting on multiple genes in multiple tissues, regulating amongst other things TAG biosynthesis and organismal energy expenditure.

REPTOR and FOXO have overlapping target genes and interact genetically

We noticed that FOXO binding sites are enriched in the genomic regions bound by REPTOR-BP (Fig S4F). Furthermore, 40% of REPTOR targets (including 4E-BP) are also FOXO targets (p score=0, using a binomial distribution) (Teleman et al. 2008). Since this suggests functional overlap between REPTOR and FOXO, we asked if REPTOR and FOXO interact genetically with each other. Indeed, whereas REPTOR and FOXO homozygous mutants animals are viable, REPTOR, FOXO double-mutant animals die as larvae (Fig 5G).

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Furthermore, REPTOR and REPTOR-BP mutant animals have strongly reduced lifespans (Fig S7E), a phenotype also observed in FOXO mutants (Slack et al., 2011).

REPTOR/REPTOR-BP mediate part of the physiological effects of TORC1

Since REPTOR is activated when TORC1 activity is reduced, this raised the possibility that REPTOR/REPTOR-BP might be mediating part of the physiological effects of reduced TORC1 activity. To this end, we tested if removing REPTOR can partly rescue TORC1 loss-of-function phenotypes. One phenotype resulting from TORC1 inhibition is increased autophagy. This could possibly be in part mediated by activation of REPTOR. However if anything, REPTOR knockout larvae have elevated, and not reduced autophagy levels (Fig S7B), suggesting this is not the case. Larvae with reduced TOR activity ($TOR^{2L1/2L19}$) (Oldham et al., 2000) are fat and small in size (Fig 6A-B). Prewandering $REPTOR^{KO}$ larvae that were well-fed during development have neither size nor metabolic phenotypes (Fig 6). Removing REPTOR in the $TOR^{2L1/2L19}$ background significantly rescued both the metabolic phenotype and, surprisingly, the size phenotype of the TOR hypomorphs (Figs 6A-B). This suggests that TOR regulates metabolism and promotes growth in part via repression of REPTOR.

DISCUSSION

We identify here two uncharacterized genes, CG13624 and CG18619, which we term REPTOR and REPTOR-BP respectively, as transcription factors mediating circa 90% of the transcriptional repression downstream of TORC1 in *Drosophila*, indicating they are major effectors of TORC1. REPTOR is inhibited by TORC1-mediated phosphorylation and cytoplasmic retention by 14-3-3 proteins (Fig 7A). Upon nutrient deprivation and low TORC1 activity, REPTOR becomes active, accumulating in the nucleus and binding target genes, a process that requires its partner, REPTOR-BP (Fig 7A).

REPTOR is repressed when TORC1 activity is high, as is the case during larval stages when animals are feeding and growing. Hence, genetic removal of REPTOR during larval stages of well-fed animals has little phenotypic consequences, including no growth defects. In contrast, REPTOR is somewhat activated in (1) pupae and adults, which eat significantly less than larvae, (2) in larvae growing in low-nutrient conditions, and (3) in S2 cells growing in standard culture conditions. Hence, under these conditions, REPTOR loss-of-function leads to transcriptional and physiological phenotypes. The strongest REPTOR phenotypes become apparent when animals are starved, as these are the conditions where TORC1 is most inactive, and hence REPTOR most active (Fig 7A).

The REPTOR regulatory system is analogous to another nutrient sensitive pathway – that of Akt and FOXO. When nutrients are low, Akt becomes inactive due to reduced systemic insulin signaling. This leads to FOXO dephosphorylation, release from 14-3-3 proteins, and nuclear accumulation, thereby activating target genes which mount a stress response (Manning and Cantley, 2007). FOXO and REPTOR can be thought of as the respective counterparts for insulin and TORC1 signaling, which sense nutrients at the systemic and cell-autonomous levels, respectively (Fig 7B). FOXO and REPTOR also have common target genes, and bind near each other in shared enhancers. In sum, FOXO and REPTOR

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appear to have overlapping functions – indeed, genetic removal of both REPTOR and FOXO is synthetic lethal, indicating that they compensate for loss of each other.

As a novel effector of TORC1 function, REPTOR mediates some of the physiological effects of reduced TORC1 activity. Body-wide inhibition of TORC1 signaling, leads to increased TAG levels (Bjedov et al., 2010; Murillo-Maldonado et al., 2011; Teleman et al., 2005) and animals of reduced size (Oldham et al., 2000; Zhang et al., 2000). These phenotypes are in part due to activation of REPTOR, since removal of REPTOR strongly rescues them. Thus, TORC1 promotes growth during development not only by activating S6K but also by keeping REPTOR/REPTOR-BP repressed. Inhibition of TORC1 also extends lifespan (Bjedov et al., 2010; Kapahi et al., 2004). This could potentially be mediated in part via activation of REPTOR/REPTOR-BP, since both REPTOR and REPTOR-BP knockout animals have significantly reduced lifespans (Fig S7E).

We quantified triglyceride levels in $TOR^{2L1/2L19}$ hypomorphic larvae and found them to be significantly elevated compared to controls (Figure 6A), in line with a number of reports from adult flies (Bjedov et al., 2010; Bohni et al., 1999; Broughton et al., 2005; Haselton et al., 2010; Murillo-Maldonado et al., 2011; Teleman et al., 2005). Our results do not fit with one report that $dTOR^{7/P}$ mutant larvae are lean (Luong et al., 2006). We do not know the reason for this discrepancy, and whether it has to do with the particular nature of the dTOR[7] and/or dTOR[P] alleles. Further work will be required to look at this carefully.

