

RESEARCH ARTICLE

A biological timer in the fat body comprising Blimp-1, β Ftz-f1 and Shade regulates pupation timing in *Drosophila melanogaster*

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ABSTRACT

During the development of multicellular organisms, many events occur with precise timing. In *Drosophila melanogaster*, pupation occurs about 12 h after puparium formation and its timing is believed to be determined by the release of a steroid hormone, ecdysone (E), from the prothoracic gland. Here, we demonstrate that the ecdysone-20-monooxygenase Shade determines pupation timing by converting E to 20-hydroxyecdysone (20E) in the fat body, which is the organ that senses nutritional status. The timing of *shade* expression is determined by its transcriptional activator β Ftz-f1. The *β ftz-f1* gene is activated after a decline in the expression of its transcriptional repressor Blimp-1, which is temporally expressed around puparium formation in response to a high titer of 20E. The expression level and stability of Blimp-1 is critical for the precise timing of pupation. Thus, we propose that Blimp-1 molecules function like sand in an hourglass in this precise developmental timer system. Furthermore, our data suggest that a biological advantage results from both the use of a transcriptional repressor for time determination and the association of developmental timing with nutritional status of the organism.

KEY WORDS: Biological timer, Developmental timing, Ecdysone, Metamorphosis, *Drosophila*

INTRODUCTION

Multicellular organisms coordinate the timing of biological events during their development and throughout their life. The most characterized systems to determine biological timing are the circadian clock (Allada and Chung, 2010) and clock-like systems (Saga, 2012). Along with the clock systems, the biological timer has been proposed to maintain a precise time period between two biological events. Although a few instances of the existence of such a timer are known (Suzuki et al., 2013), little is known about the molecular mechanisms of this biological timer, including responsive cells or organs.

During the development of insects, a pulse-release of the molting hormone ecdysone (E) from the prothoracic gland occurs several times. The E released to hemolymph is converted to 20-hydroxyecdysone (20E) by the ecdysone-20-monooxygenase

Shade in the peripheral organs, and 20E induces developmental transitions including larval molting and metamorphosis (Huang et al., 2008; Petryk et al., 2003), which is crucial for development. At the onset of metamorphosis in *Drosophila melanogaster*, a temporal increase of ecdysteroid levels induces puparium formation. A drastic change occurs in the outside shape of the fly as it transitions from a larva to a pupa. The transformed larva is called a prepupa at this stage. The ecdysteroid titer drops to a low level \sim 2–3 h after puparium formation (APF) and it rises again for a short period at \sim 10 h APF at standard rearing conditions of 25°C. This second pulse release is expected to induce pupation, the transition from a prepupa to a pupa, consistently at \sim 12 h APF (Thummel, 1996), suggesting that the fly has a biological timer to measure the time until pupation.

Many transcription factors are induced and temporally expressed due to 20E during this period. Among these transcription factors, orphan nuclear receptor β Ftz-f1 (Lavorgna et al., 1991; Ueda et al., 1990) is expressed after pulse exposure of 20E during the mid to late prepupal stage in almost all organs and is essential for pupation (Yamada et al., 2000). A Krüppel-type zinc finger factor Blimp-1 was found to bind the promoter of the *β ftz-f1* gene (Agawa et al., 2007; Kageyama et al., 1997). The *Blimp-1* transcript is induced by 20E directly at the end of larval period and disappears soon after decline of ecdysteroid level during the early prepupal period (Agawa et al., 2007; Akagi and Ueda, 2011). Blimp-1 protein works as a repressor for the *β ftz-f1* gene and contributes to determination of the expression timing of β Ftz-f1 and therefore pupation timing (Agawa et al., 2007). Furthermore, ubiquitous knockdown of *Blimp-1* results in a failure to pupate (Agawa et al., 2007). These results indicate that Blimp-1 is necessary for pupation and plays an essential role to determine pupation timing. However, the mechanism of this time-measuring system is not yet understood.

Here, we describe the molecular mechanism of a precise biological timer that determines pupation timing and specify the location of the timer in the fat body. We demonstrate that pupation timing is determined by the β Ftz-f1-dependent activation of Shade expression, which is expressed in the fat body at the late prepupal period and converts released E to 20E. We also show that the expression level and stability of Blimp-1, a repressor of *β ftz-f1*, is an important component of the precise timer system. Therefore, the quantity of Blimp-1 molecules is critical for the precise timing of pupation.

RESULTS

β Ftz-f1 expression determines pupation timing

Since we have shown that the timing of both pupation and β Ftz-f1 expression are delayed by prolonged expression of Blimp-1 during the prepupal stage (Agawa et al., 2007), we examined the relationship between β Ftz-f1 expression and pupation timing by inducing β Ftz-f1 at different times during the late prepupal period

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using the heat-inducible $\beta ftz-f1$ transgenic fly line ($hs-\beta FTZ-F1$) (Murata et al., 1996). Pupation timing was significantly advanced by approximately 30 min in $hs-\beta FTZ-F1$ prepupae compared with that of the control host line when heat shock was received at 6 h APF (Fig. 1A). No significant advancement of pupation was observed when the animals were exposed to heat shock at 7 h APF or later time points (Fig. 1A and Fig. S1). To further analyze the importance of the expression timing of $\beta Ftz-f1$ for pupation timing, we induced $\beta Ftz-f1$ at various times using $hs-\beta FTZ-F1$ in the transheterozygote $ftz-f1$ mutant background ($ftz-f1^{ex7}/ftz-f1^{(3)03649}$), which cannot transit to the pupal stage without $\beta Ftz-f1$ induction (Yamada et al., 2000). None of the prepupae pupated when $\beta Ftz-f1$ was induced at 4 or 5 h APF. However, 75 or 100% animals were able to become pupa at 12.3 or 12.6 h APF on average, when $\beta Ftz-f1$ was induced at 6 or 7 h APF, respectively (Fig. 1B), which is consistent with the timing of endogenous $\beta Ftz-f1$ expression (Yamada et al., 2000). Furthermore, pupation timing was delayed when the induction of $\beta Ftz-f1$ occurred at 8 h APF or later (Fig. 1B). The length of the delay depended on the timing of the heat shock. These results indicate that the timing of $\beta Ftz-f1$ expression contributes to the timing of pupation. We also observed the best pupation efficiency when $\beta Ftz-f1$ was induced at 7 h APF; however, the efficiency was reduced depending on advanced or delayed induction of $\beta Ftz-f1$; no animals pupated when heat shocked at 4, 5 or 16 h APF. This observation suggests that other temporal factor(s) contribute to determine the appropriate timing for pupation.

Timing of $\beta Ftz-f1$ expression determines the timing of the 20E pulse at the end of the prepupal period

To examine the possibility that the timing of $\beta Ftz-f1$ expression determines the timing of the ecdysteroid pulse which induces pupation (Thummel, 1996), we monitored the ecdysteroid pulse by detecting expression of $E75A$ ($Eip75B$ -FlyBase), which is a known 20E inducible early gene. The expression of $E75A$ was delayed about 2 h upon repression of $\beta ftz-f1$ expression via ectopic expression of $hs-Blimp-1$ at 5 h APF (Fig. 2A) (Agawa et al., 2007). This result suggests that the timing of 20E production is determined by the timing of $\beta Ftz-f1$ expression. To confirm the importance of the timing of the small ecdysteroid pulse at 10 h APF on pupation, we compared the onset of pupation in prepupae after injecting E or 20E at 8 h APF, prior to the endogenous ecdysteroid pulse. Precocious pupation was observed in 20E-injected prepupae, but E injection caused no significant change in the onset of pupation

when compared with control Ringer-injected prepupae (Fig. 2B). This result suggests that pupation timing is determined specifically by 20E, not by E.

