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Research Article

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Longevity and stress resistance are affected by activation of TOR/Myc in progenitor cells of Drosophila gut

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Abstract: Diverse physiological pathways have been shown to regulate longevity, stress resistance, fecundity and feeding rates, and metabolism in *Drosophila*. Here we tesed physiological traits in flies with Rheb and Myc-Rheb overexpressed in gut progenitor cells, known as enteroblasts (EBs). We found that activation of TOR signaling by overexpression of Rheb in EBs decreases survival and stress resistance. Additionall, we showed that Myc co-expression in EBs reduces fly fecundity and feeding rate. Rheb overexpression enhanced the level of whole body glucose. Higher relative expression of the metabolic genes *dilps, akh, tobi* and *pepck* was, however, observed. The role of TOR/Myc in the regulation of genes involved in lipid metabolism and protein synthesis was established. We showed a significant role of TOR/Myc in EBs in the regulation of the JAK/STAT, EGFR and insulin signaling pathways in *Drosophila* gut. These results highlight the importance of the balance between all different types of cells and confirm previous studies demonstrating that promotion of homeostasis in the intestine of *Drosophila* may function as a mechanism for the extension of organismal lifespan. Overall, the results demonstrate a role of TOR signaling and its downstream target Myc in EB cells in the regulation of *Drosophila* physiological processes.

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1 Introduction

Midgut homeostasis is regulated by multipotent intestinal stem cells (ISCs), which divide and give rise to immature enteroblasts (EBs) or become new stem cells [1]. Enteroblasts can differentiate to enterocytes (ECs) or enteroendocrine (EE) cells. Notch signaling plays an important role in driving EBs to become either ECs or EEs [2,3] (Fig. 1). A recent study summarized findings about maintenance and regulation of ISCs functioning drawing parallels between the fly and mammalian systems [4]. ISCs are diploid, have a small nucleus, and express Delta which is a ligand specific for the Notch receptor. EBs are also diploid with a small nucleus, and express the transcriptional reporter for Notch – Supressor of Hairless (Su(H)) [5]. ECs are polyploid cells with a large nucleus and express the transcription factor Pdm1. EEs are diploid cells with a small nucleus and express the transcription factor Prospero. It was demonstrated, that EEs are generated from Prospero-expressing ISCs, but not from EBs [6]. But another study proposed that EBs give rise to EEs of class II [7]. ECs are involved in nutrient absorption and EEs are important for hormone secretion. Consequently, the functioning of EBs and their differentiation into either ECs or EEs can affect metabolic processes, stress resistance, and aging.

Conserved metabolic pathways are involved in ISC-mediated tissue homeostasis including TOR (target of rapamycin) signaling. TOR is a highly conserved serine/threonine kinase that regulates growth and metabolism in response to nutrient availability, environmental stressors, cellular energy status [8], and aging [9]. Using an RNAi-based genetic screen, Amcheslavsky and

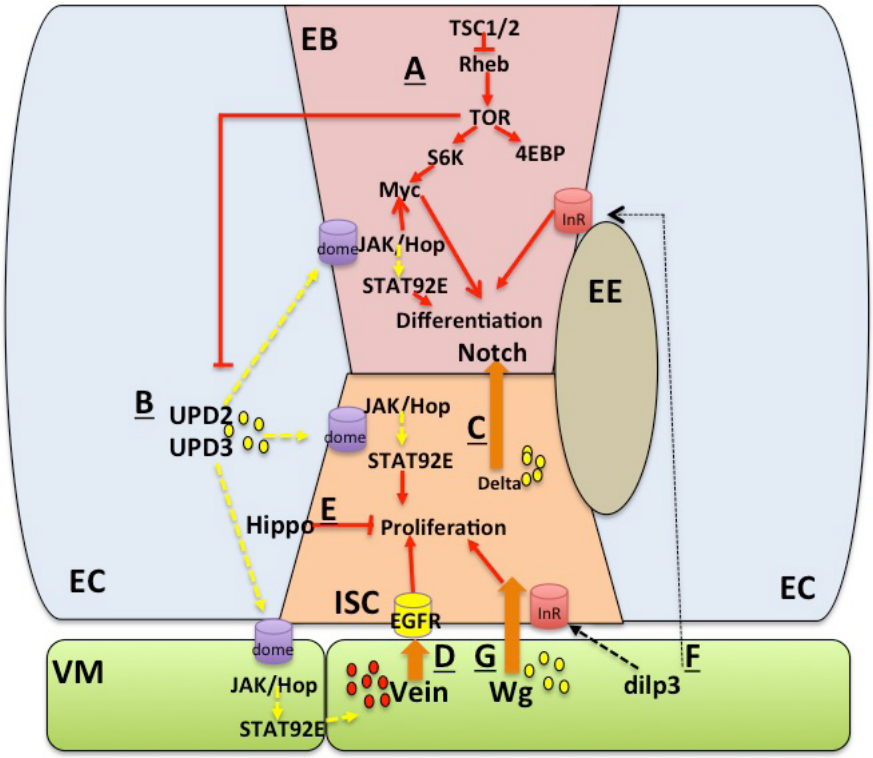

Fig. 1 A schematic of *Drosophila* ISCs lineages and signaling pathway interactions in proliferation and differentiation control.
A) TOR/Myc signaling pathways in proliferation/differentiation control. In response to TSC1/TSC2 inhibition by environmental changes GTPase Rheb becomes activated and, in turn, activates TOR kinase. JAK/STAT acts downstream of TOR signaling by inhibiting Upd secretion. But Myc is post-transcriptionally upregulated upon JAK/STAT activation [58].
B) Unparired 2 and 3 (Upd 2 and 3) activate a receptor-associated Janus Kinase (JAK) termed Hopscotch (Hop) which in turn triggers a Signal Transducer and Activator of Transcription (STAT). The downstream events lead to promoting the ISCs proliferation and EBs differentiation.
C) Ligand Delta is expressed by ISCs and activates Notch proteins in the EBs. The dissociation of Notch intracellular domain (NICD) from cell membrane is catalyzed by gamma-secretase. Then NICD translocates to the nucleus and forms a complex with Supressor of Hairless (Su(H)) protein to replace a histone deacetylase (HDAc)/corepressor (CoR) from the Su(H). Active complex triggers the transcription activation of target genes.
D) Epidermal growth factor receptor (EGFR) is activated by ligands Vein, Spitz and Kern which expressed in visceral muscle. Upon ligand stimulation EGFR activates RAS-RAF-MAPK signaling to induce an expression of target genes.
E) Hippo signaling negatively regulates ISCs proliferation.
F) Dilp 3 acts directly through insulin receptor (InR) to activate Protein kinase B (Akt), which phosphorylates and inactivates FOXO. dFOXO is a key downstream element of insulin signaling in *Drosophila*.
G) Visceral muscle produces Wingless (Wg), a Wnt ligand, which positively affects ISCs proliferation through activation of the frizzled receptor (Fzd).

colleagues [10] identified tuberous sclerosis complex (TSC), a component of TOR signaling, as an important regulator of ISC growth. Kapuria and colleagues [11] demonstrated that Notch-induced Su(H) activity dowregulates TSC2 expression in EBs. Additionally, it was shown that TSC2 is highly expressed in ISCs [10] and is downregulated in EBs [11]. A recent study demonstrated that loss of TSC1 and TSC2 function in ISCs lead to the loss of ISCs [12]. Moreover, Rheb overexpression compensates for the effect from TSC1/2 disruption in *Drosophila* ISCs [12]. A downstream target of TSC1/TSC2 is Rheb, a GTPase that becomes activated in response to TSC2 repression and directly activates TOR kinase. Together, this suggests that TOR signaling is downregulated in ISCs in order to maintain their stemness and is upregulated in EBs, which is a major step for EC differentiation.