Both REPTOR and REPTOR-BP proteins have DNA binding domains. Hence the DNA binding specificity of the REPTOR/REPTOR-BP dimer likely reflects the combined action of the two proteins. Since REPTOR-BP can also homodimerize, the REPTOR-BP homodimer might bind DNA with a distinct pattern from the REPTOR/REPTOR-BP dimer.

Many of the genes repressed by rapamycin in larvae (~80%) were no longer repressed by rapamycin in REPTOR knockout larvae, raising the possibility that these genes are also REPTOR targets. We do not think, however, this is the case for several reasons: (1) In S2 cells, almost no genes require REPTOR to be repressed by rapamycin. If REPTOR were also involved in transcriptional repression, we think we would likely see this also in S2 cells. (2) The REPTOR-induced genes are in common between S2 cells and larvae whereas the REPTOR-repressed ones are not, suggesting their regulation might result from indirect effects. (3) Transactivation assays with REPTOR and REPTOR-BP only show strong transcriptional activation of the reporters, and no repression. That said, many transcription complexes have both activating and repressive activities, so further investigation might find that REPTOR/REPTOR-BP also have repressive functions.

Surprisingly, REPTOR and REPTOR-BP have attracted little attention to date. Microarray studies found that expression of CG13624 and CG18619 are regulated by nutrient conditions in the fly (Teleman et al., 2008; Zinke et al., 2002), however no information regarding their function was available. BLASTing the human proteome with REPTOR and REPTOR-BP identifies Crebrf and Crebl2 respectively, which could potentially be human orthologs. It will be interesting to study them in light of our Drosophila data.

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In sum, this study identifies REPTOR and REPTOR-BP as dedicated transcription factors that control the transcriptional repression downstream of TORC1 in *Drosophila*. Since these transcription factors mediate part of the functional output of TORC1, it will be interesting to assess their contribution towards the role TORC1 plays in cancer, diabetes and aging.

EXPERIMENTAL PROCEDURES

Fly stocks

All chromosomes and flies used for metabolic measurements and growth experiments were backcrossed to w^{1118} at least five times and reared on density-controlled conditions before the experiment. As control flies we used isogenic w[1118] flies from Bloomington. TOR^{2L1} and TOR^{2L19} flies were described in (Oldham et al., 2000) and $FOXO^{\Delta 94}$ flies were described in (Slack et al., 2011).

Metabolic measurements

For TAG and glycogen measurements, 60 1st instar larvae were seeded per vial to grow under defined density conditions. Hatching adults were collected within a 12-hour window, and allowed to age 3 or 4 days as indicated, prior to homogenization for quantification of TAG or glycogen levels.

Rapamycin and starvation treatments

Starvation experiments were done on 0.8% Agarose/1xPBS. Rapamycin feeding was done by mixing normal food with 200μM (final) rapamycin (Santa Cruz, sc-3504A), dissolved in EtOH. Control food contained EtOH at the same concentration as rapamycin food. Adult flies were fed 3d with rapamycin, larvae for 30min-6h, depending on the experiment.

Antibodies

REPTOR, phosphoREPTOR antibodies were raised by immunizing guinea pigs and rabbits respectively with KLH-conjugated peptides. 14-3-3 antibodies were raised by immunizing full-length proteins in guinea pigs.

Cell culture and luciferase assays

Drosophila S2 cells were cultured at 25°C in Schneider's *Drosophila* Medium (GIBCO 21720), supplemented with Penicillin/Streptomycin and 10% FCS. dsRNA was generated by performing a T7 transcription reaction from an amplified genomic region of the respective gene. Gene knockdowns were done by treating S2 cells with 12μg/ml dsRNA in serum-free medium for one hour. Cells were then given serum-containing medium and allowed to grow for 5 days before analysis, to allow for the knockdown to take effect.

For luciferase assays, S2 cells were transfected overnight with indicated plasmids and then treated for 6h with either rapamycin, EtOH or Torin1. Then, cells were lysed and analysed using the Dual-Luciferase® Reporter Assay System (Promega, E1910).

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Transactivation assay

The DNA-binding domain (DBD) of Gal4 was fused to REPTOR and REPTOR-BP. Either one was expressed along with a 5×UAS>>hsp70>>FLuc reporter plasmid and a normalization control plasmid hsp70>>RLuc. Fusion protein expression was induced with Copper overnight. Samples were analyzed using the Dual-Luciferase Reporter Assay System (Promega, E1910).

Chromatin Immunoprecipitation (ChIP)

S2 cells were cross-linked with 1% formaldehyde for 10′, blocked with glycine for 5′, washed with cold PBS and lysed in shearing buffer (0.8% SDS, Tris, EDTA, protease inhibitor cocktail) for 10′ on ice. Samples were subjected to sonication in a Bioruptor (Diaenode), 23 cycles, 30″/30″ ON/OFF, intensity “HIGH” until fragment size was between 150 and 500 bp. 1% Input was taken and the rest was used for IP with indicated antibodies. Elution of IP and preparation of Input was done by incubating bead-bound chromatin and EtOH precipitated input pellet with 10% Chelex solution (Sigma, C7901) at 95°C for 15′, treatment with Proteinase K for 30′ at 50°C and another 10′ on 95°C. Chelex supernatant was diluted 1:3 before analysis by qPCR.

Data and analyses

Microarray data are deposited at NCBI Geo with Accession GSE55221. Statistical significance in the figures was calculated using student t-tests.