E is converted to 20E by the E-20-monooxygenase Shade (Petryk et al., 2003). Thus, we next examined whether the expression timing of *shade* plays any role in the determination of the pupation timing. RT-PCR revealed that *shade* transcript expression decreased after puparium formation but began to increase at 8 h APF, reaching peak levels at 9 and 10 h APF (Fig. 2C). The induction of *shade* transcripts was abolished by *ftz-f1* knockdown in $hs-FTZ-F1$ RNAi (Lam and Thummel, 2000) prepupae, but the expression level of *shade* was increased from 9 h APF in the control host line with the same heat treatment (Fig. 2D). Additionally, we observed premature expression of *shade* at 8 h APF by precocious induction of $\beta Ftz-f1$ by heat shock for 1 h at 5 h APF using the $hs-\beta FTZ-F1$ line (Fig. 2E). Together, we conclude that the expression timing of $\beta Ftz-f1$ determines the pupation timing through induction of Shade, which produces 20E.

The fat body is a crucial organ for the biological timer to determine pupation timing

We further examined which organs were responsible for this pupation timing determination pathway. Since Shade is known to be mainly expressed in the fat body (Petryk et al., 2003), which is a key tissue for post-feeding growth (Okamoto et al., 2009; Slaidina et al., 2009), we examined importance of the fat body for pupation time determination using the GAL4/UAS system. Pupation was advanced by 0.5 h or delayed by 0.3 h on average, with fat body-specific *shade* overexpression and knockdown prepupae, respectively (Fig. 3A). Slight but significant advancement of pupation was also observed when *shade* was ectopically induced in other tissues, including neurons, muscle or epidermis; however, delay of pupation was not observed by induction of *shade* RNAi (Fig. S2). These results indicate that the expression of Shade in the fat body is important to determine pupation timing, although 20E produced in multiple organs can be sufficient for pupation if Shade is expressed at a high enough level. Next, *ftz-f1* or *Blimp-1* expression was manipulated in the fat body to determine their effects on fat body-specific *shade* induction. In order to avoid the detrimental developmental effects of gene manipulation prior to the prepupal period, we harnessed the temperature-sensitive Gal4 suppressor *tub-Gal80^{ts}*. Larvae were cultured at 18°C until puparium formation, and then the culture temperature was shifted to 29°C to allow *Gal4* expression only after puparium formation. Pupation timing was advanced by

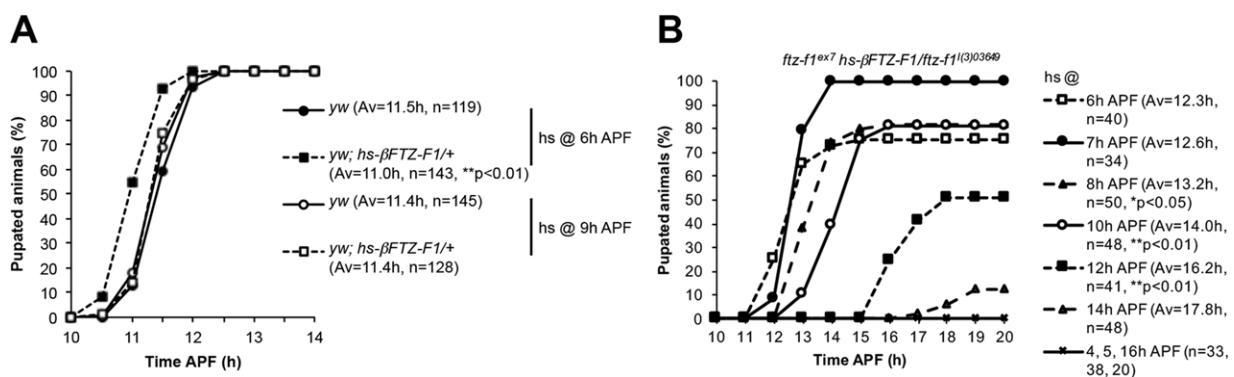


Fig. 1. Timing of $\beta Ftz-f1$ expression determines pupation timing. (A) Pupation timing was observed every 30 min after induction of $\beta Ftz-f1$. Heat shock was given at 33.5°C for 1 h from 6 or 9 h APF in prepupae of yw ; $hs-\beta FTZ-F1/+$ and yw . (B) Pupation timing was observed every 60 min after induction of $\beta Ftz-f1$ in $ftz-f1$ mutant background. Heat shock was given at 33.5°C for 1 h at various times in prepupae of $ftz-f1^{ex7}/hs-\beta FTZ-F1/ftz-f1^{(3)03649}$. * $P < 0.05$, ** $P < 0.01$ by KS-test, versus the yw control (A) and prepupae heat shocked at 7 h APF (B).