D. melanogaster Myc regulates cell growth during development [13] and together with Rheb possesses oncogenic activity [14,15]. Myc may act independently to regulate ISC growth. Loss of TSC can reduce Myc activity to control excessive ISC growth, and this allows for the restoration of ISC division [10]. In EBs TOR signaling and Myc have a strong impact on cell differentiaion into either EC or EE, which in turn influences metabolic processes, stress resistance, gut integrity, and fly survival.

Our previous study had already demonstrated that both *Myc* and *Rheb* expression in stem and progenitor cells have a great impact on *Drosophila* lifespan, stress resistance and metabolism [16]. The present study aims to show an important role of TOR/Myc signaling axis in EB cells in fruit fly. The study of signaling pathway regulation in ISCs could shed light on potential aging mechanisms. Our study presents novel evidence that TOR and Myc signaling cascades control EB functioning, which is critical for maintaining ISC homeostasis. We used a *Su(H) GBE-Gal4* driver to examine the role of ISC progeny – EBs in metabolism and lifespan determination. A *UAS-Rheb* construct was used to activate the TOR signaling pathway in *Drosophila* EBs and a *UAS-Myc-Rheb* construct was used to increase both Rheb and Myc. We found that TOR activation through Rheb overexpression in progenitor stem cells reduced life expectancy, fecundity, and feeding rates but enhanced glucose levels and expression of metabolic genes.

2 Methods

2.1 Fly husbandry and transgenic flies

Before the experiments began, the flies were cultured on standard molasses medium, composed of dry yeast (5%), corn (6.1%), molasses (7.5%), nipagin (0.18%) and propionic acid (0.4%) at 18°C for one year. During the experiments flies were kept at 25°C for two generations on the same diet.

EBs, the immature daughter ISC, can be defined using the Notch signaling reporter *Su(H)GBE-LacZ* [17], which is commonly used as an EB marker [2,18]. The *Gal4/UAS* system was used to overexpress *Rheb* or both *Myc* and *Rheb* proteins in enteroblasts. The EB-specific Gal4 fly line *Su(H)GBE-LacZ-Gal4 UAS-GFP tub-Gal80ts* (*Su(H)-Gal4ts*) and *UAS-Rheb*, *UAS-Myc-Rheb* were backcrossed for eight generations to the *w1118* background (Blommington Stock Center). Transgenic flies were obtained from the laboratory of Dr. Bruce Edgar (DKFZ, Heidelberg, Germany). *Su(H)-Gal4ts* females were mated to males of respective UAS lines. Resultant eggs were allowed to develop at 18°C, because at this temperature Gal80 inhibits the binding of Gal4 protein to UAS. Newly eclosed flies were collected and kept for a three days at 18°C for maiting. Transgenic flies were sexed and shifted to 29°C to induce *Su(H)ts*, and kept for six days. The final females, which were selected after expression induction, were used for all measurements. All physiological studies were completed at 29°C.

2.2 Lifespan and fecundity

To assess lifespan, approximately 75-100 females of each genotype were placed in 1.5L demographic cages. Plastic vials filled with 5 ml of experimental food (5% sucrose, 5% dry yeast, 1.2% agar and 0.18% nipagin) was attached to the cage. Food was changed every other day and dead flies were counted. The experiment was run twice with more than 150 flies tested per genotype.

To determine fecundity, one female and one male were randomly selected and placed into 5 ml vials with 1 ml of experimental food and reproductive rate was measured. The food was changed every day and the number of eggs laid by individual flies were recorded [19,20]. Twenty flies were tested per genotype in two biological replicates.

2.3 Feeding

Food consumption by a single fly was measured using the CAFE assay [19]. Briefly, flies were kept in vials infiltrated by a 5 µl capillary tube filled with food containing 5% yeast extract and 5% sucrose. The capillaries were changed every day and the amount of food eaten was measured over a period of four days. Vials were kept in closed boxes with distilled water to maintain high humidity. There were three control vials without flies to allow correction for any evaporation of food. Ten flies per genotype were tested.

2.4 Malnutrition, starvation and oxidative stress

For the malnutrition experiments, 85-120 flies were kept in groups of 15 in 15 ml vials with 3 ml of medium consisting of 1% sucrose, 1% autolyzed yeasts, or 0.5% of both. 0.5% sucrose was used for starvation assays [16]. Flies involved in oxidative stress resistance experiments were given a 5% sucrose medium supplemented with 20 mM menadione, a polycyclic aromatic ketone that generates intracellular reactive oxygen species (ROS). The vials were changed every 2 days and the number of dead flies recorded at 9 and 12AM and 9 PM. All experiments were run in three repeats.

2.5 Metabolites

Flies were decapitated and then centrifuged to extract hemolymph (3000g, 5 min). Resulting hemolymph was used for determination of glucose and trehalose. Pre-

weighted bodies were homogenized in 50 mM Na-buffer, centrifuged, and used for determination of glucose and glycogen levels. Measurements were performed using a glucose assay kit (Liquick Cor-Glucose diagnostic kit, Cormay, Poland, Cat. No. 2-203). The glycogen was converted into glucose by amyloglucosidase from *Aspergillus niger* (25°C, 4 hours). For triglyceride (TAG) determination, flies were weighed, homogenized in 20 mM PBST (phosphate buffered saline containing 0.05% Triton X100), boiled and centrifuged (13000g, 10 min) [21,22]. Resulting supernatants were used for the triglyceride assay with Liquick Cor-TG diagnostic kit (Cormay, Poland, Cat. No. 2-254). Flies of all genotypes were tested in 4-6 independent replicates [23].

Total whole-body protein content was measured using Coomassie blue dye according to Bradford [24]. Body supernatants were obtained by homogenization in 50 mM potassium phosphate buffer in ratio 1:10 and centrifuged (13000g, 15 min, 4°C). Serum bovine albumin was a standard. Data are expressed as miligrams of protein per gram of wet fly body weight (mg/gww).

2.6 Analysis of gut integrity

Intestinal integrity was used as an aging indicator. We examined flies expressing *Rheb* and *Myc-Rheb* in EBs that consumed non-absorbable blue food dye E133 [25]. "Smurf" flies, characterized by the visible blue food dye throughout the body, were considered to have disruption of gut integrity. Fifty females of each genotype were placed into plastic vials with food supplemented with E133 (2.5%) and after 12 hours the number of "smurf" (with dye spread throughout the body) flies was counted. About 200 flies were analysed per genotype.