Extended Experimental Procedures, including the number of technical and biological replicates for all figure panels, are available in Supplemental Material.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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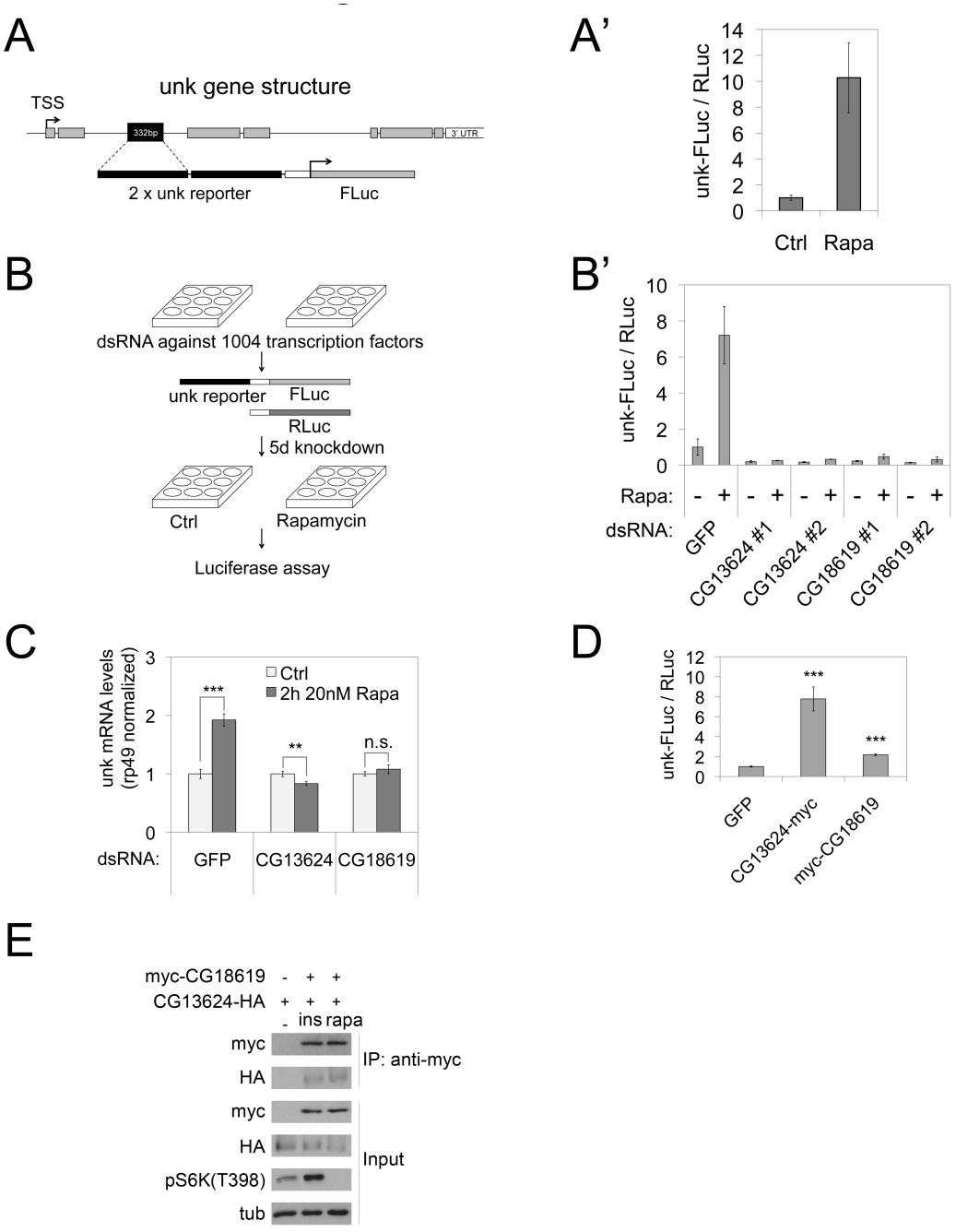

Figure 1. REPTOR and REPTOR-BP are required for induction of unk expression in response to TOR inhibition

(A) Schematic representation of the unk gene locus and unk luciferase reporter.

(A') Rapamycin (7.5nM, 6h) induces expression of the unk reporter in S2 cells.

(B) Outline of RNAi screen to identify transcription factors required for rapamycin-mediated induction of the unk reporter.

(B') CG18619 or CG13624 knockdown blunts induction of the unk reporter in response to rapamycin (20nM, 6h).

(C) CG13624 and CG18619 are required for rapamycin (20nM, 2h) to induce transcription of endogenous unk. mRNA levels measured by qRT-PCR, normalized to rp49.
(D) Overexpression of CG13624 or CG18619 induces the unk reporter in S2 cells. (E) CG13624 and CG18619 bind each other. Co-IP of CG13624-HA with myc-CG18619 in S2 cells.
Error bars: SD, *** ttest<0.001, ** ttest<0.01, * ttest<0.05.