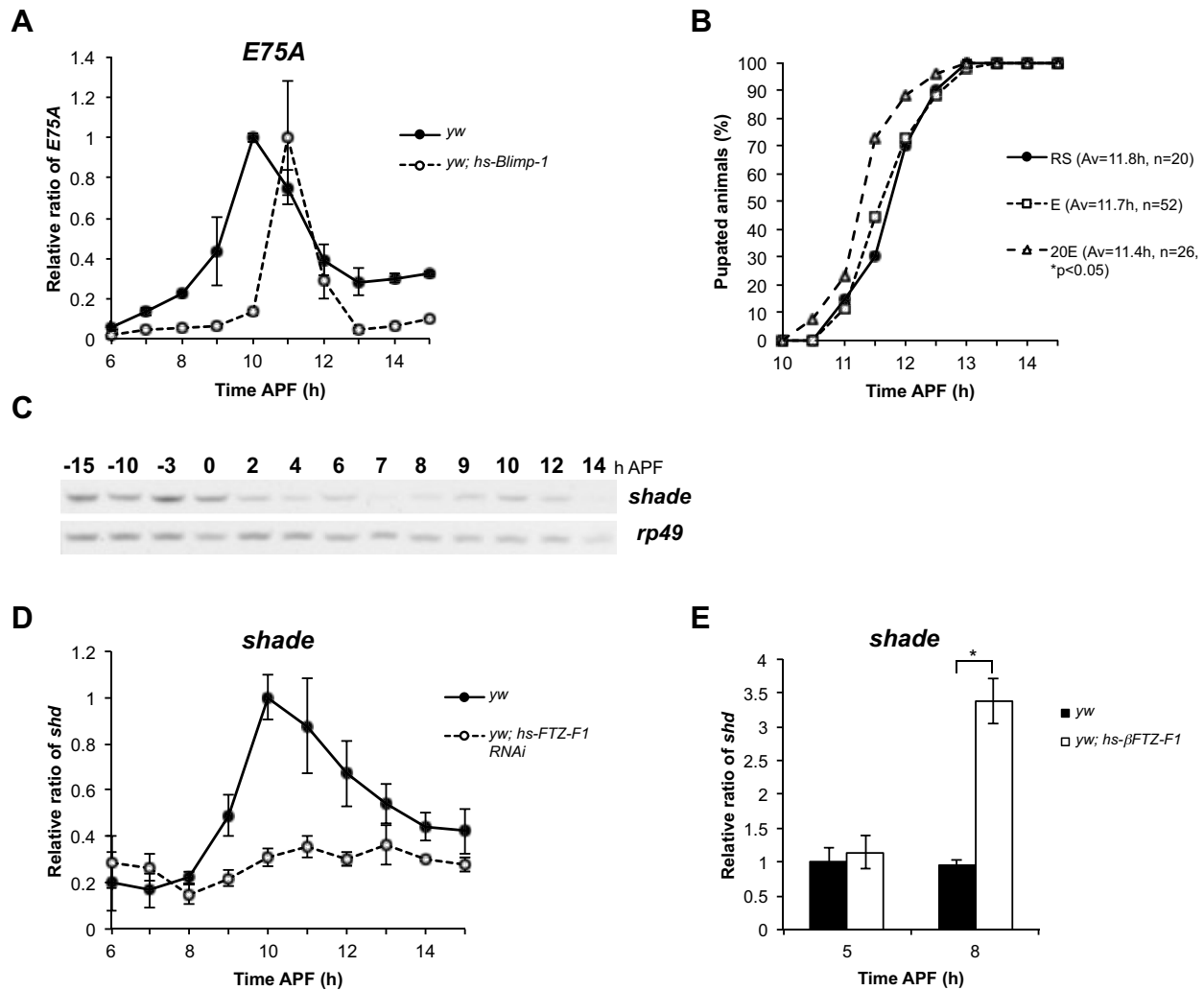


Fig. 2. Timing of β Ftz-f1 expression determines the timing of 20E pulse at the end of the prepupal period. (A) Expression patterns of *E75A* transcripts were observed by RT-PCR upon prolonged expression of *Blimp-1*. Heat shock was given at 34°C for 1 h at 5 h APF in *yw; hs-Blimp-1* and *yw* prepupae. (B) Pupation timing was observed after injection of E, 20E or their solvent Ringer solution (RS). Injection was performed at 8 h APF in *yw* prepupae. **P*<0.05 by KS-test, versus the RS-injected control. (C) Endogenous expression patterns of *shade* transcripts were observed by RT-PCR during metamorphosis in *yw*. (D) Expression patterns of *shade* transcripts were observed by RT-PCR upon β Ftz-f1 knockdown. Heat shock was given at 37°C for 1 h at 5 h APF in *yw; hs-FTZ-F1 RNAi* and *yw* prepupae. (E) Expression levels of *shade* transcripts were observed before (5 h APF) and after (8 h APF) induction of β Ftz-f1. Heat shock was given at 33.5°C for 1 h at 5 h APF in *yw; hs-βFTZ-F1* and *yw* prepupae. **P*<0.05 by Student's *t*-test. The maximum transcript expression obtained from control line was set as 1 for each transcript (A,D) and the transcripts obtained from 5 h APF in control line was set as 1 (E). *n*=5 samples for each time point and genotype. Error bars indicate mean±s.e.m. (A,D,E).

0.4 h on average in prepupae with fat body-specific β Ftz-f1 overexpression. In parallel, fat body-specific β Ftz-f1-knockdown prepupae showed delayed pupation for 0.8 h compared with control prepupae, in addition to reduction of pupation efficiency (Fig. 3B). Similarly, fat bodies overexpressing *Blimp-1* showed delayed pupation for 0.9 h and reduction of pupation efficiency, and *Blimp-1* knockdown elicited an advance in pupation by 0.6 h compared with control prepupae (Fig. 3C). We did not observe this difference in pupation timing using other tissue-specific (i.e. epidermis, muscle, glia or neurons) β Ftz-f1 overexpression or knockdown lines (Fig. S3). These results indicate that the fat body is a crucial organ for the identified pupation-timing determination pathway and this pathway works as a biological timer, because both advanced and delayed pupation were induced by overexpression or knockdown of each component. In addition, increased lethality due to delayed pupation highlights the importance of this system.

Blimp-1 stability and expression are important factors for the accurate time determination of pupation

Blimp-1 has PEST-like sequences, which are known to modulate protein degradation, located in the middle part of the *Blimp-1* protein. These sequences are consistent with the finding that *Blimp-1* protein is unstable and disappears quite rapidly, which we have previously reported (Agawa et al., 2007). Thus, we established a transgenic fly line, *hs-Blimp-1ΔPEST*, which is able to express stable *Blimp-1* upon heat treatment. While intact *Blimp-1* (*hs-Blimp-1*) was detected at a high level until 2 h after the heat shock, truncated *Blimp-1* (*hs-Blimp-1ΔPEST*) was detectable for at least 6 h after the same heat treatment, indicating that the deleted region is needed for the rapid degradation of the *Blimp-1* protein (Fig. 4A,B). We also confirmed that the truncated form of *Blimp-1* is able to work as a repressor for β Ftz-f1. Whereas β Ftz-f1 expression was delayed about 2 h in intact *Blimp-1* induced prepupae compared with the control at 5 h APF, β Ftz-f1 expression was further delayed by about 1 h in the

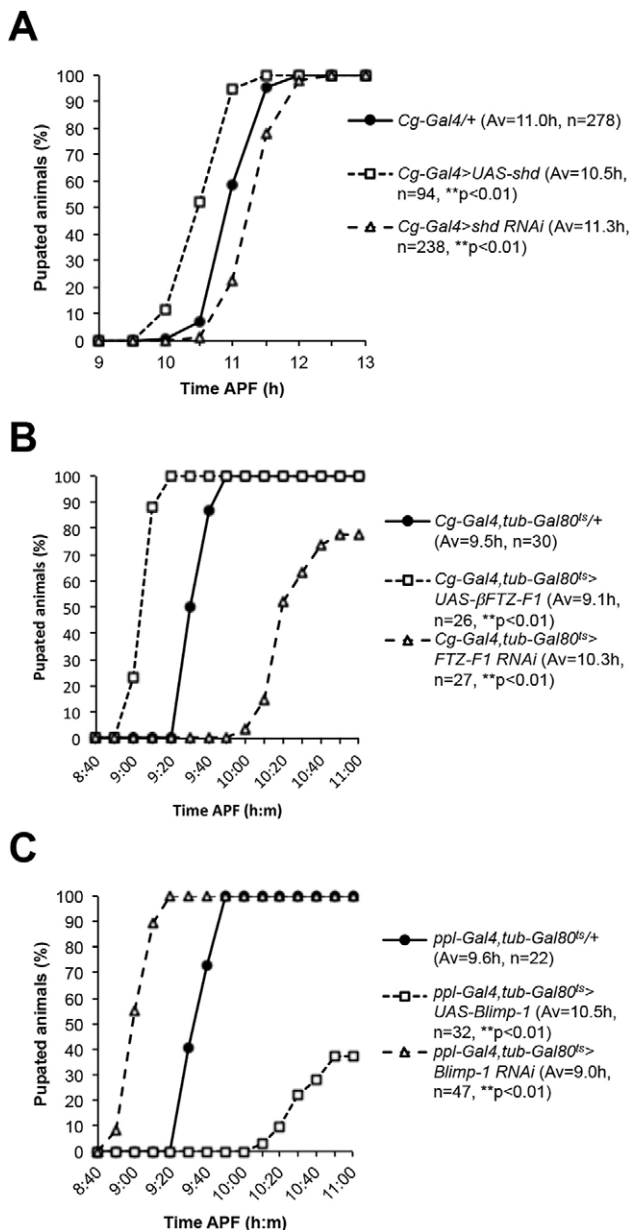


Fig. 3. The fat body is the crucial organ for determination of the timing of pupation. (A) Pupation timing was observed every 30 min in fat body-specific *shade* overexpression and knockdown prepupae. (B) Pupation timing was observed every 10 min in fat body-specific β ftz-1 overexpression and knockdown prepupae. (C) Pupation timing was observed every 10 min in fat body-specific *Blimp-1* overexpression and knockdown prepupae. Animals carrying *tub-Gal80^{ts}* were used and cultured at 18°C until puparium formation and then cultured at 29°C (B,C). ** $P < 0.01$ by KS-test, versus the *Cg-Gal4/+* control (A), the *Cg-Gal4,tub-Gal80^{ts}/+* control (B) and the *ppl-Gal4,tub-Gal80^{ts}/+* control (C).

truncated Blimp-1 induced prepupae (Fig. 4C). These results also suggest that the stability of Blimp-1 is important for the timing of β Ftz-f1 expression. Next, we examined the effect of Blimp-1 stability on pupation timing. Pupation timing was delayed by about 1 and 2 h in the endogenous Blimp-1-induced prepupae compared with the control, by heat shock at 4 and 5 h APF, respectively (Fig. 4D). Pupation timing was further delayed in the truncated Blimp-1-induced prepupae after each heat shock, although ~40% of prepupae failed to pupate after heat shock at 5 h APF (Fig. 4D). These results

suggest that stability of the Blimp-1 protein is an important factor in the determination of pupation timing and that the determination mechanism of pupation is crucial for animal survival. To address the biological meaning of the instability of Blimp-1, we examined the accuracy of the pupation timing by comparing pupation timing elicited by intact and truncated Blimp-1 inductions. Prepupae induced with intact Blimp-1 showed a small window in the dispersion of pupation timing; however, truncated Blimp-1 caused a significantly wider window in the onset of pupation (Fig. 4D). These results suggest that rapid degradation of Blimp-1 due to the function of its PEST-like sequence contributes to the precise time measurement required to induce pupation.