2.7 Gene expression

Total RNA from heads, whole flies or dissected guts was extracted with the RNeasy Plus Mini Kit (Qiagen) and converted into cDNA with QuantiTect Reverse Transcription Kit (Qiagen). Expression of genes of interest was measured using an ABI Prism 7000 instrument (Applied Biosystems), a SensiFAST SYBR Hi-ROX Kit, and a QuantiTect SYBR Green PCR Kit (Qiagen) under conditions recommended by the manufacturer. mRNA levels for *dilp2, dilp3* and *dilp 3* were defined in heads; *dilp6, akh, tobi, pepck, 4ebp* and *bmm* in whole body; *upd2, upd3, soc36, spi, krn, vn, dilp3, pepck* and *puc* in dissected guts. Each analytical and standard reaction was performed in three technical replicates. The levels of transcripts were measured using primer pairs published earlier and shown in table S2 [26-28]. The Ct method was used with *rp49* as reference gene for heads and whole flies and *crq* as the reference gene for guts.

2.8 Statistical procedures

To analyze the trends of lifespan in survival curves that were obtained from starvation, malnutrition and oxidative stress resistance assays the log-rank (Mantel-Cox) test was performed. The differences between means were analyzed using ANOVA followed by Newman-Keuls Multiple Comparison Test in Prism GraphPad.

3 Results

3.1 Lifespan and stress resistance

TOR signaling pathway and its target Myc are involved in regulation of cell growth, ribosome biogenesis and metabolism that, in turn, have impacts on lifespan. Our previous results showed significant impact of TOR/Myc activation in *Drosophila* EB on fly survival [16]. Mean lifespan of control flies (*SuH/+*) was about 34 days at 29°C. Overexpression in enteroblasts in Rheb (*SuH/rheb*) and Myc-Rheb (*SuH/Myc-Rheb*) shortened mean lifespan by 15% (log-rank test: χ^2=18, p<0.0001) and 18% (χ^2=36, p <0.0001), respectively (Fig. 2A and Table S1). Moreover, we conducted two additional controls *rheb/+* (*UAS-Rheb* fly line) and *myc-rheb/+* (*UAS-Myc-Rheb*) that were not different from *SuH/+*. These data indicated that TOR/Myc activation in EB decreased fly survival.

Nutrient accessibility is an important determinant of organismal lifespan. For this reason we tested fly survival under conditions of malnutrition or complete starvation. Under the condition of low carbohydrate diet (1% sucrose), the survival of both transgenic flies was lower by 11% in *SuH/Rheb* flies and by 20% in *SuH/Myc-Rheb* as compared to control group (Fig. 2B and Table S1). Activation of the TOR signaling pathway in enteroblasts by *Rheb* overexpression had no impact on fly resistance to malnutrition condition when flies are given a low protein diet (1% autolyzed yeast), but *Myc-Rheb* overexpression shortened lifespan by 17% (χ^2=18, p<0.0001) (Fig. 2C and Table S1). Interestingly, flies exposed to a low carbohydrate, low protein diet (0.5% sucrose and 0.5% autolyzed yeast) *Rheb* expression decreased the lifespan

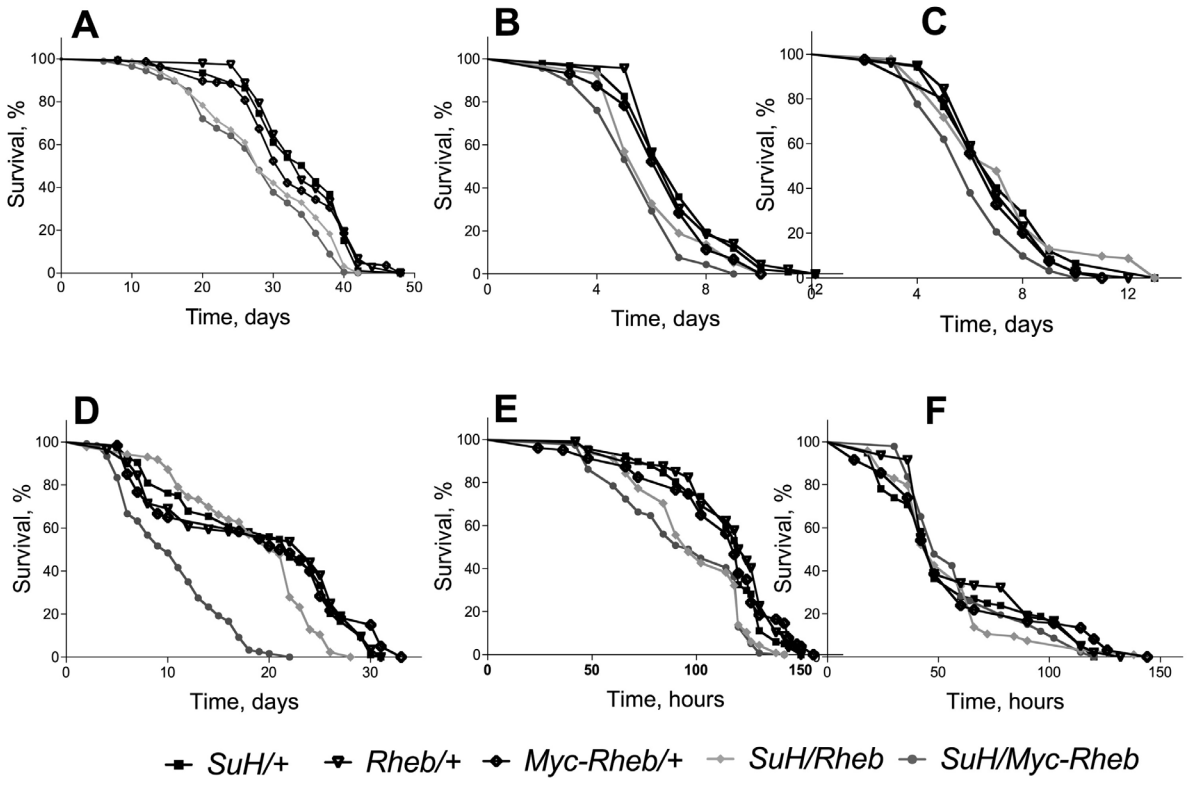

Fig. 2 Lifespan and stress resistance. Survival of flies that express *Rheb* (*SuH/Rheb*) or *Myc-Rheb* (*SuH/Myc-Rheb*) in enteroblasts (A). Survival of control and mutant flies during malnutrition: 1% sucrose (B), 1% autolyzed yeast (C), 0.5% autolyzed yeast and 0.5% sucrose (D). Survival during starvation (E) and oxidative stress (F). Each curve represents the percentage of individuals alive as a function of age. About 200 flies were tested for (A) and 90-120 for (B) – (F). The data were analyzed using a log-rank test.

by 6% (χ^2=11, p=0.0007) and Myc co-expression had an even stronger effect where those flies had a 46% reduced lifespan (χ^2=78, p<0.0001) (Fig. 2D and Table S1). *Rheb* or *Myc-Rheb* expression in enteroblasts leads to decreased resistance to complete starvation by 15-18% (Fig. 2E and Table S1).

