Molecular players affecting the biological timer system to determine pupation timing in *Drosophila*

ショウジョウバエの蛹化タイミングを決める生物タイマーシステムに影響を及ぼす分子

September 2018

Hamdy Aly Mohamed Aly

The Graduate School of Natural Science and Technology

(Doctor's Course)

Okayama University

ACKNOWLEDGMENTS

First of all, I would like to thank my supervisor ***Prof. Hitoshi Ueda*** *for giving me the opportunity of being in Japan to performing my Ph.D. in his laboratory. I am very grateful to you for advice, guidance, valuable comments, for your suggestions and for your didactic way in clarifying the ideas, because of you, I have learned how to always think with a scientific essence.*

A special appreciation is extended to the Japanese Ministry of Education, Culture, Sports, Science, and Technology for providing me the financial assistance which kept away the irritating sensation of financial instability.

In our days when role models have become fewer and fewer, I had the privilege of being shaped by an intelligent researcher, ***Dr. Moustafa Sarhan****. I gratefully acknowledge Dr. Moustafa, my mentor, and my best friend, whose passion for helping sets new standards for anyone involved in training and development, or any other endeavor in which one human being seeks to support the growth and improvement of another.*

I would also like to thank all previous and current members of our laboratory especially Haruka Takase, Shiho Koyama, Koichi Miyagawa, Mayo Nakanishi for their help and for providing useful comments during my research. Special thanks to ***Dr. Haruka Nishida*** *who helped me to set up my stay and take care of every single detail to make my life more enjoyable in Japan.*

Many thanks and great appreciations also go to my colleagues and friends especially to ***Abdullatif Elseudi*** *who have willingly helped me out by encourage and* ***Eman Taha*** *for her sincere help in the completion of this thesis.*

I would like to thank my collaborator, ***Dr. Kazutaka Akagi*** *for his useful comments and suggestions in writing my paper. I am grateful to Drs Yuji Kageyama, Masayuki Miura and Naoki Okamoto, Kyoto Stock Center, Bloomington Stock Center, and National Institute of Genetics for providing fly lines.*

I would like to thank my parents, brothers, and sisters for their love and support; I could never have done this without your faith, support and constant encouragement, I would not be the person I am without them. Moreover, finally, I am grateful to my wife ***Basma Fekry*** *for her encouragement, baked goods, understanding, and love, without which I would be lost.*

Hamdy Aly

CONTENTS

ABBREVIATIONS

20E	20-hydroxyecdysone
AEL	After egg laying
APF	After puparium formation
Bcl6	B-Cell Lymphoma 6
Blimp-1	B lymphocyte-induced maturation protein 1
Br-C	Broad-Complex
DHR	*Drosophila* hormone receptor
DRE	DNA replication-related element
E	Ecdysone
EcR	Ecdysone Receptor
ETH	Ecdysis triggering hormone
FBXO11	F-box only protein
ftz	Fushi tarazu
h APF	Hours after puparium formation
HDAC	Histone Deacetylases
HMT	Histone methyltransferase activity
hs	Heat shock
βIFN	Beta interferon
IRF	Interferon regulatory factor
mlpt	Mille-pattes
ORFs	Open reading frame
Pax5	Paired box 5
PR	Proline-rich region
Prdm1	Positive regulatory domain containing 1
Pri	Polished rice
RBX	RING-box protein 1
RING	Really interesting new gene
RP	Regulatory particle
SCF	Skp1-Cullin-F-box
Svb	Shavenbaby
tal	Tarsal-less
Ub	Ubiquitin

Ubr3	Ubiquitin protein ligase E3 component n-recognin 3
UPS	Ubiquitin-proteasome system
USP	Ultraspiracle
WPP	White prepupa

Introduction

1. GENERAL INTRODUCTION

1.1. Time control during development

Time-related transformations in body properties are a biological phenomenon found in many organisms, as illustrated by sex maturation in vertebrates, and by molting and metamorphosis, in which animals ranging from insects to crustaceans and amphibians undergo distinct morphological changes. Life begins with embryonic development, which is a four-dimensioned process, explicitly controlled by genes. The complexity and precision of development reflect the integration of spatial pattern signals with the temporal control mechanism, so that critical developmental transitions occur at the appropriate time. Time determination is essential for organisms and development without timing control would lead into a messy and disorganized life. For the developing organisms to achieve a suitable condition, they are required to coordinate various timing mechanisms. However, a little is known about how they work and interact to organize these developmental processes. In the mechanical world, we use clocks and timers: machines that tell the absolute time and can be used to measure part of the time, respectively. Do organisms possess devices that fulfill the same functions? Most animals have a biological clock; an internal mechanism that allows them to anticipate and keep in a register with the phenomena of the external world, such as day and night. Moreover, many studies have been published describing the molecular mechanisms that control how this clock works. On the other hand, numerous observations strongly support the existence of internal time measuring systems (biological timers), since many events occur at a precise time during development. How do organisms keep track of time and coordinate the proper chronology of these distinct developmental events on a molecular level? That is one of our lab's interests in research.

