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CDK8 mediates the dietary effects on developmental transition in *Drosophila*



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ABSTRACT

The complex interplay between genetic and environmental factors, such as diet and lifestyle, defines the initiation and progression of multifactorial diseases, including cancer, cardiovascular and metabolic diseases, and neurological disorders. Given that most of the studies have been performed in controlled experimental settings to ensure the consistency and reproducibility, the impacts of environmental factors, such as dietary perturbation, on the development of animals with different genotypes and the pathogenesis of these diseases remain poorly understood. By analyzing the *cdk8* and *cyclin C* (*cycC*) mutant larvae in *Drosophila*, we have previously reported that the CDK8-CycC complex coordinately regulates lipogenesis by repressing dSREBP (sterol regulatory element-binding protein)-activated transcription and developmental timing by activating EcR (ecdysone receptor)-dependent gene expression. Here we report that dietary nutrients, particularly proteins and carbohydrates, modulate the developmental timing through the CDK8/CycC/EcR pathway. We observed that *cdk8* and *cycC* mutants are sensitive to the levels of dietary proteins and seven amino acids (arginine, glutamine, isoleucine, leucine, methionine, threonine, and valine). Those mutants are also sensitive to dietary carbohydrates, and they are more sensitive to monosaccharides than disaccharides. These results suggest that CDK8-CycC mediates the dietary effects on lipid metabolism and developmental timing in *Drosophila* larvae.

1. Introduction

Deciphering the molecular mechanisms underlying multi-factorial diseases, including cancer, obesity, diabetes, cardiovascular diseases, mental disorders, and neuro-degenerative diseases, has been the major challenge of modern biomedical research. These diseases are determined by genetic, environmental, and chance factors (such as diet, temperature, and stress), as well as the complicated interactions among these factors (Berg, 2016). To mitigate the profound social and economic burdens caused by these multifactorial diseases, it is essential to gain mechanistic insights into the complex gene-environment interactions. Because the effects of genetic and environmental factors are often entangled, thereby obscuring mechanistic insights (compounded by the fact that most of developmental genetic analyses have been carried out in model organisms maintained in standard laboratory settings), the impacts of environmental exposures, with nutrient fluctuations being of major importance, on mutant phenotypes are little understood.

Drosophila represents a powerful model organism to gain insights into the mechanisms underpinning the complex gene-environment

interactions. First, the major signaling events and metabolic regulations are conserved but simpler in *Drosophila*. For example, the underlying biochemical reactions for metabolic processes, such as glycolysis, the tricarboxylic acid cycle, lipogenesis, lipolysis, and fatty acid β -oxidation, are virtually identical to those in mammals. Second, the biological processes from the molecular and cellular to organismal and behavioral levels are well characterized in *Drosophila*. Third, a rich literature about the function and regulation of most of the genes has been accumulated in the past century. Most of these studies were performed with standard diets, such as the “cornmeal, molasses and yeast medium” (CMY), at standard temperature (25 °C), and with the routine day-night cycle. Fourth, there are abundant genetic tools available, as over two-thirds of *Drosophila* genes have at least one mutant alleles maintained in stock centers or in the research community. Finally, *Drosophila* has a short life span and the environmental factors, such as diet and temperature, can be manipulated with relative ease. These combined advantages make *Drosophila* ideally suited to serve as an experimental system to gain insights into our understanding of the aforementioned multi-factorial diseases (Arrese and

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Soulages, 2010; Cox et al., 2017; Kuhnlein, 2012; Li and Tennessen, 2017; Liu and Huang, 2013; Mattila and Hietakangas, 2017; Schlegel and Stainier, 2007; Sieber and Spradling, 2017).

The developmental timing, particularly the larval-pupal transition in *Drosophila*, is an ideal phenomenon to analyze the interplay between dietary factors and genetic factors. During the larval stage, larvae keep eating and their body weight increases over 200 times within 4 days largely due to elevated *de novo* lipogenesis. In contrast, they are immobile during the pupal stage and the energy required for metamorphosis is mainly derived from lipid catabolism. The larval-pupal transition is under the control of two major hormones, juvenile hormone (JH) and steroid hormone ecdysone, and their corresponding receptors (King-Jones and Thummel, 2005; Nakagawa and Henrich, 2009; Riddiford, 1993; Riddiford et al., 2000; Yamanaka et al., 2013). Although hormonal regulation is critical for coordinating larval growth, metabolism, and environmental factors such as dietary nutrients, the underlying molecular mechanism has not yet been fully understood (Andersen et al., 2013; Danielsen et al., 2013; Rewitz et al., 2013; Tennessen and Thummel, 2011; Thummel, 2001).

Our previous studies in analyzing the *cdk8* (*cyclin-dependent kinase 8*) and *cycC* (*cyclin C*) mutants suggest that the CDK8-CycC complex may serve as a regulatory node in linking nutrient intake, i.e. feeding and starvation, to developmental timing and fat metabolism during *Drosophila* development (Xie et al., 2015). CDK8 and CycC, along with MED12 and MED13, form the kinase module of the Mediator complex, which functions as a bridge between different transcription factors and the general transcription machinery, which includes RNA Polymerase II (Clark et al., 2015; Conaway and Conaway, 2011; Kornberg, 2005; Malik and Roeder, 2005; Poss et al., 2013; Xu and Ji, 2011; Yin and Wang, 2014). Our previous work revealed that CDK8-CycC negatively regulates lipogenesis by directly inhibiting the dSREBP (sterol response element binding protein)-dependent gene expression (Zhao et al., 2012), and that CDK8-CycC can regulate the larval-pupal transition by positively regulating EcR (ecdysone receptor)-dependent gene expression (Xie et al., 2015). Interestingly, CDK8 protein levels are increased by nutrient deprivation, but decreased by refeeding during the larval stage (Xie et al., 2015). These results suggest that CDK8-CycC may serve as a regulatory node in linking nutritional perturbations to fat metabolism and developmental transitions in *Drosophila* larvae.

To further test this model, we analyzed the effects of quality of nutrient intake, i.e. different types of amino acids and sugars (monosaccharides and disaccharides), on the developmental transitions in the *cdk8* and *cycC* mutant larvae. Here we show that the *cdk8* and *cycC* mutants are sensitive to nutritional variations in diets, particularly a few types of dietary amino acids and monosaccharides. These results provide further evidence supporting a critical role of CDK8-CycC in mediating the dietary effects on lipid metabolism and developmental transition in *Drosophila* larvae.