In order to measure oxidative stress susceptibility, flies were fed with menadione in 5% sucrose [20]. There was no significant difference in oxidative stress resistance between transgenic and control flies (Fig. 1F and Table S1). It should be noted that there were no significant differences in stress resistance between two additional control groups.

3.2 Feeding and fecundity

There is a correlation between food consumption, reproduction rate and lifespan in *Drosophila*. The CAFE assay was used to identify the amount of food consumed by a single fly. Control flies consumed nearly 2 µl of food daily. TOR signaling activation through *Rheb* overexpression did not significantly change the amount of food consumed, but *Myc-Rheb* expression decreased food intake by 20% (Fig. 3A, p<0.05). Interestingly, a similar tendency was revealed when fecundity was tested. We found no effect of *Rheb* expression on fecundity but expression of both *Rheb* and *Myc* dramatically decreased reproduction by 30% (Fig. 3B, p<0.05). There was no significant difference between *SuH/+* and *Rheb/+* and *Myc-Rheb/+* in feeding and fecundity rate. Our data showed that Myc activation in fly EBs affect life-history traits: decreased feeding/fecundity rates.

3.3 Metabolism and gene expression

Glucose, glycogen and TAGs are parameters extensively used as measures of carbohydrate and fat metabolism [29]. Glucose, glycogen and TAG levels were measured in flies of all genotypes. Our results showed that activation of TOR signaling through *Rheb* overexpression in enteroblasts only increased the level of body glucose, but did not change total hemolymph glucose, glycogen, triglyceride or protein levels. Body glucose levels were 32% higher in *Rheb*-expressing flies compared to the

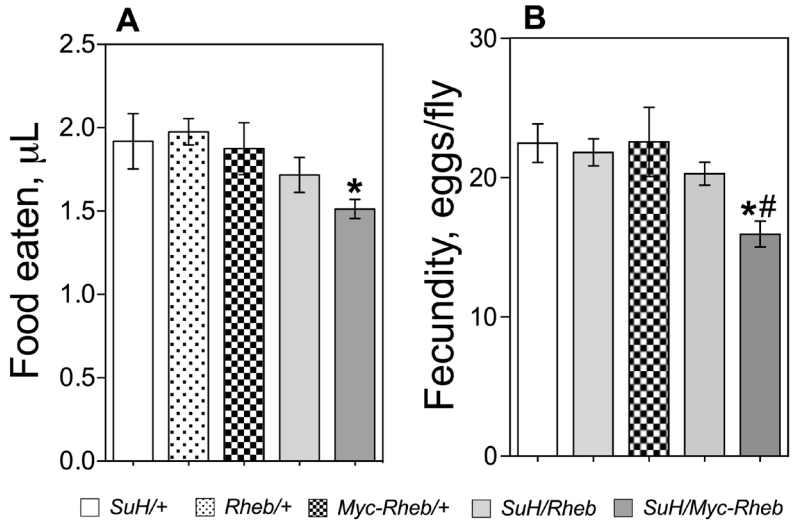

Fig. 3 Feeding and fecundity of transgenic fly lines with *Rheb* (*SuH/rheb*) and *Myc-Rheb* (*SuH/Myc-Rheb*) overexpression. Amount of food consumption (A) and eggs laid (B) of flies that express Rheb and Myc-Rheb in enteroblasts (Mean ± SEM). The differences between means were analyzed using an ANOVA followed by a Newman-Keuls Multiple Comparison Test: An asterisk (*) above the *SuH/rheb* or *SuH/myc-rheb* bar indicates a difference from the control group (*SuH/+*) (p<0.05; n=10-20); a # above the *SuH/myc-rheb* bar indicates a difference from flies that overexpress *Rheb* (p<0.05; n=10-20).

averaged control. *Myc-Rheb* co-expressing flies had 1.5-fold higher body glucose compared to the control (p<0.05) (Fig. 4A). Interestingly, there was 15% higher body glucose level in *SuH/Myc-Rheb* flies as compared to *SuH/Rheb* (p<0.05). The level of glycogen in control flies was about 60 mg/gww. None of the expressed constructs in EBs had an impact on glycogen levels in EBs (Fig. 4C). Moreover, hemolymph glucose concentration, triglyceride content and protein levels were not affected by TOR activation and *Myc* co-expression (Fig. 4B, D, and E).

To better understand the molecular mechanisms underlying the changes in metabolism resulting from *Rheb* and *Myc-Rheb* overexpression in EBs we evaluated gene expression by quantitative realtime reverse transcriptase PCR (qRT-PCR). The transcript levels of important longevity and metabolism genes were measured in *Rheb* and *Myc-Rheb* expressing flies. TOR activation through *Rheb* overexpression in *Drosophila* enteroblasts increased the expression of *dilp2, dilp3* in fly heads and *dilp6* in bodies. However, only *dilp2* and *3* transcript levels, measured in heads, were significantly upregulated in flies expresing *Myc-Rheb* (Fig. 5A, B, D, p<0.05). Additionally, there was a 3.5-4-fold increase in *akh* transcript levels when *Rheb* was overexpressed in enteroblasts (p<0.05). However, *Myc-Rheb* expression also enhanced *akh* expression (Fig. 5E). There was a 2-fold increase in *tobi* mRNA levels in the flies that expressed *Rheb* in progenitor cells (p<0.05), but *Myc-Rheb* expression had no impact on *tobi* levels (Fig. 5F). The relative expression of *brummer* (*bmm*), encoding a TAG lipase that regulates fat metabolism, was higher by 50% in flies that expressed *Rheb* and increased by 45% in *Myc-Rheb*-expressing flies (Fig. 5I, p<0.05). However, there was no significant difference in *bmm* transcript level comparing *Rheb* and *Myc-Rheb* expressing flies. Both genetic manipulations caused a nearly 4-fold increase in *pepck* transcript level compared to the control (Fig. 5G, p<0.05). Additionally, expression of *4ebp* was higher by 50% compared to control when *Rheb* was overexpressed in enteroblasts (p<0.05). Myc co-expression caused 5-fold higher transcript level of *4ebp* gene (Fig. 5H, p<0.05).

3.4 Gut-specific effects of Rheb and Myc-Rheb overexpression

A recent study showed that intestinal barrier dysfunction is an important factor in pathophysiology of aging, because loss of intestinal integrity is associated with altered metabolic signaling pathways and leads to organismal death [30]. Thus, we supplemented the fly food with an unabsorbable blue food dye for the detection of "smurf" flies [25]. We monitored this phenotype every 5 days and by experimental day 30 about 5-10% of smurf flies were observed per genotype. There was no difference between genotypes (not shown). However, the expression of gut-specific genes was highly affected by *Rheb* and *Myc-Rheb* overexpression in EBs. The transcript levels of *upd2* and *upd3,* which encode ligands for JAK/STAT signaling, were higher by 4-fold when *Rheb* was overexpressed in EBs (Fig. 6A and B, p<0.05). The transcript level of *soc36*, a target for JAK/STAT signaling, increased 1.5-fold in flies overexpressing *Rheb* (p<0.05), but *Myc* co-expression decreased *soc36* expression by 75% (Fig. 6C, p<0.05).