1.2. *Drosophila* as a model of animal development

Drosophila species are insects of the *Diptera* order, with a single pair of large wings. They are holometabolous animals that are characterized by an indirect development, with three larval stages on the way before undergoing a metamorphosis, which gives the fertile adult form. *Drosophila melanogaster* is widespread in the world, and its cosmopolitan distribution is probably related to human activities, such as increased trade, that allowed it to migrate over long distances (Lachaise et al., 1988). The female of *Drosophila* lay eggs on a medium which serve as food substrate to the emerging larvae. The female can lay several hundred of eggs

during its life. Depending on food substrate and temperature, the developmental time of the newly laid individuals will vary, indicating the impact of environmental contribution for control of *Drosophila* development. For instance, the entire development from embryo to adult at 25°C usually is about ten days; it lengthens to 20 days if the temperature is lowered to 18°C (Thompson et al., 1977). Among many of the model organisms, *Drosophila* is one which is extensively studied to find essential genes and mechanisms. The regulation mechanism of development has been studied continuously for the past several decades, and recent discoveries using *Drosophila* molecular genetics have further proceeded our information. The availability of genome sequences, the ease of genetic manipulation and the vast collection of mutants, all make *Drosophila* a handy model animal for studying and understanding the mechanisms regulating development.

1.3. Ecdysone pulses control developmental timing in *Drosophila*

Holometabolous insects provide an excellent model system of hormonal control of gene expression. In insect metamorphosis, larval tissues are destroyed and replaced by adult tissues. The differentiation of imaginal cells into adult structures is achieved by controlled cell-fate determination and cell movement behaviors. Metamorphosis is driven by intermittent pulse releases of the steroid hormone ecdysone (E), which can activate the signaling cascade required for these developmental events. Steroid hormones are a large family of cholesterol derived compounds that act as critical developmental regulators in many organisms. In vertebrates, steroids, such as adrenal cortical steroid hormone and neuro-steroids, play essential roles in various processes including reproduction, differentiation, metabolic homeostasis and brain function (Hosie et al., 2006). In most insects, steroidogenesis occurs in the cells of the prothoracic gland resulting in the conversion of food derived cholesterol to E. The glands are stimulated to undergo steroidogenesis in discrete and periodic surges, and this is reflected in the peaks of ecdysteroids observed in larvae and pupae (Warren et al., 2006). Pulses of ecdysteroids direct each of the major developmental transitions in the life cycle of *Drosophila* (Fig. 1) (Riddiford and Truman, 1993). The active form of E is 20-hydroxyecdysone (hereafter referred to as 20E) (Ashburner, 1974; Kozlova and Thummel, 2003; Riddiford and Truman, 1993; Shi et al., 1996).