2. Results

2.1. *cdk8* and *cycC* mutants are sensitive to dietary perturbations

Based on our previous work, regarding the effects of starvation and refeeding on the levels of CDK8 and developmental transition as well as the effects of CDK8-CycC in regulating EcR and dSREBP-dependent gene expression, we have proposed that CDK8-CycC serves as a regulatory node linking nutrient availability and the larval-pupal transition in *Drosophila* (Xie et al., 2015). In this model (Fig. 1A), the CDK8 activity is inhibited by insulin/TOR (Target of Rapamycin) signaling during the larval stage, which allows dSREBP to activate the expression of lipogenic genes, thereby promoting lipid accumulation. The wandering behavior triggered by a pulse of ecdysteroids allows the accumulation of CDK8-CycC, which inhibits dSREBP but activates EcR-dependent gene expression, thereby blocking *de novo* lipogenesis while promoting metamorphosis during the pupal stage.

Although the data based on the starvation and re-feeding of early third instar larvae, as well as developmental genetic analyses of the phenotypes of the *cdk8* and *cycC* mutants, support this model (Xie et al., 2015), we sought to further validate this model using dietary perturbations because of their effects on insulin/mTOR signaling and growth. Initially, we analyzed the effects on developmental transition by feeding both the *cdk8* or *cycC* mutants and the control larvae with either a low sugar diet (LSD, see below), or a high sugar diet (HSD, i.e., LSD with 20% of sucrose). The ingredients of LSD are better defined than the CMY recipe, and the HSD has also previously been shown to delay the larval-pupal transition by prolonging the period of activation of insulin signaling (Musselman et al., 2011; Na et al., 2013; Pasco and Leopold, 2012). As expected, HSD dramatically delays the developmental timing in the control larvae (*w¹¹¹⁸*; Fig. 1B). In control animals fed with the LSD, the levels of CDK8, CycC, EcR and USP proteins were increased and the mature nuclear dSREBP level was reduced at the onset of the larval-pupal transition at the end of day 5 after egg laying (AEL, Fig. 1C). In contrast, the levels of CDK8 and CycC remained low in animals fed on a HSD until day 9 AEL, when pupariation was initiated (Fig. 1B, Fig. 1C). The effect of HSD on CDK8 and CycC was evident in steady-state mRNA levels of *cdk8* and *cycC*, which were significantly lower in HSD-fed animals during day 5 and day 6 AEL (Fig. 2A, Fig. 2B). The protein levels of EcR and USP also closely mirrored their mRNA levels (Fig. 1C, Fig. 2C, Fig. 2D) and EcR activity, as monitored by the levels of EcR target genes (Fig. 2E, Fig. 2F). Similarly, reduced CDK8-CycC levels in HSD-fed animals were correlated with increased levels of nuclear dSREBP (Fig. 1C) and dSREBP activity, which were monitored by expression of dSREBP target genes including *dFAS* (Fig. S1A), *dACC* (Fig. S1B), and *dACS* (Fig. S1C). The mRNA level of *dSREBP* was not significantly affected by HSD (Fig. S1D), indicating that HSD affects dSREBP protein level. Importantly, low CDK8 and CycC levels are correlated with high mature dSREBP and low EcR and USP levels (Fig. 1C). These observations suggest that the regulation of dSREBP and EcR by CDK8 is delayed upon HSD feeding, consistent with the model in which CDK8-CycC integrates diet and feeding with the timing of pupariation (Fig. 1A).

If the CDK8-CycC complex is required for the dietary effects on developmental transitions, then *cdk8* (*cdk8^{K185}* homozygous) and *cycC* (*cycC^{Y5}* homozygous) mutants are expected to be more sensitive in response to HSD than the control. Indeed, *cycC* mutant larvae fed with HSD took ~7 more days to pupariate than mutant larvae fed with a LSD, which is significantly longer than the ~4.5 day delay in the control (Fig. 1B). Strikingly, when fed with either HSD or LSD, no *cdk8* mutant larvae could pupariate and they died either before or during the third instar, even 18 days AEL (Fig. 1B). These observations suggest that both the *cdk8* and the *cycC* mutants are particularly sensitive to nutrient conditions, but it is unexpected and puzzling as to why the *cdk8* mutant larvae become hypersensitive when fed with either a HSD or a LSD.

2.2. *cdk8* mutants are sensitive to dietary protein levels

To understand why *cdk8* mutants are hypersensitive to both a HSD and a LSD, we tested a different HSD recipe by analyzing the timing of pupariation using the standard “cornmeal, molasses and yeast” medium (referred to as the “CMY-LSD” diet in this work) to the same medium with additional 20% sucrose (designated as the “CMY-HSD” diet). Compared to larvae fed with CMY-LSD, feeding with CMY-HSD significantly delayed the larval-pupal transition for ~4.5 days and ~3.5 days in *cdk8* and *cycC* mutants, respectively, while the control animals were delayed for ~2.5 days (Fig. 3A). Consistent with this observation, EcR target genes such as *E74* and *E78* are reduced in wild-type larvae fed by the CMY-HSD, and they are also lower in *cdk8* mutants than the wild-type control (Fig. S2A and Fig. S2B). Increasing dietary sucrose has little effects on the mRNA levels of *cdk8* and *cycC* in wild-type larvae (Fig. S2C). These observations differ from the data that the *cdk8*