The relative expression of EGFR signaling genes s*pi* (Spitz), *krn* (Keren), *vn* (Vein) were measured. Relative gene expression of *spi* increased when TOR signaling was activated through *Rheb* overexpression in EBs (Fig. 6D, $p<0.05$). However, no significant difference in *krn* transcript levels were observed when *Rheb* and *Myc-Rheb* were expressed (Fig. 6E). Expression of *vn* markedly increased (5-fold) in *Rheb*-expressing flies (Fig. 6F, $p<0.05$).

Drosophila intestine expresses two insulin-like peptides, Dilp3 and Dilp7. Dilp3 is expressed in the midgut and foregut muscles [31] and acts directly to induce proliferation and midgut growth via asymmetric and symmetric division [32]. We observed 12-fold higher *dilp3* transcript levels in *Rheb*-expressing flies and an increase of about 4-fold when *Rheb* and *Myc* were co-expressed (Fig. 6G, $p<0.05$). Next, we examined the ability of the TOR/Myc pathway to modulate gluconeogenesis in fly gut cells. Fig. 5H demonstrates 3.5-fold higher *pepck* transcript levels in *Rheb-Myc*-expressing flies ($p<0.05$). Given the clear role of *puc* (puckered) in controlling tissue homeostasis [62], we examined relative *puc* expression in fly gut to see if this might also be correlated with organismal survival. *Rheb* overexpression in EB led to a 4.5-fold increase in *puc* transcript levels in *Drosophila* gut cells (Fig 5I, $p<0.05$).

4 Discussion

The TOR pathway is an important regulatory system for stem cell protection, maintenance and proliferation [11]. It is a key nutrient sensing pathway that can increase lifespan if inhibited [33]. However our study showed that TOR signaling activation through *Rheb* overexpression in EBs shortens fly lifespan. The inhibition of TOR in EBs is sufficient to change their commitment from an EC fate to an EE fate [11]. TOR activation in intestinal stem cell progeny favors the differentiation to ECs [11], and this differentiation event may be critical for shortening organismal lifespan. Consequently, an imbalance between ECs and EEs decreases fly lifespan. Intestinal stem cell proliferation increases during aging, which causes an accumulation of ISC progenitor cells that have impaired

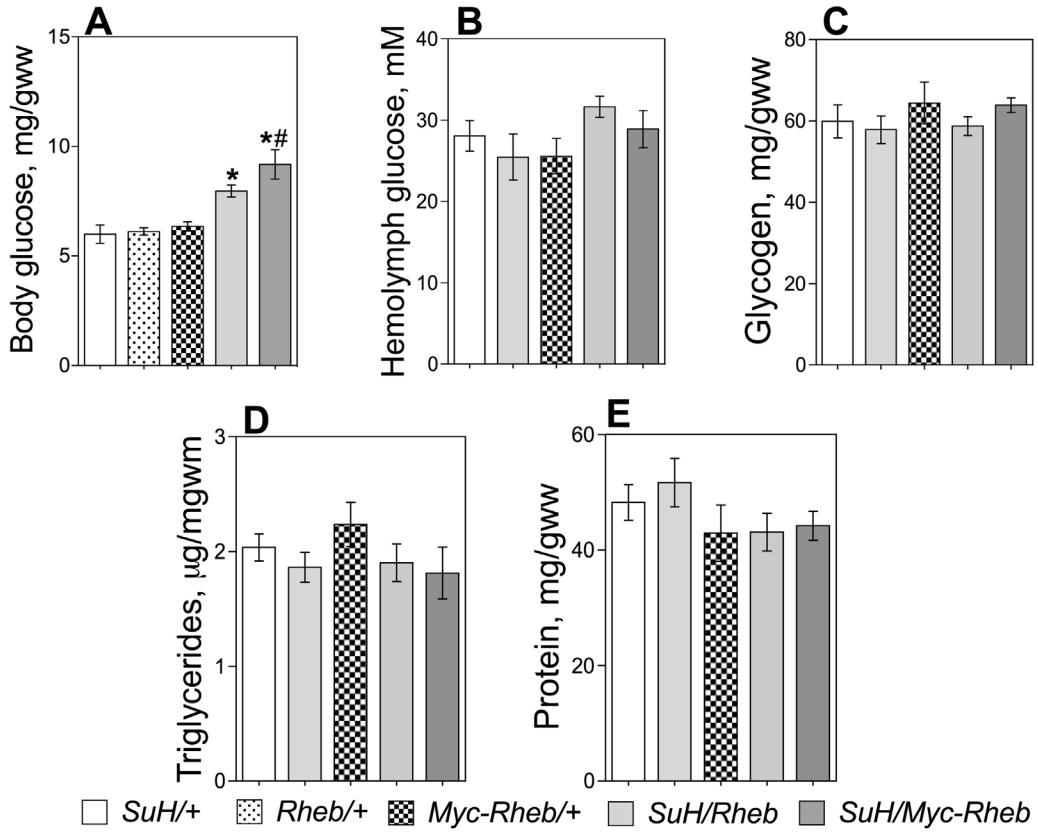

Fig. 4 Metabolism of transgenic fly lines with *Myc* (*SuH/rheb*) and *Myc-Rheb* (*SuH/myc-rheb*) overexpression. Levels of body glucose (A), hemolymph glucose (B), glycogen (C), triglycerides (D) and protein content (E) in flies that express *Rheb* and *Myc-Rheb* in enteroblasts. An *asterisk* (*) above the *SuH/rheb* or *SuH/myc-rheb* bar indicates a difference from the control group (*SuH/+*) ($p<0.05$; n=4-6); a # above the SuH/myc-rheb bar indicates difference from flies that overexpress *Rheb* ($p<0.05$; n=4-6).

terminal differentiation pathways [34]. Our previous study showed that the lifespan of *Drosophila* was decreased when *Rheb* was overexpressed in ISCs and lifespan was even shorter when *Myc* was coexpressed [16]. Present results propose a critical role for TOR/Myc signaling pathway in progenitor stem cells, EBs, which in turn can have a strong impact on *Drosophila* stress resistance and lifespan.