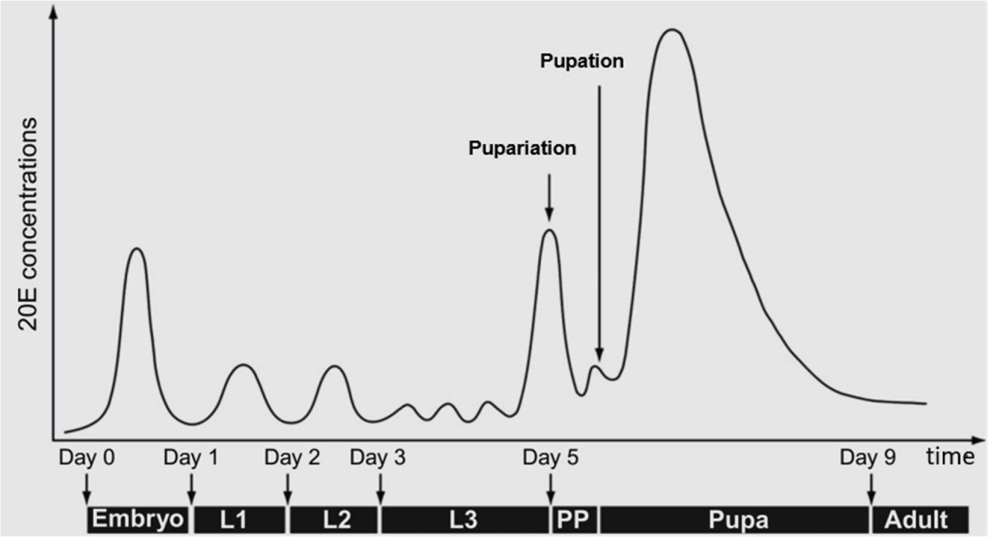


Figure 1. Pulses of ecdysone trigger each of the major developmental transitions in *Drosophila* life cycle. Ecdysone pulses during the first and second larval instars signal molting of the cuticle, defining the duration of each instar. A high titer ecdysone pulse at the end of the third instar triggers puparium formation, initiating the prepupal stage of development. At the end of prepupal period a low titer ecdysone pulse triggers pupation marked by everting the head from inside the thorax. The result of these sequential ecdysone-triggered responses at the onset of metamorphosis is the transformation of a crawling larva into an immature adult fly. The ecdysone titer profile is depicted as 20E equivalents in whole body homogenates and the developmental transitions are marked by arrows. Adapted from Ou and King-Jones, (2013).

The first increase in 20E level occurs at the onset of metamorphosis and promotes the change from larvae to prepupae in the larval-prepupal transition. In this stage, the larval salivary glands secrete a mixture of glue proteins that are used to affix the animal to a solid surface, the cuticle then transforms into a puparium carrying pupal shape, which is initially soft and white. We call this transformation as puparium formation, and the transformed animal as white prepupa hereafter referred to as WPP. Because this transformation is an excellent developmental marker, and we represent the developmental stage from here by after puparium formation or APF by making short. Soon after puparium formation, the soft and white body hardens more, turning tan and eventually brown and brittle. During the prepupal stage, a subset of larval tissues, including the midgut, initiate programmed cell death, eliminating some of the larval tissues (Robertson, 1936). In the meantime, the leg and wing imaginal discs evert and elongate to form the coming adult appendages. The second ecdysteroid pulse occurs 10-12 h APF, leading to the prepupal-pupal transition. The morphological marks of this stage are the gas bubble

originates in the middle followed by truncation from the posterior to the anterior part of the pupal case, and the eversion of the pupal head from the larval thorax (Thummel, 1996; Woodard et al., 1994). These critical events form the body plan of the adult fly, with a head, thorax, abdomen, and appendages. Terminal differentiation then occurs over the following ~3.5 days of pupal development, followed by eclosion of the adult insect (Fig. 1). Thus, each of the ecdysteroid pulses triggers changes throughout the animal to control development, and it seems that *Drosophila* is endowed with a time measuring system to measure the time for each developmental transition including pupation.

1.4. Ecdysone initiates genetic cascades of gene expression to control temporal development in *Drosophila*

In order to regulate gene expression, 20E must bind to ecdysone receptor (EcR), which transduces this signal as a non-covalent heterodimer with *Drosophila* retinoid X receptor ortholog, Ultraspiracle (USP) (Bond et al., 2011; Koelle et al., 1991; Kozlova and Thummel, 2003; Yao et al., 1993). Damage or loss of either 20E or its receptors was found to be lethal to the animal (Kozlova and Thummel, 2003). Since ecdysteroids affect arthropod life from early embryogenesis to reproduction and adult life, it is not surprising that practically every organ is a target organ for ecdysteroids (Riddiford et al., 2000). The increase in 20E levels leads to an increase of receptors bounded by the hormone, which in turn leads to the transcription of genes that activate the transcriptional cascade (Bond et al., 2011).

Ecdysteroid pulse occurring late during the third instar phase causes the transcription of the primary response "early genes" such as *E74*, *E75A*, and *Broad-Complex* (*Br-C*). These genes are is directly induced by 20E and are expressed from around 6 hours (h) before puparium formation to around 2 h APF. The molecular characterization of these three genes revealed that all of them encode transcription factors, although belonging to different DNA-binding protein families (Thummel et al., 1990). The protein products of these early genes induce the transcription of "late genes" which locate downstream, and late genes, in turn, execute the appropriate biological effects at the onset of metamorphosis (Ashburner, 1974). Mutations that disrupt all BR-C functions result in larval lethality, indicating that *Br-C* is an essential gene for the entry into metamorphosis (Kiss et al., 1988). The *E74* gene encodes two overlapping transcription factors, *E74A* and *E74B*, whose function is required for both pupariation, pupation, and metamorphosis of both larval and imaginal tissues. *E74B* mutants are defective in puparium formation and head eversion and die as prepupae or crypto cephalic pupae, while