Next, we examined whether not only the stability but also the expression level of Blimp-1 contributes to pupation timing. We used *Blimp-1* mutant lines, which are homozygous lethal as they carry a P-element insertion in the first intron of the gene, in order to examine the effect of the Blimp-1 expression level on pupation timing. Precocious pupation was observed in the prepupae from two independent *Blimp-1* heterozygous mutant lines, *Blimp-1^{P14751/+}* (Agawa et al., 2007) and *Blimp-1^{P09531/+}* (Fig. 4E and Fig. S4A). Furthermore, the observed precocious pupation was recovered in the prepupae of the revertant lines, *Blimp-1^{P14751ΔP1/+}* and *Blimp-1^{P14751ΔP2/+}*, which are obtained from *Blimp-1^{P14751}* by P-element excision (Fig. 4E and Fig. S4A). To see the effect of *Blimp-1* gene dose on the expression timing of the *ftz-f1* gene, we examined the expression pattern of β Ftz-f1 in the heterozygous *Blimp-1^{P14751}* mutant and its revertant *Blimp-1^{P14751ΔP1}*. β Ftz-f1 expression was advanced about 0.5 h in the *Blimp-1^{P14751}* heterozygous mutant prepupae compared with the control line and the timing of β Ftz-f1 expression was restored in the revertant prepupae (Fig. S4B). The importance of the Blimp-1 expression level was also indicated by induction of Blimp-1 from different copy numbers of the *hs-Blimp-1ΔPEST* transgene (Fig. S4C). These results indicate that the expression level of *Blimp-1* is an important factor for the determination of pupation timing through the control of timing of β Ftz-f1 expression.

DISCUSSION

Here, we show that the gene regulatory pathway consisting of *Blimp-1*, β ftz-f1 and *shade* works as a biological timer to measure a specific period during the prepupal period (Fig. 5). Our results suggest that a biological advantage results from use of a transcriptional repressor for the precise timer system. The timing of gene expression could be determined by either induction of its transcriptional activator or depletion of its transcriptional repressor. Accuracy of the induction timing of a gene depends on the rate of accumulation and disappearance of its transcriptional activator and repressor, respectively. It is possible to increase the amounts of repressor molecules produced to elongate the time without changing their rate of degradation. Thus, if the affinity of DNA binding by transcription factors is at the same level, the repressor (e.g. Blimp-1) can maintain high accuracy to determine the timing of expression of the target gene after receiving the upstream induction signal. We further demonstrated the importance of the expression level and stability of the transcriptional repressor Blimp-1 for the time measurement system. Accordingly, we propose that Blimp-1 molecules work like sand in an accurate hourglass to determine pupation timing (Fig. 5).

Blimp-1 is a conserved factor among Metazoa and contributes to cell fate decision in many organs during development (Bikoff et al., 2009). A recent report showed that degradation of Blimp-1 by DRE-1/FBXO11 coordinates developmental timing in *Caenorhabditis elegans* and this protein interaction is conserved in mammals (Horn

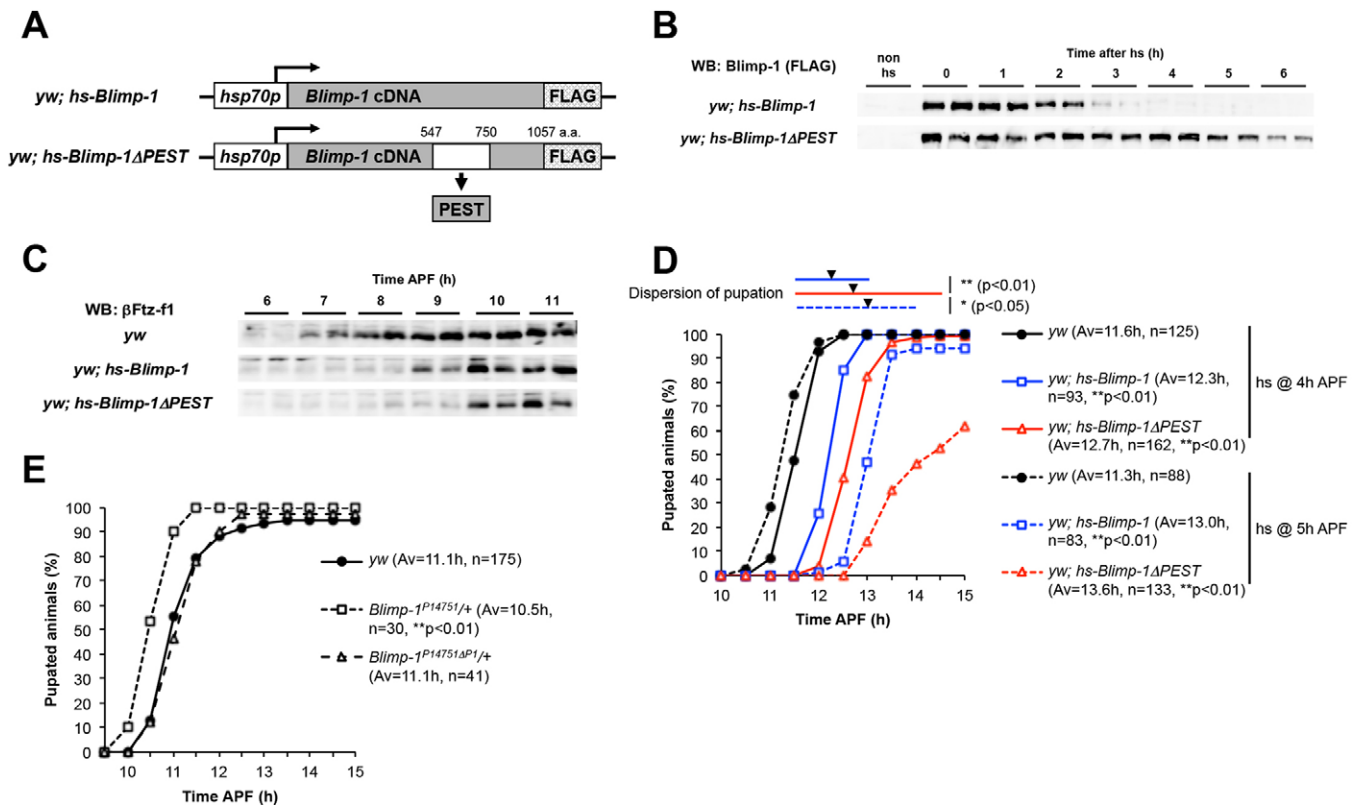


Fig. 4. Effect of Blimp-1 stability and expression level on pupation timing. (A) Structure of *yw; hs-Blimp-1ΔPEST* transgene. To express stabilized Blimp-1, the PEST-like sequence was removed from the full-length *hs-Blimp-1* construct (Agawa et al., 2007). (B) Intact and truncated Blimp-1 was induced by heat shock at 37°C for 1 h at 0 h APF and the expression levels of the induced protein were detected every hour by western blotting using FLAG antibody. (C) The expression timing of β Ftz-f1 was measured by western blotting after induction of intact Blimp-1 or Blimp-1 Δ PEST by heat shock at 34°C for 1 h at 5 h APF. (D) Pupation timing was observed every 30 min after induction of unstable Blimp-1 and stable Blimp-1. Heat shock was given at 34°C for 1 h from 4 or 5 h APF in prepupae of *yw; hs-Blimp-1/+*, *yw; hs-Blimp-1ΔPEST/+* and *yw*. ** $P < 0.01$ by KS-test, versus the *yw* control with the same heat treatment. A window in the dispersion of pupation is displayed at the top. Bars represent the period of time that the majority of animals, in the indicated genotype as well as heat treatment, are pupated. Arrowheads indicate the average point of pupation timing. ** $P < 0.01$, * $P < 0.05$ by *F*-test. (E) Pupation timing observed every 30 min in heterozygotes of *Blimp-1* mutant *Blimp-1^{P14751}* and its revertant line *Blimp-1^{P14751ΔP1}*. ** $P < 0.01$ by KS-test, versus the *yw* control.

et al., 2014), suggesting the conserved importance of the degradation of Blimp-1 for the timing decision during the development.