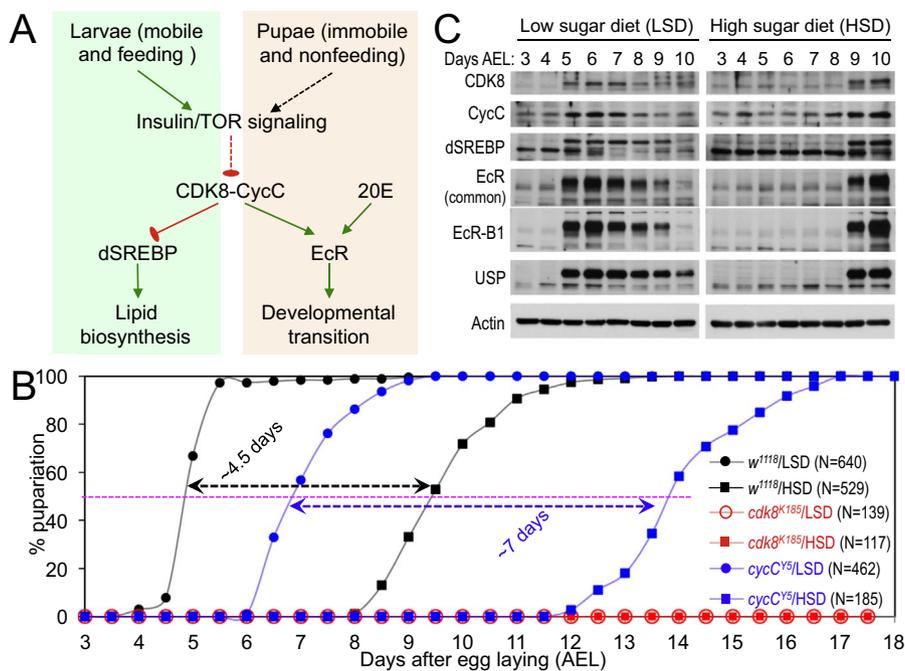


Fig. 1. CDK8-CycC couples nutrition, lipid biosynthesis, and developmental timing. (A) Model: CDK8-CycC coordinately regulates lipogenesis by directly repressing dSREBP-activated gene expression and developmental timing by activating EcR-activated gene expression during the larval and pupal stages. Arrows represent activation, and blunt arrows represent inhibition. (B) Percentage of pupariated animals raised on either the low sugar diet (LSD) or the high sugar diet (HSD) foods between 3 and 18 days AEL. (C) The protein levels of CDK8, CycC, dSREBP, EcR-B1 and USP (upper band is the 54 kDa full-length USP) in wild-type larvae cultured on either the LSD or the HSD between 3 and 10 days AEL. For dSREBP, the lower band (~49 kDa) is the mature nuclear form, while the upper band (53–54 kDa) is the N-terminal fragment of dSREBP after cleavage by the S1P (Xie et al., 2015).

larvae fail to pupariate when fed with either a HSD or a LSD (Fig. 1B), indicating that other dietary components may contribute to the larval lethality under both HSD and LSD.

The LSD is also known as the “German food” or sick fly formulation (Backhaus et al., 1984). Compared to standard CMY-LSD medium, the recipe of LSD is featured with ~5X higher levels of protein from extra yeast (~12% in LSD or “German food” vs. 2.5% yeast in CMY-LSD medium). If a higher level of total protein in a LSD causes the larval lethality of the *cdk8* mutants, then increasing protein levels in the CMY-LSD medium is expected to have similar effects on the larval-pupal transition. Inactive dried yeast is the major protein component of the LSD (Table S1), and it also provides low levels of carbohydrates,

salts, vitamins, and cholesterol. Indeed, when feeding with the CMY-LSD medium with 5% extra yeast, ~60% of the *cdk8* mutants never pupariate, and they die in the third instar larval stage (Fig. 3B). Strikingly, all of the *cdk8* mutants are larval lethal when fed with the CMY-LSD medium with 10% extra yeast and they never pupariate (Fig. 3B), similar to animals fed with LSD (Fig. 1B). Similar analyses using *cycC* mutant larvae showed that CMY-LSD with 5% extra yeast had a weak effect on the pupariation curve, but feeding the *cycC* mutants with CMY-LSD with 10% extra yeast caused ~80% larval lethality (Fig. 3C). Further increasing the levels of yeast to 20% in the CMY medium caused complete larval lethality in both *cdk8* and *cycC* mutants (data not shown). These observations suggest that the larval

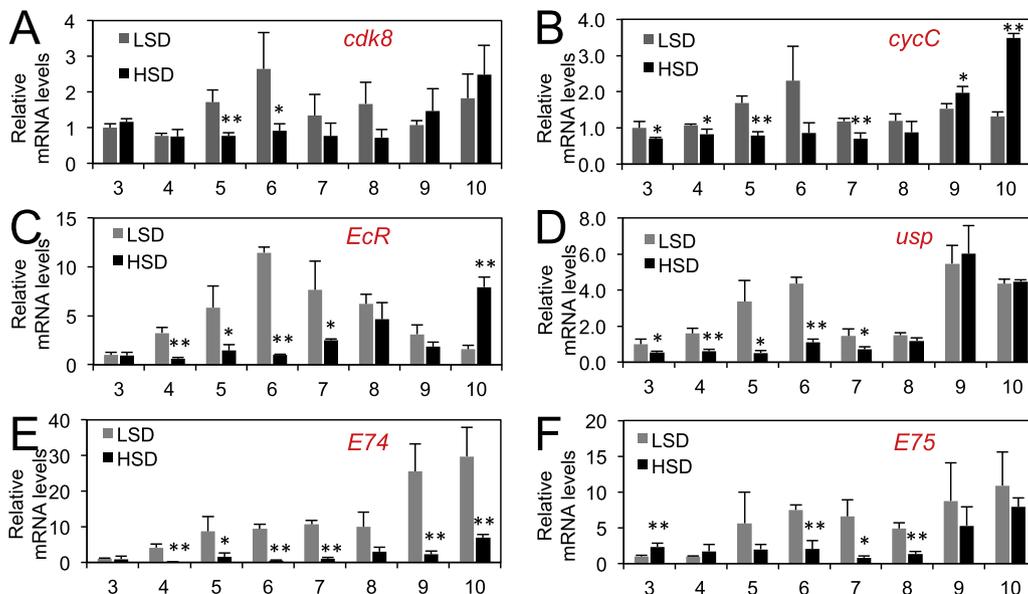


Fig. 2. The relative mRNA levels of different genes in LSD- or HSD-fed larvae between 3 and 10 days AEL as assayed using qRT-PCR. HSD refers to LSD with addition of 20% of sucrose. **p* < 0.05; ***p* < 0.01; ****p* < 0.001 based on *t*-tests.