E74A mutants pupariate normally and die either as prepupae or pharate adults (Andres et al., 1993). *E75* encodes three mRNA isoforms, designated *E75A*, *E75B*, and *E75C* (Segraves and Hogness, 1990). *E75A* mutants die primarily during larval stages with a reduced ecdysteroid titer, while *E75B* mutants are viable, and *E75C* mutants die as adults (Bialecki et al., 2002).

In addition to early genes, the *Blimp-1* gene is also one of the primary response genes of 20E, and is expressed in the high ecdysteroid periods during the embryonic and prepupal periods (Agawa et al., 2007), although this gene has not been reported as an early gene. It has shown that the timing of the end of the expression of Blimp-1 determines the timing of the expression of βFTZ-F1 and the length of the prepupal period (Agawa et al., 2007; Akagi et al., 2016), indicating that the mechanism to terminate the expression of the Blimp-1 gene is important for development.

Another group of genes induced at metamorphoses is "early-late genes," which can be defined as genes that require both the 20E-bound EcR and an early gene product for maximal transcriptional induction. Early-late genes are induced just before puparium formation until mid-prepupal stage, including *DHR3*, *DHR4, E78,* and *DHR39*. *DHR3* and *DHR4* are expressed at the beginning of the prepupal stage, when the expression of early genes such as *Br-C*, *E74A*, and *E75A* is declining, and *βftz-f1* expression is about to be induced. Both *DHR3* and *DHR4* are sufficient to repress the early genes and are required for maximal *βftz-f1* expression in the mid-prepupal period (King-Jones et al., 2005; Lam et al., 1997); thus, these two factors are acting in concert to regulate the early genes and *βftz-f1*. Furthermore, *DHR4* mutants are small and display precocious wandering, followed by early puparium formation. Thus, *DHR4* plays an essential role in defining the end of larval growth and the timing of pupariation (King-Jones et al., 2005). *E78* and *DHR39* are not essential for viability or fertility (Ayer et al., 1993; Horner and Thummel, 1997; Horner MA, Chen T, 1995; Ohno and Petkovich, 1993; Russell et al., 1996).

The late genes are the group of genes which is, as mentioned above, induced by the early gene products. Among them, *L71* gene family is activated just before puparium formation to around 10 h APF and requires the activity of the *Br-C* and *E74* early genes for transcriptional activation (Gene et al., 1991; Karim et al., 1993; J. C. Fletcher and Thummel, 1995; Jennifer C Fletcher and Thummel, 1995). The L71 proteins play a role in antimicrobial cell lysis during metamorphosis (Wright et al., 1996).

The next group of genes is the "mid-prepupal genes," which are induced only after the decline of ecdysone levels. One of them is *ftz-f1* that encodes a nuclear receptor-type transcription factor (Lavorgna et al., 1991; Ueda et al., 1990). The *ftz-f1* gene product is

expressed not only during the mid-prepupal period at the onset of metamorphosis but also during late embryogenesis, just before larval ecdysis and eclosion (Sullivan and Thummel, 2003; Ueda et al., 1990; Woodard et al., 1994; Yamada et al., 2000; Lavorgna et al. 1991, 1993). This transcription factor is the only one that has been identified as the mid-prepupal gene products and is necessary for the induction of late prepupal genes (Broadus et al., 1999; Woodard et al., 1994).

Finally, "late prepupal genes" are directly induced by the small pulse of 20E just before pupation (Richards, 1976a). Molecular analysis has revealed that some of the genes expected to be early genes based on puffing pattern and direct induction by 20E, are late prepupal genes (Andres and Thummel, 1992; Kozlova and Thummel, 2003).

The extensive research in *Drosophila* has led to the identification of the ecdysteroid functions in the developmental transitions by triggering cascades of gene expression. However, almost no understanding has been achieved regarding how these transitions including pupation are determined at a specific time.