We found that the pupation timer is composed of *Blimp-1*, β Ftz-f1 and *shade*; however, the delays in pupation by induction of *Blimp-1* or knockdown of either β Ftz-f1 or *shade* were different (Fig. 3). The difference may be caused by the expression levels of each transgene, including the RNAi efficiency and the minimum requirement of each protein for timing determination. Other organs may also contribute to this pathway, because relatively low levels of *shade* expression are detected in the midgut and the Malpighian tubules in addition to the fat body (Petryk et al., 2003). This multi-organ contribution could be one of the reasons that fat body-specific knockdown of *shade* did not cause lethality (Fig. 3A).

Although we found that the identified timer system is crucial to determine pupation timing, several observations reveal that pupation timing is restricted only to a specific period (Fig. 1B, Fig. 2B, Fig. 3B,C and Fig. 4D), suggesting that other factor(s) provide competence for pupation. In addition to these results, we could not rescue the pupation deficiency by injecting 20E in the *ftz-f1* mutant prepupae (data not shown), suggesting that β Ftz-f1 controls not only 20E production through Shade but also expression of other factors necessary for pupation. Ecdysis triggering hormone (ETH), a peptide hormone that regulates the pupation behavior, is potentially one of these factors, as β Ftz-f1 is essential for release of ETH from the *inca* cells (Cho et al., 2014). The timing of E

production, which is triggered by prothoracicotrophic hormone (PTTH) (McBrayer et al., 2007), is an important factor for pupation; we observed that pupation timing was delayed for several hours (data not shown) in prepupae where PTTH-producing neurons are ablated. However, neither ectopic *Blimp-1* induction nor β Ftz-f1 knockdown affected the expression of either *ptth* or *eth* transcripts (Fig. S5). It has been reported that the chromatin remodeling protein INO80 has an effect on the pupation timing by regulating the regression of ecdysone-regulated genes including β Ftz-f1 (Neuman et al., 2014). These results suggest the presence of other mechanisms, acting independently of our identified timer system, to restrict pupation timing.

We identified the fat body as an essential tissue necessary to drive this developmental timer system. Several reports have shown a link between nutrient status and developmental timing, and it has been suggested that the fat body is the central tissue for coordinating this link (Colombani et al., 2003; Géminard et al., 2009; Rewitz et al., 2013). Thus, we expect that the fat body incorporates the nutritional status of the animal and sends a cue for the final decision of pupation independent of the timer system.

A recent publication proposed that *shade* gene expression could be finely tuned by acetylation of Ftz-f1 (Borsos et al., 2015). This result supports the idea that β Ftz-f1 directly regulates the *shade* gene. On the other hand, β Ftz-f1 is expressed after decline of 20E level in almost all organs at the late embryonic stage and each larval

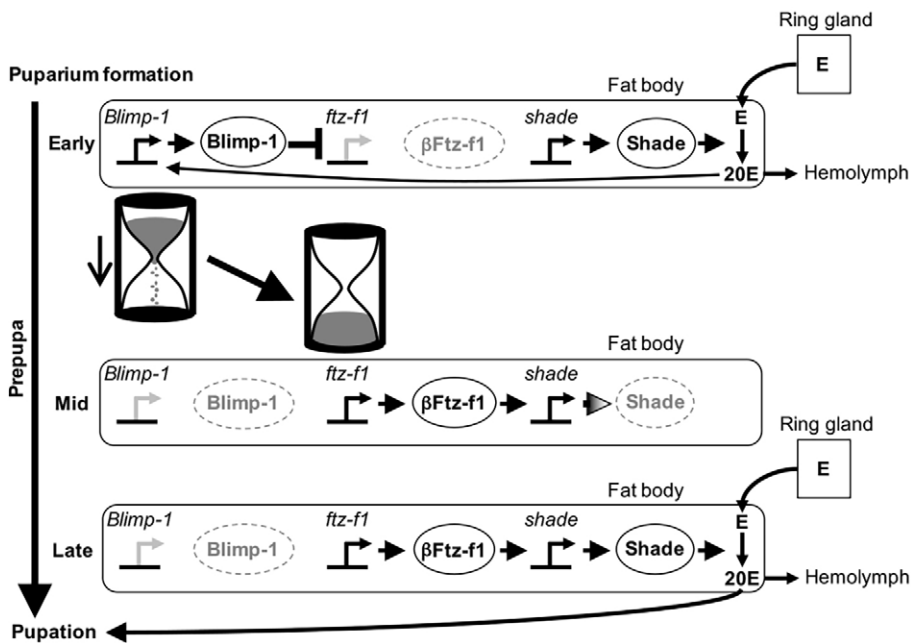


Fig. 5. An hourglass model of the molecular mechanism to determine pupation timing. At early prepupal stage, the *ftz-f1* gene is repressed by Blimp-1 when levels of 20E are high. The *shade* gene is expressed independent of β Ftz-f1 and disappears by mid-prepupal stage. After a decline of the level of 20E, Blimp-1 and its mRNA degrade rapidly (Agawa et al., 2007; Akagi and Ueda, 2011) allowing expression of β Ftz-f1, which induces *Shade* in the fat body in the late prepupal period. Then, *Shade* converts released E to 20E, which induces pupation.

and pupal stage during development (Sultan et al., 2014; Yamada et al., 2000), but the upregulation of *shade* is limited around the high ecdysteroid period for puparium formation (Fig. 2C). These results suggest that the activation of the *shade* gene by β Ftz-f1 is restricted only in the prepupal period. Further studies are needed to understand the regulation mechanism of the *shade* gene, including epigenetic regulation of the time-measuring mechanism.

In this transcriptional cascade, the mechanism for initiating the timer is crucial to drive the system. The regulatory mechanism required to determine the period of ecdysone pulse, which induces puparium formation, has been unveiled recently at the molecular level (Moeller et al., 2013; Rewitz et al., 2013). This system potentially works as a switch for the Blimp-1 timer to determine the specific period after the decline of the 20E level.

MATERIALS AND METHODS

Fly culture and stocks

Flies were raised at 25°C on 10% glucose, 8% commeal, 4% yeast extracts and 0.7% agar medium containing propionic acid and butyl-*p*-hydroxybenzoate as antifungal agents. *hs-FFi-24* obtained from Dr. Carl S. Thummel (University of Utah, UT, USA) was used for *hs-ftz-f1 RNAi* line; *ppl-Gal4* was obtained from Dr. Masayuki Miura (University of Tokyo, Tokyo, Japan); *UAS-dicer*; *Cg-Gal4*, *Repo-Gal4*, *Mef-Gal4* and *C855a-Gal4* were obtained from Dr. Naoki Okamoto (University of California, Riverside, CA, USA); and *Gal80^s*, *elav-Gal4* and *24B-Gal4* lines were obtained from Dr. Hideki Nakagoshi (Okayama University, Okayama, Japan). *UAS-shd* lines were obtained from Dr. Hajime Ono (Kyoto University, Kyoto, Japan) and *UAS-shd-RNAi* lines were obtained from National Institute of Genetics in Japan. Double homozygotic line of *UAS-shd-RNAi-1* and *UAS-shd-RNAi-2* was used for Fig. 3A in order to increase RNAi efficiency.

Establishment of transgenic flies

For *UAS-Blimp-1* fly lines, a 3.1 kb *EcoRI* and *NotI* fragment obtained during construction of *hs-Blimp-1* gene (Agawa et al., 2007) was inserted between *EcoRI* and *NotI* sites of pUAST vector. For *UAS- β ftz-f1* fly lines, a 2.5 kb *EcoRI* fragment obtained during construction of *hs- β ftz-f1* gene (Murata et al., 1996) was inserted into pUAST vector using the *EcoRI* site. Transgenic fly lines were established as previously described (Murata et al., 1996) using embryos of *y¹Df(1)w^{67cl}* (*yw*) as the host strain.