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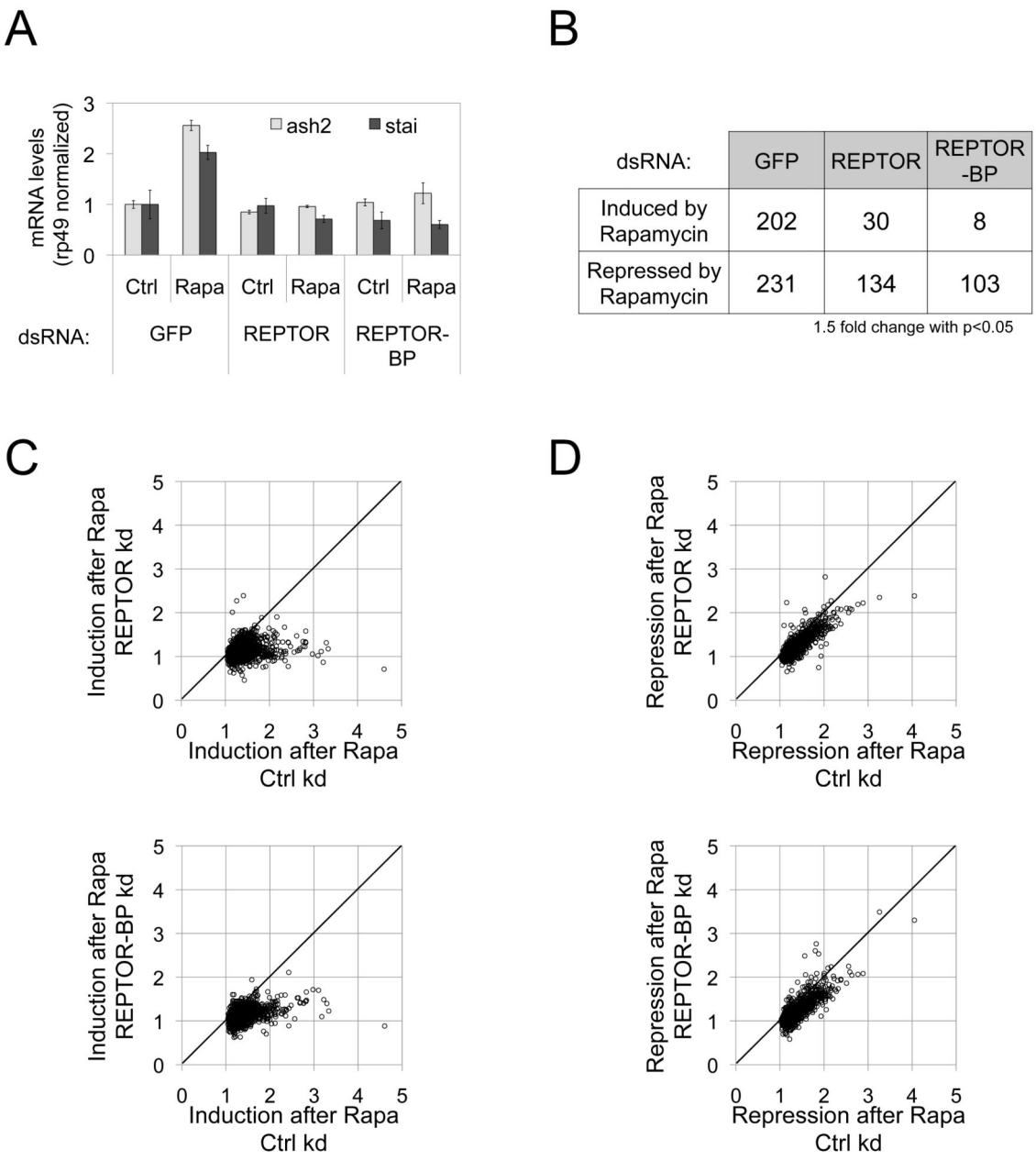

Figure 2. REPTOR and REPTOR-BP are required for activation of almost all rapamycin-induced genes in S2 cells

(A) REPTOR and REPTOR-BP are required for transcriptional activation of *ash2* and *stai* after rapamycin treatment (20nM, 2h). mRNA levels measured by qRT-PCR, normalized to rp49. Error bars: SD.

(B) Knockdown of REPTOR or REPTOR-BP prevents induction of almost all rapamycin-induced genes in S2 cells. Table shows all genes that are induced or repressed at least 1.5-fold (with $p<0.05$) after rapamycin treatment (20nM, 2h) in each knockdown condition (from microarray analysis).

(C-D) REPTOR or REPTOR-BP knockdown affects genes whose expression is induced by rapamycin (C) but not genes whose expression is repressed by rapamycin (20mM, 2h) (D). All genes whose expression changes upon rapamycin treatment in control S2 cells with $p<0.05$ are shown (x-axis). y-axis: corresponding fold change in the REPTOR or REPTOR-BP knockdown cells.