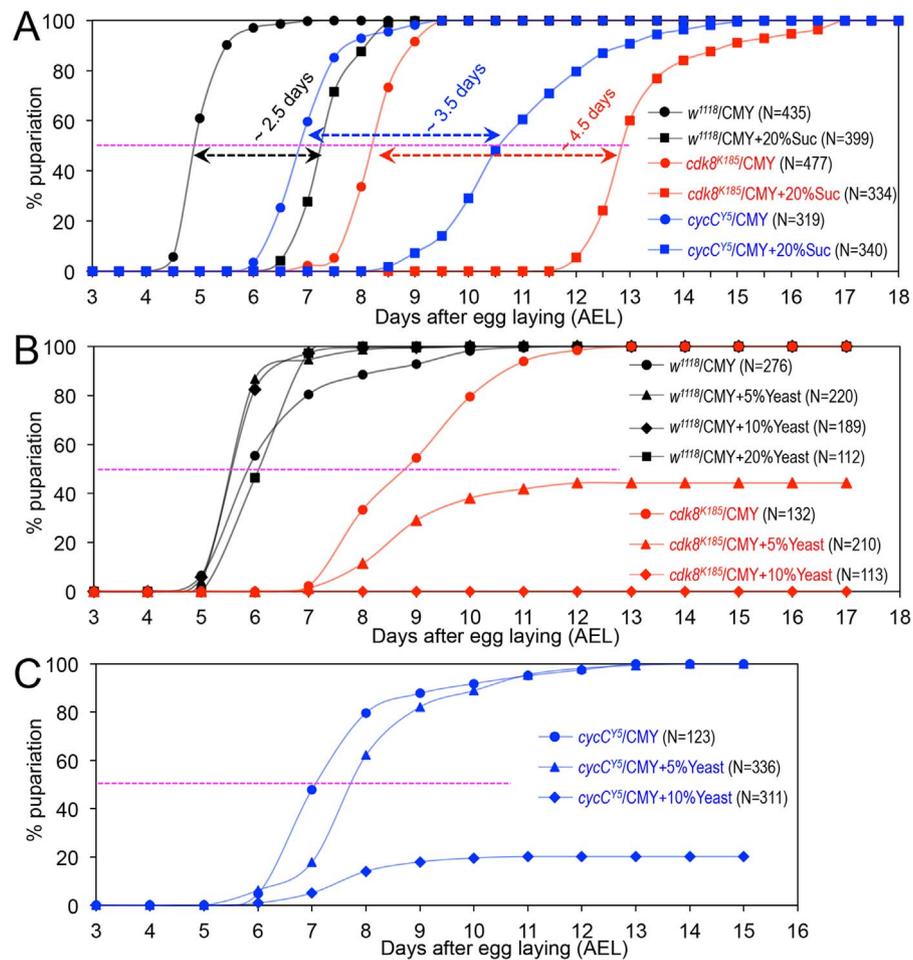


Fig. 3. The effects of dietary protein levels on the timing for pupariation of the *cdk8* and *cycC* mutants. (A) Percentage of pupariated *cdk8* and *cycC* mutants raised on the CMY (referred to as “CMY-LSD”) or CMY-HSD (CMY with 20% sucrose) foods between 3 and 18 days AEL. Percentage of pupariated *cdk8* (B) and *cycC* (C) mutants raised on the CMY with increasing levels of yeast.

lethality of *cdk8* and *cycC* mutants induced by feeding LSD and HSD may be caused by a higher level of proteins in the LSD and HSD recipes compared to the CMY-LSD medium.

2.3. *cdk8* mutants are sensitive to several specific amino acids

To further validate the effects of dietary protein in lethal stage of the *cdk8* mutants, we asked whether reducing proteins levels in LSD can alleviate the effects of LSD on larval lethality and enable them to go through the larval-pupal transition. To test this, we fed both the *cdk8* mutant larvae and the control (*w¹¹¹⁸*) in LSD containing reduced the levels of dry yeast by 50% and 75%, without altering other ingredients in the LSD recipe. As shown in Fig. 4A, about 20% of the *cdk8* mutant larvae pupariated when fed with LSD containing 50% less dry yeast (“LSD-50%Yeast”), while 60% of *cdk8* mutants pupariated when fed with LSD with a 75% reduction of dry yeast (“LSD-75%Yeast”). These observations indicate that a higher level of protein in LSD may cause the larval lethality of the *cdk8* mutants. Together with the results showed in Fig. 3, these observations suggest that the *cdk8* mutant larvae are particularly sensitive to the levels of dietary proteins.

Proteins and amino acids in diets are known to stimulate the activity of TOR, an atypical protein kinase that plays important roles in controlling growth and metabolism (Bar-Peled and Sabatini, 2014; Jewell et al., 2013; Loewith and Hall, 2011; Sabatini, 2017). Activation of TOR signaling in prothoracic gland (PG) by nutrients regulates biosynthesis of ecdysone and the larval-pupal transition (Layalle et al., 2008). Interestingly, mammalian TOR (mTOR) was recently shown to

inhibit CDK8-CycC in mammalian cells (Feng et al., 2015). Therefore, we asked whether feeding the *cdk8* mutant larvae with Rapamycin, a compound known to inhibit mTOR activity (Ballou and Lin, 2008; Benjamin et al., 2011), could alleviate the effects of extra yeast on the viability of the *cdk8* mutant larvae. As shown in Fig. 4B, addition of 20 nM of Rapamycin in diets with 5% or 10% extra yeast increased the pupariation rates of the *cdk8* mutant larvae by ~20%, indicating that the effects of extra yeast on pupariation rate is in part through mTOR.

Since mTOR is specifically activated by certain amino acids, such as Leu (leucine) and Arg (arginine) (Bar-Peled and Sabatini, 2014; Jewell et al., 2013; Kim, 2009), we asked whether different types of amino acids have the same or different impacts on the development of the *cdk8* mutant larvae. Specifically, we tested each of the 20 amino acids for their effects on the pupation course of *cdk8* mutants using the concentration of the 20 amino acids as 10 times higher as described previously (Grandison et al., 2009). As shown in Fig. 4C, increasing the concentration of seven amino acids, including Arg, Gln (glutamine), Ile (isoleucine), Leu, Met (methionine), Thr (threonine), and Val (valine), in a diet strongly induced the larval lethality of the *cdk8* mutants. In contrast, addition of the other 13 amino acids had no obvious effect on the viability of the *cdk8* mutants (Fig. 4C and data not shown). The mRNA levels of EcR target gene such as *E74* and *E78* are not significantly altered in wild-type larvae fed with CMY diet with increased Val, but their levels are reduced in *cdk8* mutants as expected (Fig. S3A, Fig. S3B). Consistent with this observation, increasing these amino acids in diet does not affect the growth nor the developmental transitions of the wild-type larvae (Fig. S4 and data not shown). Thus,