Measurement of the prepupal period

Newly formed white prepupae were picked up every 10, 30 or 60 min and transferred to a plastic dish with moist paper. Pupation timing was observed every 10, 30 or 60 min. The Kolmogorov–Smirnov test (KS-test) was used to evaluate differences between pupation timings and determine *P* values. The *F*-test was used to evaluate the variance of pupation timings. We used the Prism software package (GraphPad Software) to carry out statistical analysis.

Detection and quantification of transcripts

For semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR), total RNA was extracted from five animals at each time point using the NucleoSpin RNA Kit (TaKaRa). cDNAs were synthesized from the RNA using oligo(dT)15 primer (Novagen) and ReverTra Ace (Toyobo). PCR was performed in three independent biological replicates using OneTaq polymerase (New England Biolabs). The following synthetic oligonucleotides were used for detecting transcripts: *Blimp-1-F*:5'-CGCA-CCTCCAGAAGCATCAT, *Blimp-1-R*:5'-GGGCAGAGATCAGGCA-TA; *E75A-F*:5'-AGCCGCAGCAGCAAATG, *E75A-R*:5'-ACCCGAGTG-GTGCAGAT; *shade-F*:5'-GATGACGAGGCTGCTGGATTAC, *shade-R*:5'-AGCACCGGGATCTCCAGTAACA; *pth-F*:5'-TGAGGATCTGGTG-ACCACCAAACGCA, *pth-R*:5'-TTCCAGTGGCCTGCAATTGGATCC-A; *eth-F*:5'-AGGCGAGAAGCTTGGCCATAA, *eth-R*:5'-ACCACGTTAA-GTTCCTGTCTC; *rp49-F*:5'-CCACCAGTCGGATCGATATG, *rp49-R*:5'-CACGTTGTGCACCAGGAAGT.

The reaction products were resolved on 8% Tris/borate/ethylenediaminetetraacetic acid (TBE) polyacrylamide gels and the amounts of the PCR products were quantified by LAS-4000mini (Fujifilm) and MultiGaugeVer.3 (Fujifilm). Samples were normalized with an endogenous control, *ribosomal protein 49* (*rp49*). The Student's *t*-test (two tailed) was used to perform statistical analysis.

Western blotting

Western blot analysis was performed as previously described (Murata et al., 1996). M2 antibody (Sigma, F3165, 1:2000 dilution) and goat anti-mouse IgG HRP (Cayman, 10004302, 1:5000 dilution) was used to detect Flag-tagged proteins.

Acknowledgements

We thank Drs C. S. Thummel, H. Ono, H. Nakagoshi, M. Miura, M. O'Connor, N. Okamoto, R. Niwa and the Fly Stocks of Kyoto Stock Center and National Institute of Genetics for providing the fly strains. We also thank Drs N. Bond, S. D. Katewa and J. Meyer for a critical reading of the manuscripts.

Competing interests

The authors declare no competing or financial interests.

Author contributions

K.A., M.S. and H.U. designed experiments. K.A., M.S., A.-R.S., H.N., A.K. and T.N. performed experiments. K.A. and H.U. wrote the manuscript.

Funding

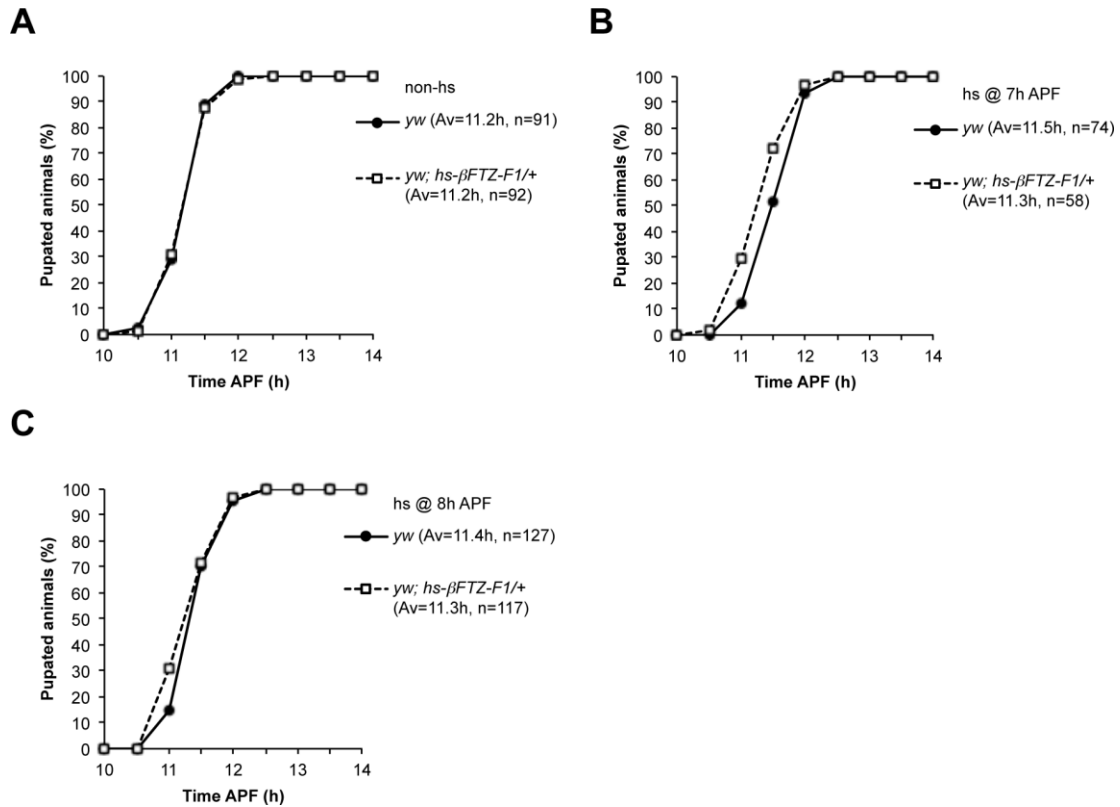
This work was partially supported by Grants in-Aid for Scientific Research from the Japanese Ministry of Education, Culture, Sports, Science and Technology. A. S. was supported by the Japanese Ministry of Education, Culture, Sports, Science and Technology [20570204, 25440110]. M. S. was supported by Cultural Affairs and Missions Sector, Ministry of Higher Education, Egyptian government and Japan Society for the Promotion of Science.

Supplementary information

Supplementary information available online at <http://dev.biologists.org/lookup/doi/10.1242/dev.133595.supplemental>