We also found that food availability significantly impacts fly survival. Flies that expressed *Rheb* and *Myc-Rheb* in EBs were less resistant to starvation and malnutrition. But our previous results showed that low carbohydrate or low protein diets increase lifespan of *Rheb* and *Myc-Rheb*-expressing flies [16]. Choi and colleagues [35] demonstrated that starvation caused a delay in EC growth and a prolonged contact between ISCs and their progenitors. *Drosophila* adults that fed on a protein-poor diet had an increased number of stem

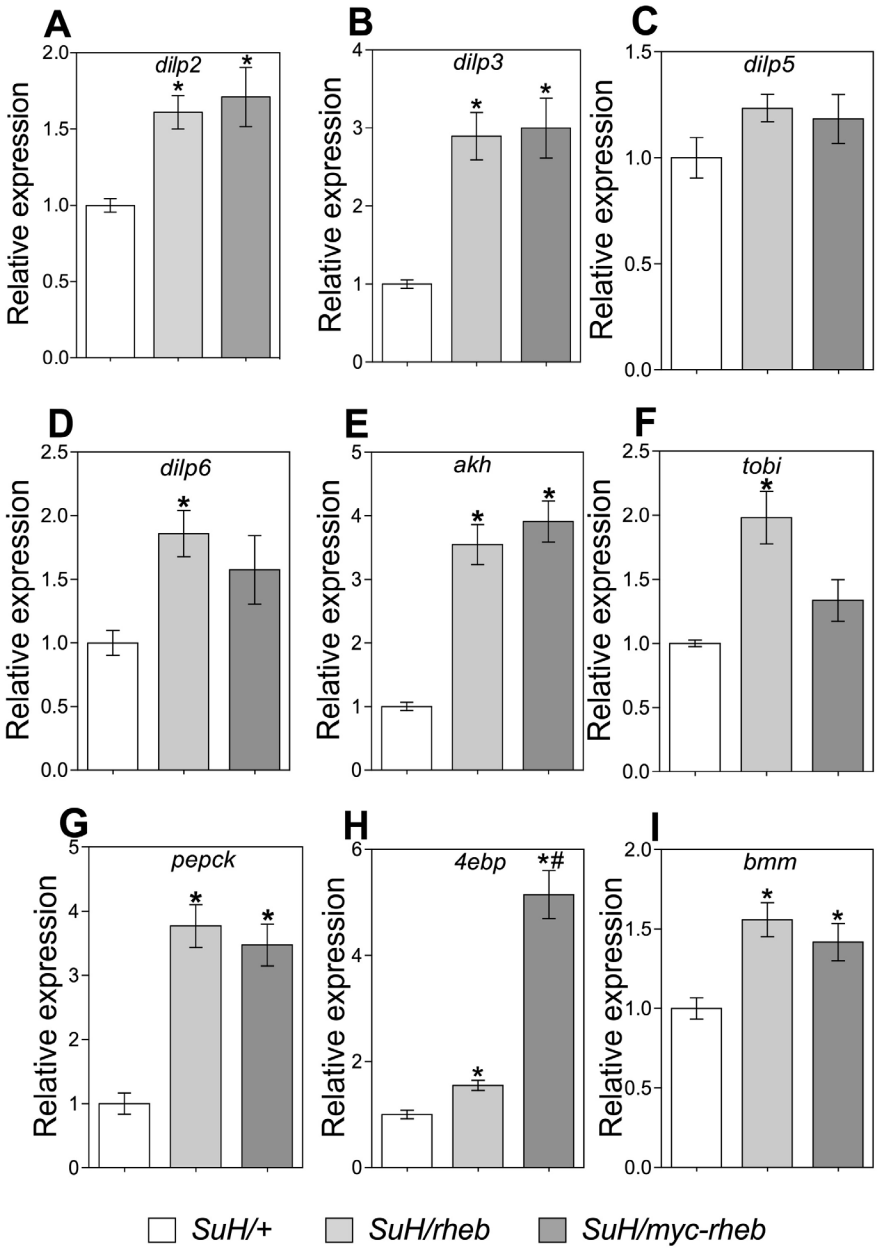

Fig. 5 Expression of longevity and metabolic genes. The transcript levels of *dilp2* (A), *dilp3* (B), *dilp5* (C) in fly heads, and *dilp6* (D), *akh* (E), *tobi* (F), *pepck* (G), *4ebp* (H), *bmm* (I) in whole bodies of transgenic fly lines with *Rheb* (*SuH/rheb*) and *Myc-Rheb* (*SuH/myc-rheb*) overexpression. An *asterisk* (*) above the *SuH/rheb* or *SuH/myc-rheb* bar indicates a difference from the control group (*SuH/+*) ($p<0.05$; n=3-4); a # above the *SuH/myc-rheb* bar indicates a difference from flies that overexpress *Rheb* ($p<0.05$; n=3-4).

cell progeny and a slower proliferation rate, indicating that nutrition influences ISC proliferation [2]. Similarly, Choi and colleagues observed a strong reduction in EC reduplication in flies fed a low protein diet [35]. Flies with defects in ISC growth had a thinner gut epithelium and were more sensitive to food quality.

Starvation and oxidative stress resistance are mediated through 4E-BP, a downstream target of TOR [36]. Furthermore, Tettweiler and colleagues demonstrated that 4E-BP has significant impact on lifespan in that *4ebp* overexpression is a protection against starvation and oxidative stress [36]. In response to stressful agents such as pathogens and ROS-inducing compounds, ISCs increase proliferative rates, which allows for the repair of damaged cells [37]. This fly phenotype closely resembles the aging phenotype. Treatment with stressful agents, such as paraquat exposure which induces oxidative stress, has been shown to increase ISC number and activity in the guts