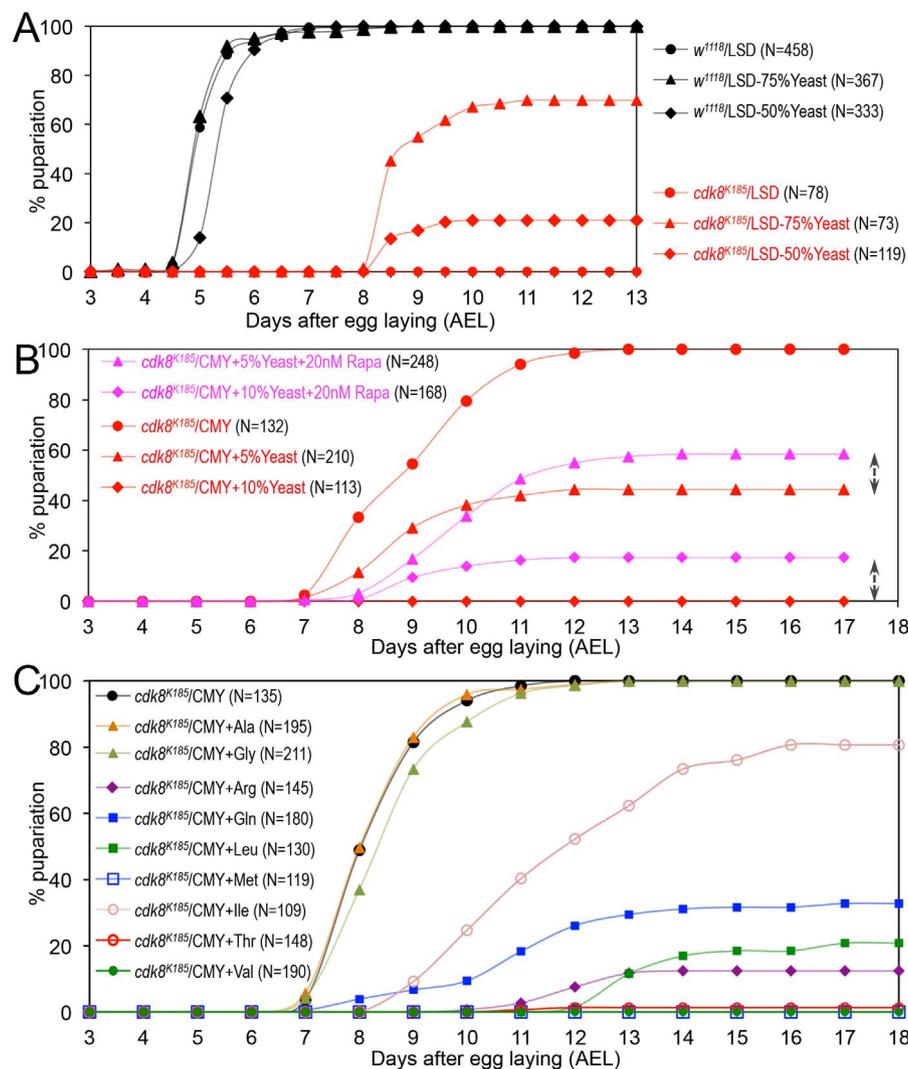


Fig. 4. The effects of dietary levels of protein, rapamycin, and amino acids on the timing for pupariation of the *cdk8* mutants. (A) Percentage of pupariated wild-type and *cdk8* mutants raised on the LSD or LSD with reduced levels of yeast: “LSD-50%Yeast” represents the diet with 50% reduction of the yeast in the LSD recipe; “LSD-75%Yeast” is the diet with 75% reduction of yeast in the LSD recipe (see Table S1 for details). (B) Percentage of pupariated *cdk8* mutants raised on the CMY diet with 5% or 10% of extra yeast, with or without rapamycin (Rapa). (C) Percentage of pupariated *cdk8* mutants raised on the CMY diet with extra amino acids as indicated.

cdk8 mutants are particularly sensitive to specific types of dietary amino acids. Increased dietary Val also does not affect the mRNA levels of *cdk8* and *cycC* in wild-type larvae (Fig. S3C), suggesting the effect likely occurs at the CDK8-CycC protein level.

2.4. The effects of different types of dietary sugars on timing of pupariation of the *cdk8* mutants

Although the larval viability of the *cdk8* and *cycC* mutants is sensitive to the levels of dietary protein, the mutant larvae that did pupariate made the larval-pupal transition between day 7 and day 9 AEL when fed with CMY diets with or without extra yeast (Fig. 3B, Fig. 3C). When fed with high protein diets (CMY with 5%, 10% or 20% of yeast), the control (w^{1118}) larvae grow well and pupariate more synchronously at the normal timing for the larval-pupal transition (Fig. 3B). These observations suggest that the extra sucrose in CMY-HSD delays larval-pupal transition in the *cdk8* and *cycC* mutants but does not affect the viability of the mutant larvae, while extra protein from yeast affects the larval viability but has a weaker effect on the timing for pupariation than the levels of dietary sugars.

To characterize the effects of different types of sugars on developmental timing for the *cdk8* and *cycC* mutants, we first tested sucrose

and trehalose, the two types of disaccharides. As shown in Fig. 5A, adding 5% of sucrose and trehalose in CMY diet does not affect the timing of pupariation for the wild-type animals (the control is shown in Fig. 5B). 5% of sucrose in CMY food does not further delay the timing for pupariation of the *cdk8* mutants, but less than 10% of *cdk8* mutants can pupariate when fed with 5% of trehalose in CMY food. Most of these mutants die as larvae (Fig. 5A). In contrast, 5% of trehalose or sucrose does not have obvious effects on the timing for pupariation for *cycC* mutant animals (Fig. 5A), consistent with the notion that *cdk8* mutants display more severe defects than *cycC* mutants (Xie et al., 2015). These observations suggest that the *cdk8* mutants display different sensitivity to different dietary disaccharides.

Given that dietary polysaccharides need to be digested into monosaccharides to be absorbed, we asked whether different types of monosaccharides could impact the developmental transition in the same or different fashions in wild-type and the *cdk8* or *cycC* mutants. For this reason, we tested three types of monosaccharides, glucose (Fig. 5B), fructose (Fig. 5C), and galactose (Fig. 5D). We have observed that 20% of glucose more severely delayed the timing for pupariation of the wild-type animals than the 20% of sucrose (Fig. 5B vs. Fig. 3A). While 5% of glucose in CMY food does not obviously affect the developmental timing for the control animals (Figs. 5B), 5% of glucose