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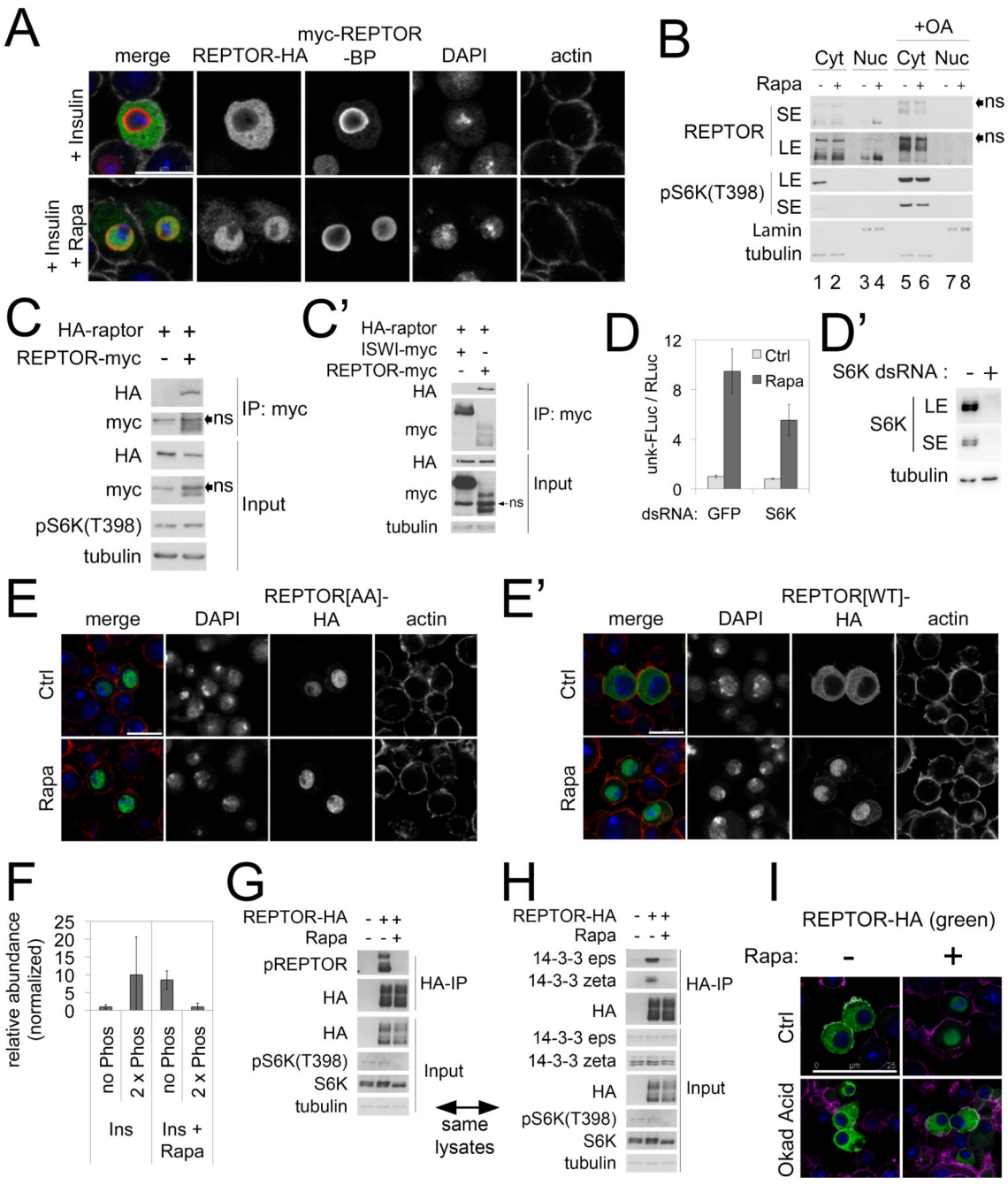

Figure 3. REPTOR is sequestered in the cytoplasm by TORC1-mediated phosphorylation

(A) REPTOR shuttles into the nucleus upon rapamycin treatment (20nM, 30min). REPTOR-HA and myc-REPTOR-BP co-transfected in S2 cells and analysed by immunostaining.

(B) Endogenous REPTOR accumulates in the nucleus upon rapamycin treatment (20nM, 30min). S2 Cells fractionated into cytoplasmic (Cyt) and nuclear fractions (Nuc) prior to immunoblotting. +OA: 50nM okadaic acid added 15min prior to rapamycin addition (lanes 5-8). LE: long exposure. SE: short exposure.

(C-C') REPTOR interacts with TORC1. HA-raptor co-IPs with REPTOR-myc in S2 cells.

(D-D') unk reporter activation is independent of S6K. (D) S2 cells efficiently depleted of S6K (D'), transfected with unk reporter and treated with rapamycin (7.5nM, 6h) (D). Error bars: SD. LE: long exposure. SE: short exposure.

(E-E') Ser527 and Ser530 are required for cytoplasmic retention of REPTOR. Mutation of Ser527 and Ser530 to alanine (REPTOR[AA]-HA) results in constitutive nuclear localization (S2 cells, rapamycin 20nM for 30min).

(F-G) S527/S530 are both phosphorylated and become dephosphorylated upon rapamycin treatment (20nM, 30min).

(F) Relative levels of doubly-phosphorylated pS527/pS530 detected by mass spectrometry on immunoprecipitated REPTOR-HA. Each treatment condition normalized separately. Error bars: SD.

(G) Double-phosphorylation on S527/S530, detected using a phospho-specific antibody (pREPTOR) on immunoprecipitated REPTOR-HA, is abolished upon rapamycin treatment.

(H) REPTOR interacts with 14-3-3 proteins only when TORC1 is active. REPTOR-HA immunoprecipitates immunoblotted to detect endogenous 14-3-3 epsilon and zeta.

(I) Dephosphorylation of REPTOR-HA by PP2A is required for nuclear accumulation upon rapamycin treatment (20nM, 30min). S2 cells transfected with REPTOR-HA, treated with 50nM okadaic acid 15 min prior to rapamycin treatment.