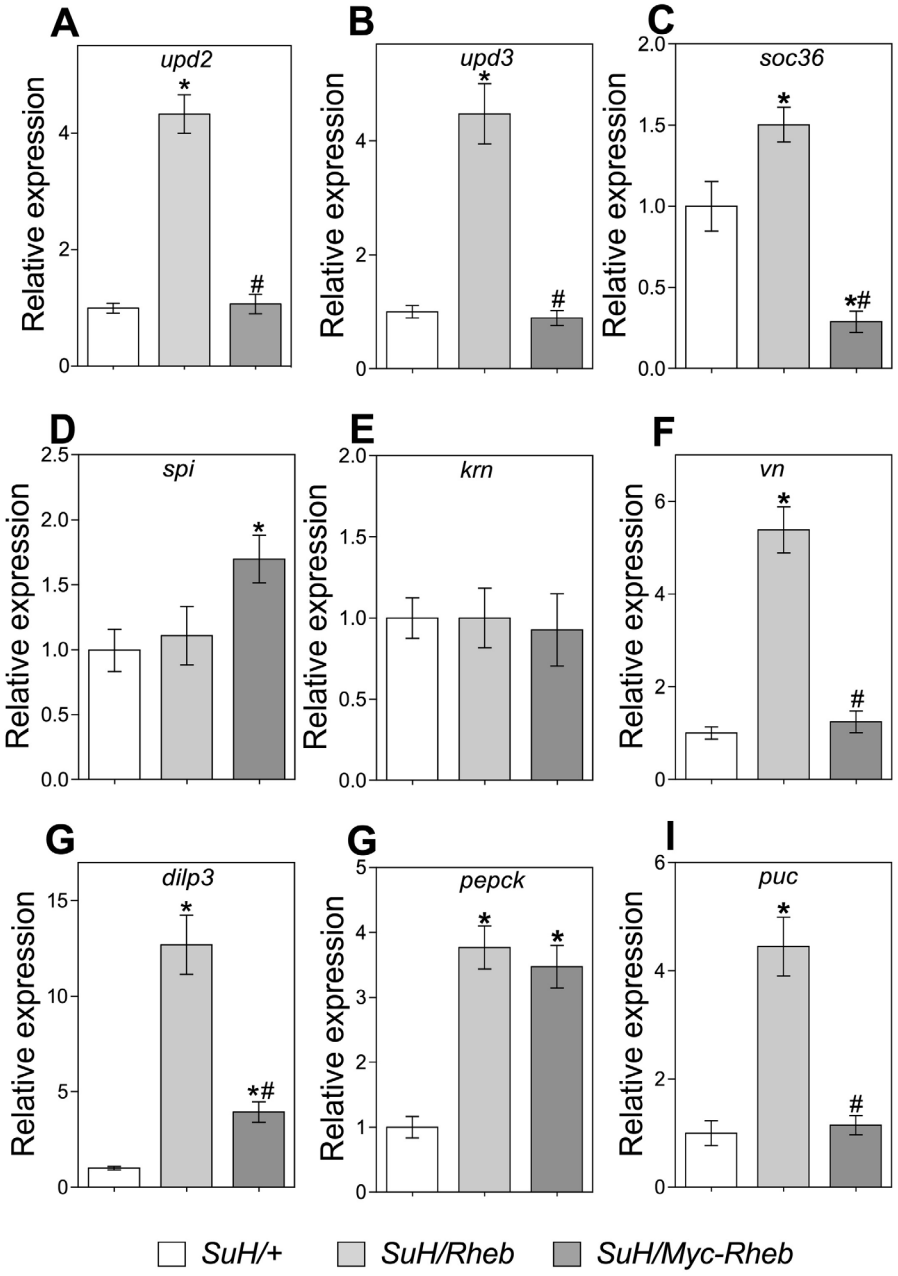

Fig. 6 Gut-specific effects of *Rheb* and *Myc-Rheb* overexpression. Transcript levels of *upd2* (A), *upd3* (B), *soc36* (C), *spi* (D), *krn* (E), *vn* (F), *dilp3* (G), *pepck* (H), *puc* (I) in fly guts. Mean values (± SEM) were compared with Student's *t*-test. An *asterisk* (*) above the *SuH/rheb* or *SuH/myc-rheb* bar indicates a difference from the control group (*SuH/+*) ($p<0.05$; n=3-4); a # above the *SuH/myc-rheb* bar indicates a difference from flies that overexpress *Rheb* ($p<0.05$; n=3-4).

of aged flies [38]. Our data demonstrated that menadione exposure shortened the lifespan of all experimental flies. Furthermore, TOR/Myc activation in EBs had no impact on organismal oxidative stress response. However, the lower resistance to oxidative stress when TOR/Myc was activated in ISCs [11], suggesting a critical role of midgut stem cells, but not their progenitors, in the regulation of stress resistance.

Of significance, this work also uncovered the role of TOR/Myc in *Drosophila* EBs on food consumption and its impact on fecundity rates. The conserved TOR signaling pathway plays a central role in the regulation of nutrient intake and maintaining nutrient balance [39]. Balance in food consumption is important for organismal growth, development, and survival. Feeding rate and egg production are interrelated biological processes in *Drosophila*. Feeding rate is controlled by a molecular pathway which regulates egg production through an ejaculate "sex peptide" [40]. Previous studies have shown that increases in female feeding correlated with higher reproductive rates reflected by higher egg production [41]. Our results demonstrated that TOR activation through *Rheb* overexpression together with *Myc* co-expression decreased the amount of food consumed by a single fly. A similar effect was observed when *Myc-Rheb* was overexpressed in ISCs [16]. Simultaneously, this group of transgenic flies showed lower fecundity. These data indicate that Myc, as a transcription factor, in *Drosophila* enteroblasts may play an important role in linking nutrition and reproduction. Moreover, *Su(H)*-driver which is expressed in EBs has an impact on the expression of sex determinants, which may have significant impact on fecundity rate.

In addition to the important role of TOR in regulating growth and aging, it also regulates various aspects of cellular metabolism. In particular, we were interested in how TOR and Myc activation in EBs affect energy metabolism. We observed higher levels of whole body glucose and *akh* transcript levels in *Rheb* and *Myc-Rheb* expressing flies. Previous studies have demonstrated a hypothetical link between glucose levels and glucagon-like peptide AKH levels [41,42]. DILPs are important insulin signaling molecules that display antagonistic properties to AKH in regulation of *Drosophila* metabolism [41]. Insulin signaling pathways regulate ISC proliferation in *Drosophila* [37] and DILP3 in particular is known to promote ISC differentiation. Activation of TOR signaling pathways through *Rheb* overexpression in EBs lead to higher *dilp2, dilp3, dilp6* transcript levels in fly heads. Previous studies have shown that insulin-producing cells sense nutritional signals for DILP production in *Drosophila* [43,44]. Additionally, Okamoto and Nishmura [43] illustrated the existence of a feedback mechanism between DILPs and FOXO. Fly intestine dilp3 transcript levels increased 12-fold and 4-fold, respectively, in response to *Rheb* and *Myc-Rheb* expression. According to these results, a possible feedback mechanism may exist between DILP3 and Rheb in fly guts. Specifically, *Rheb* overexpression in EBs controls metabolic processes in *Drosophila* gut by regulating dilp3 expression. Elevated *dilp3* levels in the gut may be important for maintaining tissue homeostasis. Moreover, DILPs and AKH have an impact on *tobi* gene expression which was elevated in *Rheb* expressing flies [45]. This increase suggests that TOR activated flies have more insulin signaling in their peripheral tissues compared to control flies. A possible reason why insulin and AKH regulatory signals increased simultaneously could be that transgenic flies have a faster metabolism or that these outputs are regulated by peptide synthesis or release. *Drosophila* PEPCK is involved in gluconeogenesis and glycerogenesis [46]. Interestingly, elevated *pepck* levels correspond with elevated whole body glucose levels in *Rheb* and *Myc-Rheb* expressing flies. Although there are many studies to support this theory [47,48], some evidence suggests that fluctuating levels of *pepck* do not affect levels of plasma glucose in mice [47]. Future studies directly assessing the effect of changes in *pepck* expression on glucose levels are warranted. Only *Myc-Rheb* expression in EBs enhance *pepck* expression in *Drosophila* gut cells, suggesting that *Myc* may directly regulate gluconeogenesis and glycerogenesis. Previous study also demonstrated the involvement of TOR/Myc in ISCs in regulation of metabolities content [16].