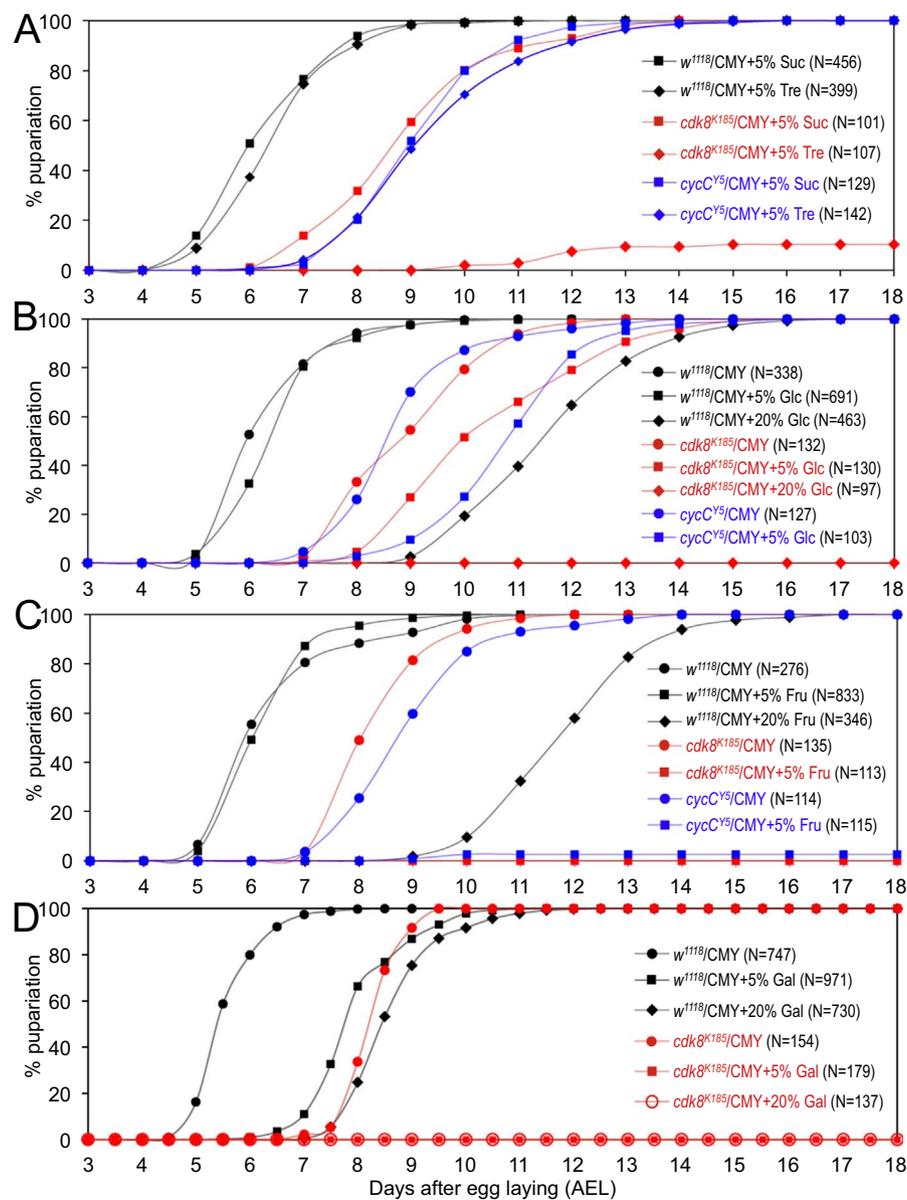


Fig. 5. The effects of dietary carbohydrate levels on the timing for pupariation of the *cdk8* and *cycC* mutants. (A) Percentage of pupariated wild-type, *cdk8*, and *cycC* mutants raised on the CMY with 5% of sucrose (Suc) or trehalose (Tre). (B) Percentage of pupariated wild-type, *cdk8*, and *cycC* mutants raised on the CMY with 5% or 20% of glucose (Glc). The data for *cycC* mutants on 20% of Glc are identical to those of *cdk8* mutants on 20% of Glc in CMY diet, thus not shown. (C) Percentage of pupariated wild-type, *cdk8*, and *cycC* mutants raised on the CMY with 5% of fructose (Fru). (D) Percentage of pupariated wild-type and *cdk8* mutants raised on the CMY with 5% or 20% of galactose (Gla).

in CMY further delays the timing for pupariation of both *cdk8* and *cycC* mutants, and all these mutants die as larvae when fed with CMY food with 20% glucose (Fig. 5B shows the data for *cdk8* mutants; identical data for *cycC* mutants are not shown). These observations suggest that glucose has a stronger impact on timing of pupariation in the wild-type animals than disaccharides, and both *cdk8* and *cycC* mutants are more sensitive than the control when tested with the same type of dietary sugars.

Similar analyses were performed to test the dietary effects of fructose and galactose. For wild-type animals, both 5% and 20% of fructose (Fig. 5C) had the same effects on the timing for pupariation as glucose (Fig. 5B), while 5% and 20% galactose has stronger effect in delaying this transition (Fig. 5D vs. Fig. 5B/5C). In contrast, 5% (or 20%) of either fructose (Fig. 5C) or galactose (Fig. 5D) caused larval lethality for both *cdk8* and *cycC* mutants, and the mutants never pupariate (data for *cycC* mutants are not shown). These observations show that different types of monosaccharides have different impacts on the wild-type animals, and fructose and galactose have stronger effects

than glucose on developmental transition for both *cdk8* and *cycC* mutants. Taken together, these results show that different types of dietary sugars have different effects on timing of pupariation in *Drosophila*. Monosaccharides have stronger effects than disaccharides in delaying the timing of pupariation, and both the *cdk8* and *cycC* mutants are more sensitive than the wild-type when fed with the same diets. These observations support the model that CDK8 and CycC play important roles in linking the dietary perturbations and developmental transitions in *Drosophila* (Fig. 6, see below).

3. Discussion

Studies in the past two decades have significantly advanced our understanding of how dietary nutrients regulate organismal growth and developmental transitions in *Drosophila*. At the tissue and organ levels, crosstalks among the fat body, brain, prothoracic gland (PG), imaginal discs, and gut integrate the nutrient conditions with organismal growth and biosynthesis and release of the major hormone

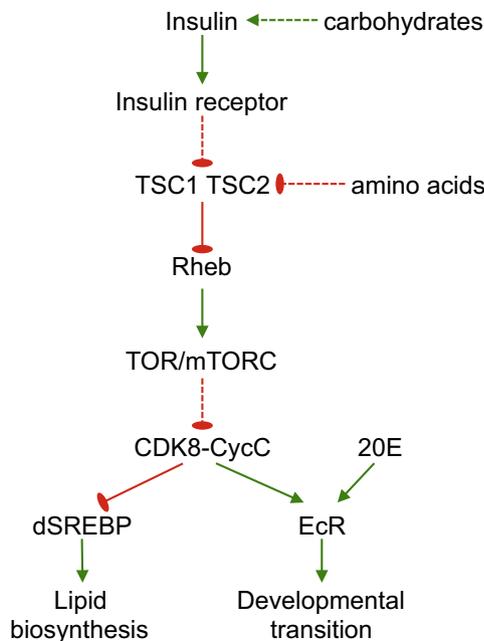


Fig. 6. CDK8-CycC mediates the dietary effects on lipid metabolism and developmental timing in *Drosophila*. The model schematizes the effects of dietary factors such as carbohydrates and amino acids through the insulin/TOR pathway and CDK8-CycC complex, which coordinate the activities of transcription factors such as dSREBP and EcR during the larval-pupal transition.