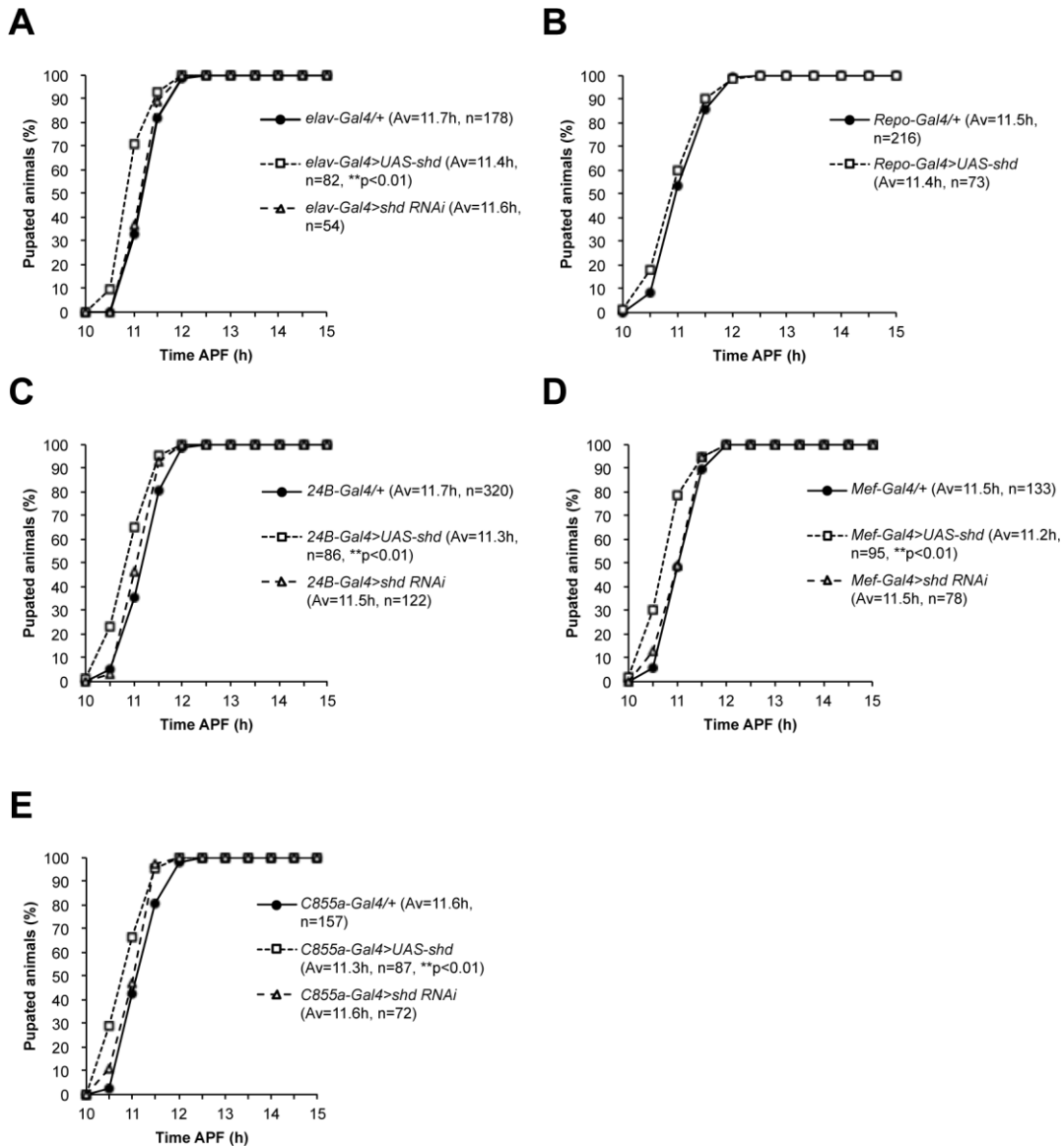
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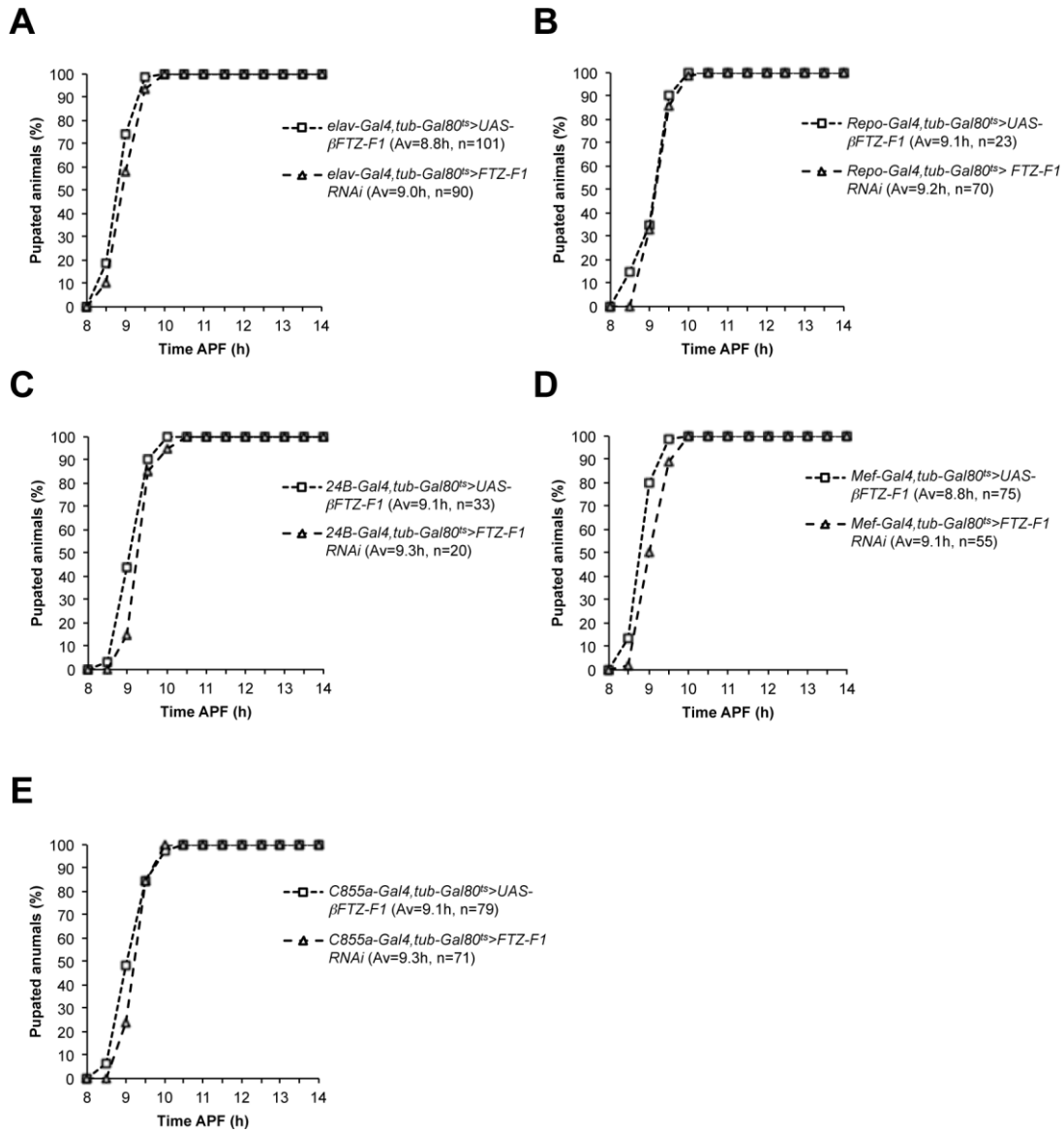
Supplementary Fig. 1. Ectopic expression of β FTZ-F1 at endogenous β FTZ-F1 expression timing does not alter pupation timing.

(A-C) Pupation timing was observed every 30 minutes after induction of β FTZ-F1. Heat shock was given at 33.5 °C for 1 hour from 7 (B) or 8 hours (C) APF in prepupae of *yw; hs-βFTZ-F1/+* and *yw*. (A) Pupation timing without heat shock in both lines are shown. No significant difference in pupation timing was observed in each condition.