1.5. Spatiotemporal players coordinate the biological timer to determine pupation timing

The drastic change from larval shape to pupal shape occurs at larval-pupal transition followed by pupation about 11 h APF at standard rearing condition at 25˚C (Riddiford and Truman, 1993), indicating that flies have a well-controlled timer system to determine pupation timing. The complexity of components and mechanisms involved in this specific biological timer was the core of our lab's research work for the last several years, and many of them already uncovered (Fig. 2) (Agawa et al., 2007; Akagi et al., 2016). It has shown that the two ecdysone-inducible transcription factors, Blimp-1 (Agawa et al., 2007) and βFTZ-F1 (Sun et al., 1994; Ueda et al., 1990), play a crucial role for the determination of pupation timing (Akagi et al., 2016). According to these analyses, the first trigger of these events is the increase of 20E level at the end of the larval period, which induces *Blimp-1* expression. After around 2-3 h APF, 20E level decreases and Blimp-1 production stops because of the termination of *Blimp-1* mRNA production and rapid degradation of it (Akagi and Ueda, 2011). Moreover, Blimp-1 is a labile protein; therefore, the expressed Blimp-1 disappears rapidly (Agawa et al., 2007). Blimp-1 works as a repressor for the *βftz-f1* which encodes transcriptional activator (Agawa et al., 2007). βFTZ-F1 is induced after Blimp-1 disappearance at around 6 h APF and activates *Shade* which encodes E to 20E conversion enzyme, ecdysone-20-monooxygenase, in the fat body. Hence, pupation timing is determined by a biological timer in the fat body comprising Blimp-1, βFTZ-

F1, and Shade (Akagi et al., 2016), although it has been thought that pupation timing is determined by secretion timing of E from the prothoracic gland just before pupation (Thummel, 1996).

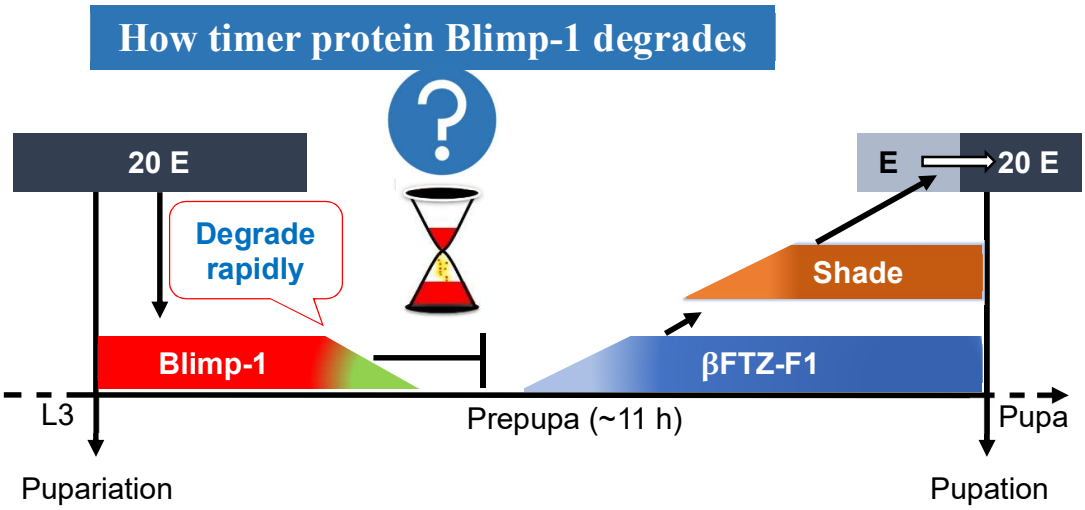


Figure 2. Mechanism of time measuring system for pupation. After 2 h APF, induced Blimp-1 by 20E disappears rapidly soon after decline of 20E level and controls expression timing of βFTZ-F1. βFTZ-F1 induces *shade*, which encodes the conversion enzyme of released E to 20E in fat body, and produced 20E induces pupation.

1.5.1. Blimp-1

Murine Blimp-1 contains 856 amino acids and is predicted to be a 95,835 Da protein. The five C_2H_2 zinc finger motifs in the C-terminal region of Blimp-1 were clearly implicated as the DNA-binding domain. However, further study showed that only the first two finger motifs are sufficient for recognition of the PRDI region in the *IFNβ* promoter (Keller and Maniatis, 1991). The protein possesses a proline-rich region (PR), five zinc fingers, and a SET domain known to have a role in histone methyltransferase activity (HMT). However, the SET domain of Blimp-1 may not have demonstrable HMT activity, because Blimp-1 does not contain the NHSC(I) sequence, which is a conserved motif locates in the C-terminal half of the SET domain, defined as a catalytic core in HMT active proteins (Kouzarides, 2002; Marmorstein, 2003). The PR region is present between SET and zinc finger domains, and this region along with the zinc fingers in some cases is required for transcriptional repression (Ren et al., 1999; Yu et al., 2000). Several mechanisms were identified using mammalian tissue culture system to understand the transcriptional repression activity of Blimp-1. In most of these mechanisms,