ecdysone (Andersen et al., 2013; Danielsen et al., 2013; Edgar, 2006; Nijhout et al., 2014; Tennessen and Thummel, 2011). Coordination of these processes among different tissues depends on hormonal regulation within the larva. Several major signaling pathways have been identified to play critical roles in those processes, including the insulin/insulin-like growth factor signaling (IIS) pathway regulated by carbohydrates, the TOR signaling pathway regulated by amino acids in both fat body and discs, the Upd2-mediated crosstalk between the fat body and the brain in response to the HSD, and the prothoracicotropic hormone (PTTH)-mediated crosstalk between the brain and PG (see below). These elegant studies have established an intellectual framework to understand how larval growth and developmental timing are coordinated with environmental factors such as dietary nutrients and photoperiod, but the exact molecular mechanisms underlying these crosstalks are still not fully understood. It is important to identify additional players and elucidate how they are involved in these processes. In this report, we summarize our characterization of effects of different diets on the timing for pupariation of the *cdk8* and *cycC* mutant larvae, and our results suggest that CDK8-CycC is necessary for mediating the dietary effects on the developmental timing during the larval stage.

This study has raised more questions than it can answer. A key question related to these results is: why do the *cdk8* mutant larvae display different sensitivities to excessive levels of different types of carbohydrates and amino acids? This is a complex question, and here we discuss potential models to answer it from the following aspects, all of which are centered on the idea that the *cdk8* mutant larvae may be defective in multiple signaling pathways critical in coordinating the inter-organ crosstalks in linking dietary effects on developmental timing.

Given the essential roles of CDK8-CycC and the Mediator complexes in regulating RNA Pol II-dependent gene expression, mutations in CDK8 may disrupt the outputs of multiple signaling pathways involved in coordinating the inter-organ crosstalks. For example, the single insulin-like receptor expressed in various tissues mediates the effects of eight *Drosophila* insulin-like peptides secreted by insulin-producing cells in the larval brain, fat body, or impaired growth

imaginal discs (Kannan and Fridell, 2013). Our previous work suggests that CDK8-CycC is negatively regulated by insulin signaling (Zhao et al., 2012). Consistent with this notion, CDK8-CycC is down-regulated by mTORC1 in mouse liver and cultured HEK293T cells (Feng et al., 2015), and the larval fat body in *Drosophila* (Tang et al., 2018). In addition, embryos without the maternal *cycC* do not hatch, suggesting that the pupal lethality of the *cdk8* and *cycC* mutant is due to gradual depletion of maternal CDK8-CycC (Xie et al., 2015). Thus, one possibility is that the residual maternal CDK8 (or CycC) in the *cdk8* (or *cycC*) mutant larvae is further down-regulated by IIS signaling activated by dietary carbohydrates, which may further postpone the regulatory network (Fig. 1A, Fig. 6). This model may explain why the *cdk8* and *cycC* mutant larvae display further delay than the control upon feeding on diets overloaded with carbohydrates. Along this line, why do *cdk8* mutants respond differently to different types of carbohydrates? It has been proposed that different tricarboxylic acid intermediates from the fat body, such as fumarate, isocitrate, and malate, can prompt the release of ecdysone from the PG by stimulating the synthesis of PTTH in the brain (Xu et al., 2012). Thus, we speculate that different carbohydrates may be absorbed and metabolized at different rates, thus affecting the dynamics of different tricarboxylic acid intermediates and resulting in different effects on ecdysone release. It will be intriguing to apply metabolomic approaches to analyze the larvae feed with different levels of carbohydrates in the future (Cox et al., 2017).

The crosstalk between the IIS pathway and TOR, the major sensor for amino acids, is conserved between *Drosophila* and mammals (Fig. 6) (Bar-Peled and Sabatini, 2014; Dennis et al., 2011; Edgar, 2006; Sabatini, 2017). It is interesting to note that *cdk8* mutant larvae seem to be particularly sensitive to increased dietary levels of seven amino acids: Arg, Gln, Ile, Leu, Met, Thr, and Val. Of these, Leu, Ile, and Val are branched-chain amino acids, which are known to stimulate mTORC1 activities (Lynch and Adams, 2014; Neishabouri et al., 2015; Yoon, 2016; Zhang et al., 2017). Besides these branched-chain amino acids, the other four amino acids (Arg, Gln, Met, and Thr) can also stimulate TOR in different experimental systems (for examples, (Feng et al., 2013; Gallinetti et al., 2013; Lansard et al., 2011; Sabatini, 2017; Zhai et al., 2015)). Recently, it was reported that the abundance of CDK8-CycC is down-regulated by mTORC1 activation in *Drosophila*, as well as cultured mammalian cells and mouse liver (Feng et al., 2015; Tang et al., 2018). Therefore, it is likely that increased dietary amino acids may decrease the residual maternal CDK8 and CycC through the activation of TOR, which may explain why *cdk8* mutants are particularly sensitive to these amino acids (Fig. 6). However, it is also possible that these amino acids may feed into the CDK8 regulatory network in distinct modes. For example, besides activating TOR, Leu and Ile can also stimulate the secretion of the insulin-like peptides from insulin-producing cells in the larval brain, thereby indirectly activating TOR (Geminard et al., 2009). To understand the different dietary effects of amino acids on *cdk8* mutants, further analyses are necessary to distinguish these scenarios.