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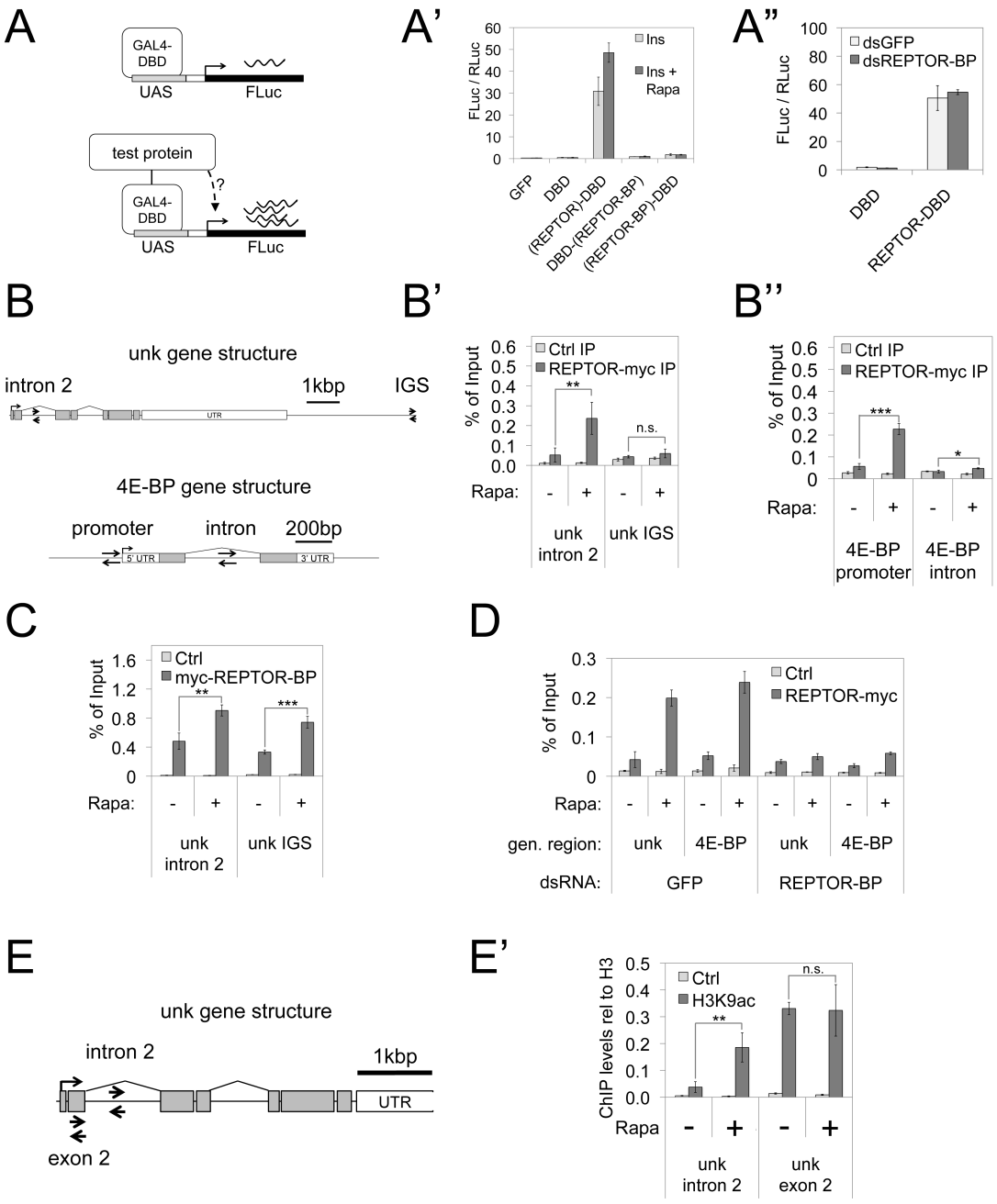

Figure 4. REPTOR directly binds and transactivates target genes

(A) Schematic of the transactivation assay used in A'-A".

(A') REPTOR, but not REPTOR-BP, shows transactivation activity when fused to the GAL4 DNA binding domain (DBD) (insulin 6h 10μg/ml, rapamycin 6h 20nM).

(A") REPTOR transactivation activity does not require REPTOR-BP. S2 cells treated with dsRNA against GFP or REPTOR-BP 5d before assay.

(B-B") Rapamycin treatment (20nM, 30min) strongly increases REPTOR binding to target genes.

(B) Diagram of qPCR oligos used for ChIP analysis in B'-D.
(B'-B") REPTOR-myc chromatin IP, analysed by qPCR.
(C) REPTOR-BP binds constitutively to DNA, also in the absence of rapamycin (20nM, 30min). ChIP using myc antibody from S2 cells stably transfected with myc-REPTOR-BP.
(D) REPTOR-BP recruits REPTOR to target genes after rapamycin treatment (20nM, 30min). ChIP against REPTOR-myc after 5d knockdown of REPTOR-BP.
(E-E') H3K9ac is specifically enriched in the unk reporter region after rapamycin treatment (20nM, 30min) compared to control treatment (vehicle only - EtOH). ChIP with H3 and H3K9ac antibodies from S2 cells. Control ChIP is beads with no primary antibody.
Error bars: SD *** ttest<0.001, ** ttest<0.01, * ttest<0.05.