Finally, we were interested in the role of TOR and Myc activation in the regulation of lipid metabolism. Brummer lipase (*bmm*) is responsible for TAG mobilization from fly fat bodies [49]. TOR signaling activation through *Rheb* overexpression as well as *Rheb-Myc* expression were found to lead to higher *bmm* expression, but *bmm* expression was not different between *Rheb* and *Myc-Rheb* expressing flies. These results suggest that glucose metabolism, insulin signaling, and TAG mobilization are dependent on TOR activation in EBs. We also observed elevated *4ebp* transcript levels in flies that expressed *Rheb*, and a much more dramatic increase in flies that co-expressed *Myc* transcription factor. It was shown that 4E-BP is activated during starvation and oxidative stress conditions, but its absence impairs fly survival [49]. The study of Tahmasebi and colleagues [50] showed that 4E-BP has a complex impact on the reprogramming process of embryonic fibroblasts into pluripotent stem cells in mice. Moreover, enhanced reprogramming is a

result of increased Myc and Sox2 translation [50]. *Myc* can be considered a downstream target of TOR because its activity is in part controlled by other downstream TOR targets such as S6K [51,52]. TOR and Myc are involved in ribosome biogenesis and protein synthesis [53]. Therefore, the observation of elevated *4ebp* expression in *Rheb*- and *Myc-Rheb*-expressing flies prompted the analysis of total protein levels in transgenic *Drosophila* lines. Interestingly, neither *Rheb* nor *Myc-Rheb* overexpression in EBs influenced protein level in fly body. But we expected that increased *4ebp* expression would increase whole body protein levels because activated (phosphorylated) 4EBP allows translation to proceed. However, we have also found in our study that TOR activation through *Rheb* overexpression may reduce feeding or appetite and does not help survival when flies are given low-nutrient diets. Perhaps the increase in expression of *4ebp* during TOR activation is not in response to TOR activation alone, but the other phenotypes it induces. For future studies, it would be interesting to analyze relative levels of phospho-4EBP and unphosphorylated 4EBP to determine which processes are actually occurring. Another possible explanation of our observation that total protein levels did not differ between experimental fly lines is the existence of compensatory mechanism of protein synthesis by *4ebp* overexpression in response to TOR signaling activation, but in a TOR-independent manner. However, these suggestions need additional experiments to make more specific conclusions.

We were interested whether death of *Rheb* and *Myc-Rheb*-expressing flies is the result of disruption of gut integrity. Smurf assays did not help us to confirm or deny this theory. Aging is associated with reduced cellular regeneration and defects in tissue homeostasis. We found no difference in the number of transgenic "Smurf" flies compared to the control group, indicating that the unabsorbable blue dye did not penetrate the intestinal wall in either of the experimental fly groups. Similar trends were obtained when we expressed *Rheb* and *Myc-Rheb* in ISCs [16]. Therefore, we were interested in observing the effects of TOR and Myc activation on the molecular pathways that control EB viability and proliferation. Intestinal stem cell homeostasis is regulated by Notch, JAK/STAT, EGF, Hippo, insulin, and Wnt signaling pathways [54]. Consequently, the transcript levels of genes that encode ligands for EGFR (*spi, krn, vn*), JAK/STAT (*upd2, upd3*, and downstream gene *soc36*), and insulin signaling (*dilp3*) were measured in *Drosophila* gut. The JAK/STAT signaling pathway is highly conserved from flies to mammals and plays essential roles during development. It also serves as a regulator of stem cell differentiation. Signaling ligands for this pathway, Upd2 and 3, activate the JAK/STAT pathway activity in ISCs [55]. We found higher *Upd2* and *Upd3* transcript levels when *Rheb* is overexpressed in *Drosophila* EBs, indicating that the JAK/STAT pathway is activated. Recent data revealed that these cytokines are controlled by *dTOR* expression, indicating TOR as a possible regulator of JAK/STAT [56]. We also show that Rheb-expressing flies have an elevated transcription of *soc36*, a JAK/STAT target gene. Thus, our findings suggest that TOR signaling activation in EBs leads to increased JAK/STAT activity. We recently showed that *Rheb* overexpression in *Drosophila* ISCs enhanced the relative expression of *upd2* and *soc36* in fly guts [16]. Moreover, the study of Wang and Huang indicated that JAK/STAT acts downstream of TOR signaling by inhibiting Upd secretion [57]. But Myc is post-transcriptionally upregulated upon JAK/STAT activation [58] (Fig. 1A). Inhibition of JAK/STAT signaling promotes gut homeostasis and extends lifespan [59]. Interestingly, *soc36* acts as a pathway repressor via feedback inhibition. Moreover, soc36 suppresses EGFR signaling [60] and can interact with multiple signaling pathways to maintain appropriate stem cell functionality.

During normal tissue homeostasis in the *Drosophila* midgut JAK/STAT signaling acts as a part of a greater regulatory network. It acts together with EGFR and Wnt signaling. This coordination is necessary for ISC maintenance and promotes differentiation of EBs. Specifically, EGFR signaling is required for ISC proliferation induced by JAK/STAT [54], and both JAK/STAT and EGFR are required for the regeneration of midgut epithelium [60]. McNeill and colleagues [61] illustrated the existence of crosstalk between TOR and EGFR signaling. Moreover, TOR controls the expression of several EGFR components, such as *argos*, *rho*, and *pntP2* [61]. We found elevated levels of *vn* transcripts in *Rheb*-expressing flies suggesting that vein is regulated by TOR signaling. Our study also revealed increased *spi* transcript levels when *Myc-Rheb* is overexpressed in *Drosophila* EBs, indicating that *Myc* takes part in the regulation of *spi* expression. Together, the data suggests that TOR/Myc activation influences EGFR activity in fly EBs.

Puckered (puc) is also known to be involved in maintaining the balance between cell differentiation and proliferation [62]. It plays an important role in controlling cell viability and may act as a tumor suppressor gene. We found that TOR activation through *Rheb* overexpression enhanced the relative expression of *puc*. This data is interesting since Rheb is known to have oncogenic properties [14,15]. Therefore, a balance between Rheb and puc expression may be critical for fly survival, and TOR/Myc signaling is likely to be a key regulator of ISC

proliferation and differentiation. Together, our data concerning several key metabolic pathways suggests that TOR/Myc activation has an important role in maintaining cell viability in the fly intestine.

5 Conclusions

Stem cells play a critical role in maintaining tissue homeostasis, which is necessary for organismal survival. Moreover, the TOR/Myc signaling pathway has been shown to be a conserved regulator of longevity. Here, we specifically focus on how activation of TOR and Myc signaling in progenitors cells regulate different aspects of fly metabolism, stress resistance, and aging. Our results demonstrate a significant decrease in lifespan and stress resistance when TOR is activated in *Drosophila* EBs. *Myc* overexpression leads to lower fecundity and feeding levels. Finally, we show that TOR/Myc signaling controls fly metabolism by differentially regulating the expression of key metabolic genes. Our results highlight the importance of TOR/Myc signaling in the regulation of various physiological processes *in Drosophila* progenitor cells. Identification of changes in the expression of specific genes encoding signal transduction proteins involved in proliferation and differentiation suggest that TOR/Myc activation is critical for maintaining *Drosophila* gut homeostasis.

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Abbreviations

TOR, target of rapamycin; EB, enteroblast; JAK/STAT, Janus kinase and signal transducer and activator of transcription; EGFR, epidermal growth factor receptor; ISC, intestinal stem cell; EC, enterocyte; EE, enteroendocrine; Su(H), suppressor of hairless; TSC, tuberous sclerosis complex; CAFE, capillary feeding assay; ROS, reactive oxygen species; TAG, triglycerides; DILP, *Drosophila* insulin-like peptide; AKH, adipokinetic hormone; PEPCK, phosphoenolpyruvate carboxykinase; TOBI, target of brain insulin; 4EBP, 4E binding protein.

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