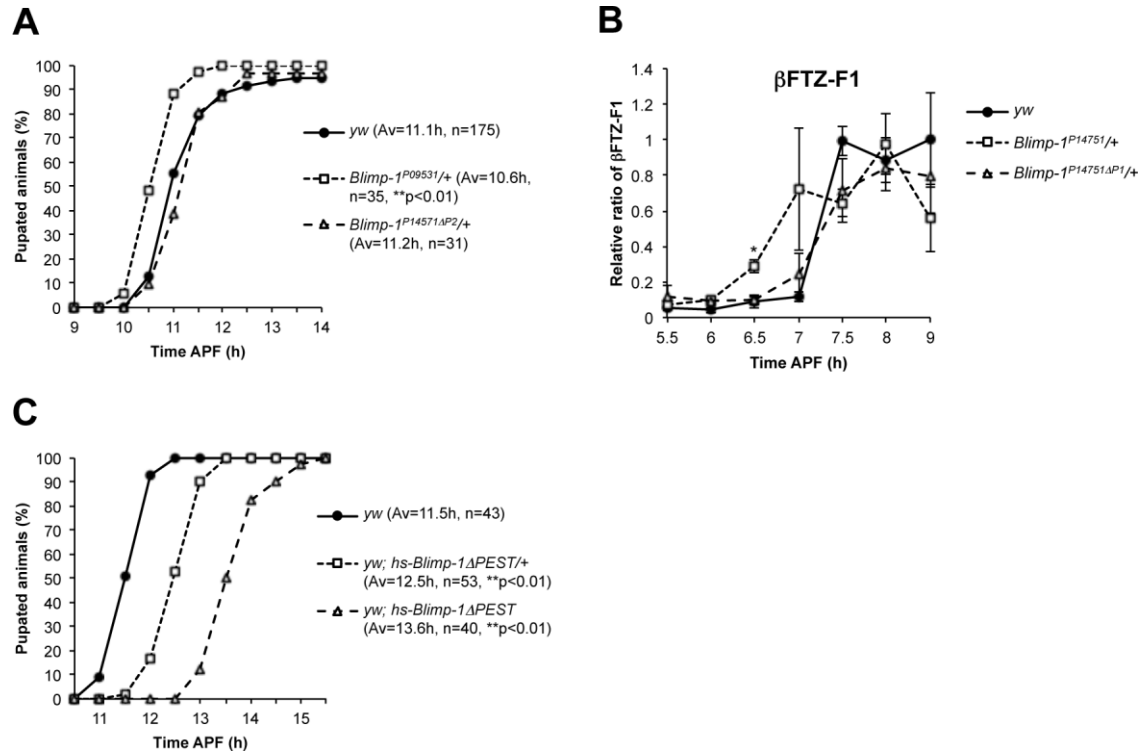
Supplementary Fig. 2. The effects of organ specific induction or knockdown of *shade* in neurons, glia, muscle or epidermis on pupation timing.

(A-E) Pupation timing was observed every 30 minutes in neurons (A), glia (B), muscle (C), muscle (D) and epidermis (E) specific *shade* overexpression (A-E) and knockdown (A, C, D and E) prepupae, respectively. No significant difference in pupation timing was observed in *shade* knockdown prepupae in different tissues (A, C, D and E) as well as the glia cell-specific *shade* overexpression (B). Significant advance in pupation timing was observed by the ectopic induction of *shade* in neuron, muscle or epidermis (A, C, D and E). ** $p < 0.01$ by KS-test, versus the Gal4 control prepupae, respectively.



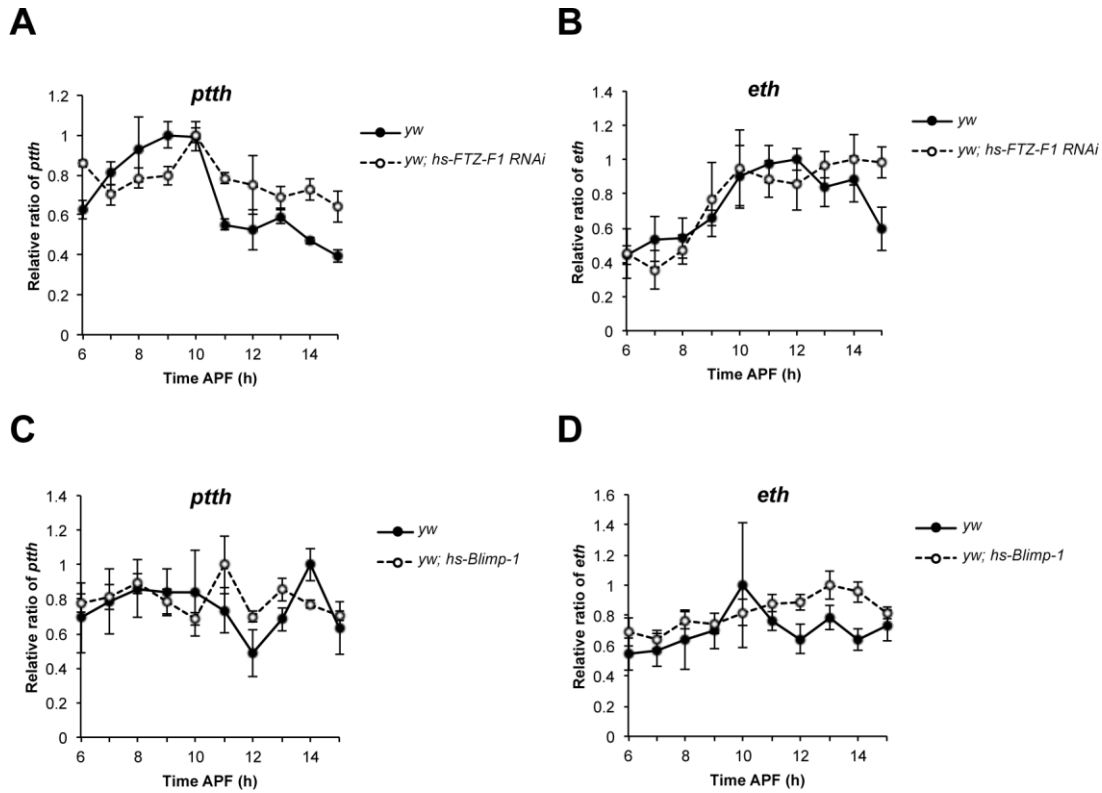
Supplementary Fig. 3. Organ specific induction or knockdown of βFTZ-F1 in neurons, glia, muscle or epidermis does not alter pupation timing.

(A-E) Pupation timing was observed every 30 minutes in neurons (A), glia (B), muscle (C), muscle (D) and epidermis (E) specific *βFTZ-F1* overexpression and knockdown prepupae, respectively. Animals carrying *tub-Gal80^{ts}* were used and cultured at 18 °C until puparium formation and then cultured at 29 °C. No significant difference in pupation timing between overexpression and knockdown were observed in each condition.



Supplementary Fig. 4. The effect of Blimp-1 expression level on the timing of β FTZ-F1 expression and pupation.

(A) The effect of endogenous gene dose of *Blimp-1* on pupation timing. Pupation timing was observed every 30 minutes in heterozygote *Blimp-1^{P09531}* mutant line and *Blimp-1^{P14751ΔP2}* revertant line. Advanced pupation was also observed in this second *Blimp-1* mutant line. **p<0.01 by KS-test, versus the *yw* control. (B) The effect of endogenous gene dose of *Blimp-1* on expression timing of β FTZ-F1. Expression pattern of β FTZ-F1 in heterozygote *Blimp-1^{P14751}* mutant line and its revertant line *Blimp-1^{P14751ΔP1}* were observed by Western blotting. β FTZ-F1 protein band intensities were quantified by LAS-4000mini (Fujifilm) and MultiGaugeVer.3 (Fujifilm). Significantly advanced β FTZ-F1 expression was observed in *Blimp-1* mutant line. *p<0.05 by *t*-test, versus the *yw* control at 6.5 hours APF. (C) The effect of the Blimp-1 expression level on pupation. Pupation timing was observed every 30 minutes in homo- or hetero-zygote of *yw; hs-Blimp-1ΔPEST* prepupae. Heat shock was given at 34 °C for one hour at 3 hours APF. While pupation timing was delayed for 1 hour on average in prepupae expressing a single copy of the *hs-Blimp-1ΔPEST* transgene compared to the control host strain with the same heat-treatment, 2 hours delay in pupation timing on average was observed in prepupae expressing two copies of transgene compared to the control prepupae. **p<0.01 by KS-test, versus the *yw* control.



Supplementary Fig. 5. β FTZ-F1 may not regulate the *pth* or *eth* gene during prepupal period.

(A and B) Expression patterns of *pth* (A) and *eth* (B) transcripts were observed by RT-PCR upon *FTZ-F1* knockdown. Heat shock was given at 37°C for 1 hour at 5 hour APF in *yw; hs-FTZ-F1 RNAi* and *yw* prepupae. (C and D) Expression patterns of *pth* (C) and *eth* (D) transcripts were observed by RT-PCR upon prolonged expression of Blimp-1. Heat shock was given at 34°C for 1 hour at 5 hour APF in *yw; hs-Blimp-1* and *yw* prepupae. The maximum transcript expression obtained from control line was set as 1 for each transcript (A-D). Error bars indicate mean \pm s.e.m.