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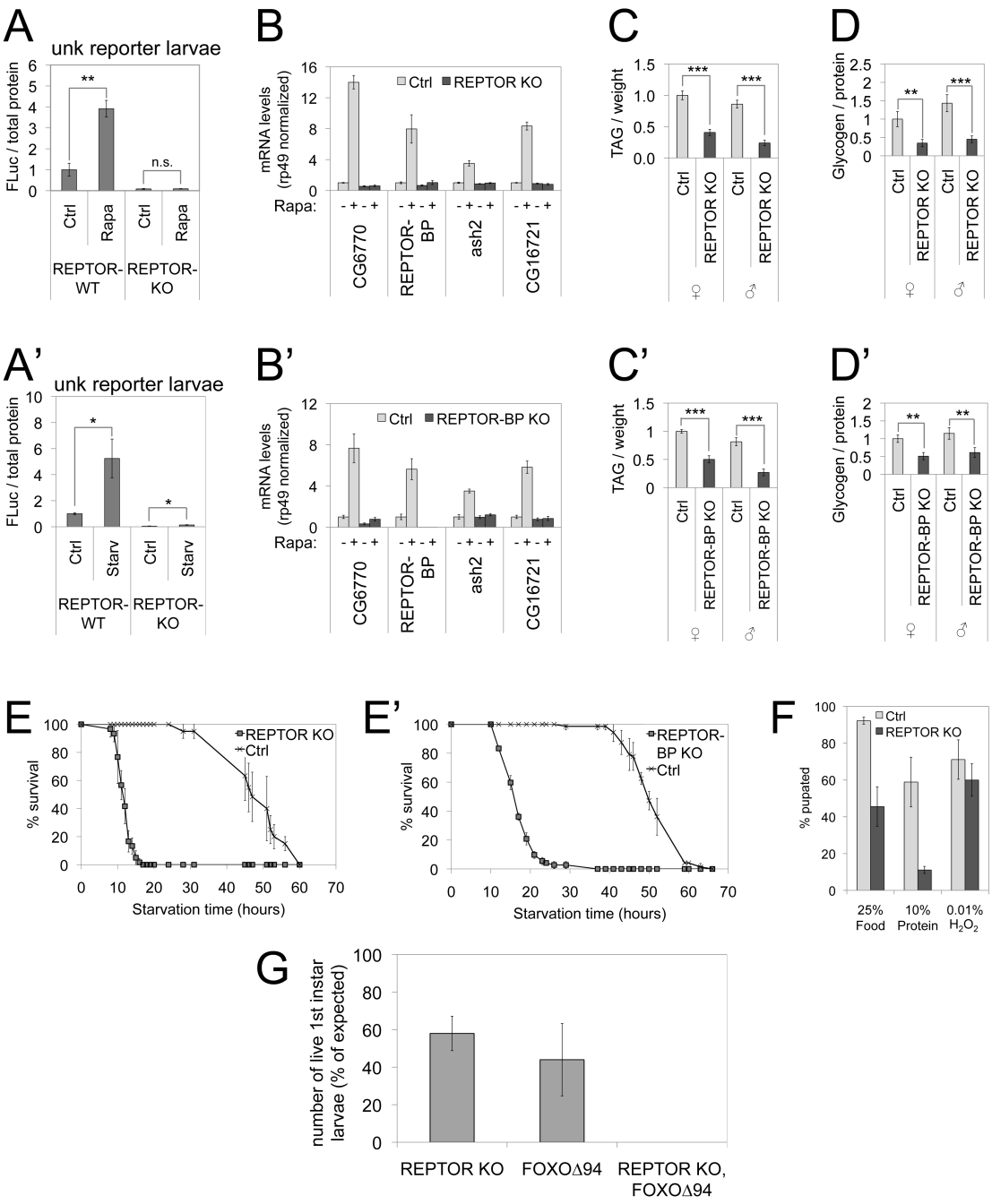

Figure 5. REPTOR and REPTOR-BP are required for rapamycin induced transcription *in vivo* and regulate metabolism in *Drosophila*

(A-A') Unk reporter activity is induced *in vivo* upon nutrient withdrawl or TORC1 inhibition in a REPTOR-dependent fashion. Unk-reporter luciferase activity, normalized to total protein, in 24h prewandering control and REPTOR-knockout larvae fed with rapamycin (200μM, 5h) (A) or starved (0.8% agarose/PBS, 4h) (A').

(B-B') REPTOR (B) and REPTOR-BP (B') are required *in vivo* in larvae for induction of genes in response to rapamycin. 24h prewandering control or knockout larvae fed with rapamycin (200μM, 5h). mRNA levels measured by qRT-PCR, normalized to rp49.

(C-C') REPTOR (C) and REPTOR-BP (C') knockout flies have very low triglyceride stores (4d old adults, normalized to weight).
(D-D') REPTOR (D) and REPTOR-BP (D') KO flies have reduced glycogen stores. Glycogen levels of 2d (D) and 6d (D') old flies, normalized to protein content.
(E-E') REPTOR (E) and REPTOR-BP (E') KO flies are extremely sensitive to starvation. 4d old male flies starved on 0.8 % agarose/PBS.
(F) REPTOR KO larvae are sensitive to nutrient stress. Larvae were grown on food diluted to 25% the normal concentration in PBS/agarose, or on food containing only 10% protein of the standard food or food containing 0.01% H_2O_2.
(G) FOXO and REPTOR mutations are synthetic lethal. Number of live, homozygous L1 larvae compared to number of expected homozygous animals under growth controlled conditions.
Error bars: SD. ***ttest<0.001, **ttest<0.01, *ttest<0.05.

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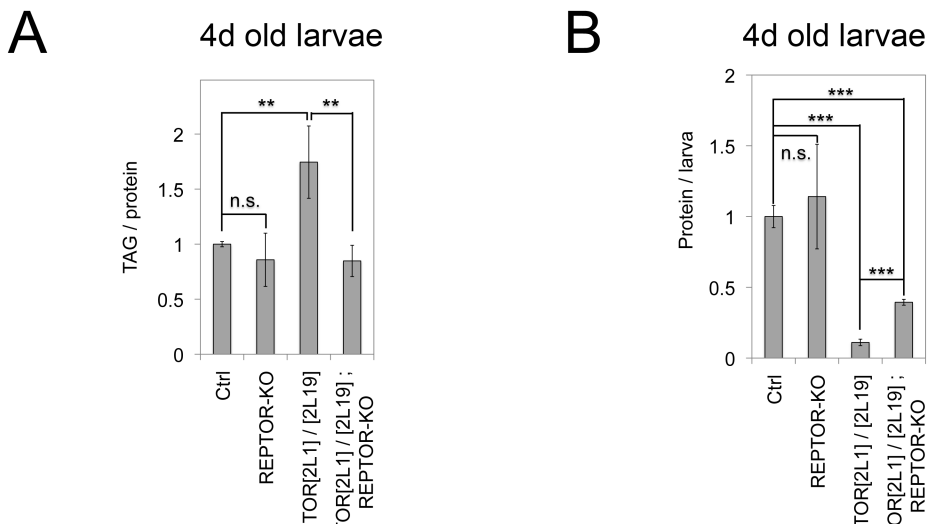