Besides the dietary effects on CDK8-CycC through the IIS pathway and TOR, defective functions of CDK8 may also disrupt the activities of other signaling pathways involved in coordinating different organs and growth control in response to dietary perturbations. For example, the transforming growth factor β (TGF β)/Activin signaling pathway activates the expression of *Insulin-like receptor* and *torso* in the PG, a process required for inducing biosynthesis of ecdysteroids and the larval-pupal transition (Gibbens et al., 2011). In mammalian systems, CDK8 and CDK9 can positively regulate the activity of SMADs, the key transcription factors downstream of TGF β signaling (Alarcon et al., 2009). CDK8 and CDK9 play similar role in activating Mad-dependent gene expression in *Drosophila* (Aleman et al., 2014; Li et al., 2018), thus the PTTH-regulated synthesis and release of ecdysone may be affected in *cdk8* mutants. Consistent with this prediction, the expression of a few Halloween genes, regulated by PTTH and its receptor

Torso (Rewitz et al., 2009), are lower in *cdk8* and *cycC* mutants than the control, accompanied with reduced levels of ecdysteroids in these mutant larvae (Xie et al., 2015). Similarly, the JAK-STAT signaling pathway is shown to mediate the crosstalk between the fat body and insulin-producing cells in response to dietary fats and sugars (Rajan and Perrimon, 2012). Interestingly, CDK8 can directly phosphorylate the transactivation domain of mammalian STAT1, STAT3, and STAT5 (Ser727 of STAT1) and modulate the expression of STAT-target genes in IFN- γ -induced antiviral responses (Bancerek et al., 2013). Although the mammalian STAT1-Ser727 is not conserved in Stat92E (not shown), the STAT1 homolog in *Drosophila*, we could not exclude the possibility that dietary components modulate the activity of JAK-STAT signaling through CDK8 in *Drosophila*.

Finally, all of our analyses are at the organismal level, and it is unknown whether the status of CDK8 and CycC affects cellular responses to different nutrients. Given that mutations of subunits of the CDK8 module have been identified in a variety of human diseases including cancer and cardiovascular diseases (Clark et al., 2015; Schiano et al., 2014a, 2014b; Spaeth et al., 2011; Xu and Ji, 2011), future studies are needed to explore the cellular effects of dietary perturbations on the survival and proliferation of cancer cells with mutations in these subunits. This will advance our understanding of how dysregulation of the CDK8 module contributes to tumorigenesis, and impacts of fluctuations of dietary ingredients on this process. These may also aid cancer treatment when considering the dietary factors.

In conclusion, our study reveals that CDK8-CycC plays important roles in mediating the dietary effects on the larval-pupal transition in *Drosophila*. The *cdk8* and *cycC* mutants display different sensitivities to varied levels of dietary proteins, amino acids, and carbohydrates. Given that both CDK8 and CycC are either mutated or amplified in a variety of human cancers and cardiovascular diseases, understanding the impact and the underpinning mechanisms of dietary perturbations on CDK8-CycC and gene expression may have important clinical implications for the design of strategies to treat these diseases.

4. Materials and methods

4.1. *Drosophila* stocks and reagents

The null alleles of *cdk8* (*cdk8*^{K185}) and *cycC* (*cycC*^{Y5}) were described previously (Loncle et al., 2007; Xie et al., 2015). The flies were kept at 25 °C with a 12:12 h on/off light cycle. Inactivated dry yeast, yeast extract (YE), different amino acids, and sugars were purchased from VWR Life Science. Inactive dry yeast and *Drosophila* agar were purchased from Genesee Scientific.

4.2. Preparation of fly food

The LSD used in this work is the semi-defined medium (<https://bdsc.indiana.edu/information/recipes/germanfood.html>) and was prepared according to description by Na et al. (2013). HSD food was prepared by adding 20% sucrose into the LSD food. Table S1 includes the detailed recipes for preparation of 500 ml of media, which were stored in the fridge at 4 °C until use. The standard Cornmeal-Molasses-Yeast (CMY) recipe was used to prepare CMY food (<https://bdsc.indiana.edu/information/recipes/molassesfood.html>). Additional yeast or different types of sugars were added to the CMY food. For example, “CMY+20% sucrose” food was prepared by mixing 20 g sucrose to 100 ml of the CMY medium. To increase the concentration of the 20 different amino acids (supplied by Alfa Aesar, purchased from VWR Life Science), we used CMY food with addition of each amino acid at the final concentrations as 10X higher as described previously (Grandison et al., 2009).

4.3. Analyses of the timing for the larval-pupal transition

Flies at the 5–10 days of age, maintained on standard CMY diet, were used to collect embryos. After 1–2 h of pre-collections on CMY food, the flies were transferred into bottles containing different diets and embryos were collected for another 2–3 h. The mid-point of this collection time was considered as the beginning point to count the pupation timing. The number of pupariated animals of specific genotypes was scored once every 12 or 24 h according to respective experiment. The pupariation curves were plotted using Microsoft Excel.

4.4. Western blot analysis and antibodies

Western blot analysis was performed as previously described (Xie et al., 2015). Briefly, larvae were collected and homogenized in a buffer containing NaCl (420 mM), Tris HCl (50 mM, pH 8.0), EDTA (0.1 mM), NP-40 (0.5%), glycerol (10%), dithiothreitol (DTT, 1 mM), phenylmethanesulfonyl fluoride (PMSF, 2.5 mM), the cOMplete protease inhibitor and the PhosSTOP phosphatase inhibitor cocktail tablets (Roche Applied Science). After centrifugation at 2000 \times g for 15 min at 4 °C, supernatants were collected and protein concentrations were measured with a Bradford protein assay kit (Bio-Rad), followed by the standard sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Each experiment was repeated at least three times and the representative results were shown.

The following antibodies were used in this work: anti-EcR-common DDA2.7 (mouse, 1:250, Developmental Studies Hybridoma Bank), anti-EcR-common D4.4 (mouse, 1:250, Developmental Studies Hybridoma Bank), anti-USP (guinea pig, 1:2000; (Xie et al., 2015)), anti-CDK8 (guinea pig, 1:500; obtained from Dr. Henri-Marc Bourbon), anti-CycC (rabbit, 1:2000; provided by Dr. Terry Orr-Weaver), anti-dSREBP (rabbit, 1:100; (Xie et al., 2015)), and anti-actin monoclonal antibody (mouse 1:4000, Thermo Scientific MA5-11869).

4.5. Analysis of the mRNA levels by quantitative Reverse Transcription PCR (qRT-PCR) Assay and Statistical analyses

This assay and the specific primer sequences were described in detail previously (Xie et al., 2015; Zhao et al., 2012). Briefly, whole larvae were homogenized in TRIzol reagent (Invitrogen) followed by RNA isolation as described by manufacturer's instructions. Isolated RNAs were quantified and then the cDNAs were prepared using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The quantitative PCR (qPCR) was performed using SYBR Green (Applied Biosystems) as described previously. Three independent biological repeats of each genotype or treatment were conducted. Standard deviation and *t*-tests were performed using Microsoft Excel. Statistical significance was shown in figures and all error bars indicate standard deviation. Statistical significance was shown in figures: **p* < 0.05; ***p* < 0.01; ****p* < 0.001.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ydbio.2018.10.001.

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