Figure 6. REPTOR partially mediates TOR mutant phenotypes

(A-B) Loss of REPTOR rescues the obesity (A) and partially rescues the growth defect (B) of TOR mutant larvae. Measurements performed on larvae that were synchronized at first instar and grown under density-controlled conditions for 4 days to prewandering 3rd instar. Error bars: SD. ***ttest<0.001, **ttest<0.01

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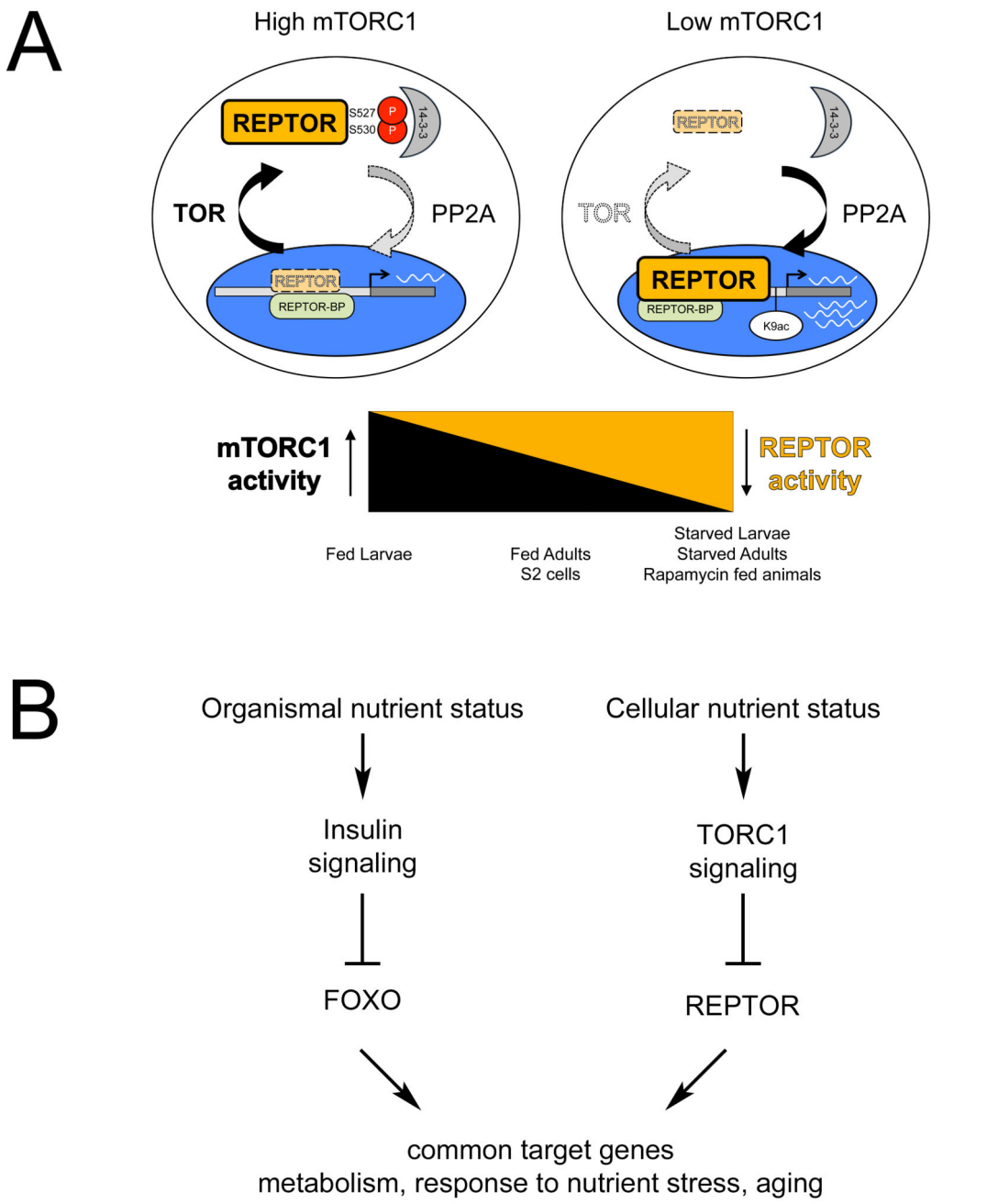

Figure 7. Model of REPTOR regulation

(A) When TORC1 is high REPTOR activity is low, as is the case in feeding larvae. REPTOR activity increases during development of the fly and becomes fully activated upon nutrient withdrawal or rapamycin treatment. REPTOR activity is kept in check by TORC1 phosphorylation and subsequent 14-3-3 mediated cytoplasmic retention. When TORC1 activity drops, REPTOR becomes de-phosphorylated by PP2A, accumulates in the nucleus, binds target genes together with REPTOR-BP, and induces their transcription.

(B) FOXO and REPTOR are the respective counterparts for insulin and TORC1 signaling, which sense nutrients at the organismal and cellular levels, respectively.

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