repression activity of Blimp-1 depends upon its capability of directly recruiting multiple chromatin-modifying enzymes to target promoters that create a repressive or inactive chromatin structure (Ancelin et al., 2006; Gyory et al., 2004; Ren et al., 1999; Yu et al., 2000). The mechanism of action of Blimp-1 requires more study to identify real corepressors that required for its activity (Martins et al., 2008). Also, no transcriptional activation capability has been demonstrated for Blimp-1, although it remains a theoretical possibility (Savitsky and Calame, 2006).

Experientially, it has been shown that Blimp-1 regulates directly or indirectly 250 different target genes in mammalian B-lymphocyte cells (Shaffer et al., 2002). Blimp-1 also plays an essential role in the terminal differentiation of B cells through direct transcriptional silencing of several transcription activators such as *Pax5*, *Bcl6*, and *c-myc* (Sciammas et al., 2006; Sciammas and Davis, 2005; Su et al., 2009; Yu et al., 2000). Additionally, Blimp-1 is a key regulator for the differentiation of myeloid lineage (Chang et al., 2000) and determination of germ cell lineage (Ohinata et al., 2005; Vincent et al., 2005). It has been reported that Blimp-1 function to regulate cell growth through repression of tumor suppressor *p53* transcription (Yan et al., 2007). In addition to the functions of Blimp-1 in mammals, it has also been shown that it plays important roles in many other groups of animals. In Xenopus, the frog homolog of Blimp-1, X-Blimp-1, controls anterior mesodermal fate (De Souza et al., 1999). In Zebrafish, the homolog of Blimp-1 promotes the cell fate specification of both neural crest cells and Rohon-Beard sensory neurons (Hernandez-Lagunas et al., 2005) and differentiation of the embryonic slow muscle lineage (Baxendale et al., 2004). It is also necessary for the Zebrafish embryo patterning and organogenesis (Wilm and Solnica-Krezel, 2005). Collectively, these studies indicate that Blimp-1 plays a crucial role in many cellular differentiation processes.

In *Drosophila*, only a few reports are focusing on the role of Blimp-1 in the developmental control, up to date. The first one published in 1997 during the analysis of the *cis*-regulatory region of the *ftz-f1* gene to understand how *ftz-f1* expression is temporally regulated, Blimp-1 was found binding to the upstream region of the transcriptional start site of the *ftz-f1* gene and designated as factor I-4 (Kageyama et al., 1997). The binding site of factor I-4 was determined (Kageyama 1997 thesis, data published later), and using its specific binding site as a bait, factor I-4 was purified. Based on the information of the molecular mass of the purified protein, the corresponding gene was identified as Blimp-1 (Agawa 2002 thesis, data published later). Protein sequence homology search revealed that this protein is an ortholog of mammalian Blimp-1, because only these two factors have both a SET domain and five zinc fingers in addition to a strong amino acid sequence homology in these regions (Keller and Maniatis, 1991;

Ren et al., 1999; Turner et al., 1994). Furthermore, it has been shown that the mammalian Blimp-1 binds to a similar sequence (Keller and Maniatis, 1991) to that recognized by factor I-4. The second report revealed the important function of *Drosophila* Blimp-1 in the terminal differentiation of the tracheal system (Ng et al., 2006). Detection of *Drosophila* Blimp-1 in both mRNA and protein levels revealed that they are expressed during the mid-embryonic period at 9-15 h after egg laying (AEL). The third report demonstrated that Blimp-1 is expressed at the onset of metamorphosis, from around 3 h before puparium formation to 4 h APF and can be detected again at the end of prepupal period 10 to 14 h APF (Agawa et al., 2007). All these periods correspond to the increase of ecdysteroid titers, and indeed, it has been shown that *Drosophila* Blimp-1 is induced directly by 20E as described above. *Drosophila* Blimp-1 was found to work as a repressor for the *ftz-f1* gene. Two different approaches have confirmed this repression function; first, knock down this factor at the onset of metamorphosis leads to premature expression of βFTZ-F1. Second, prolonged expression of Blimp-1 by its induction after the endogenous expression period under the control of the heat shock promoter from the *hs-Blimp-1* transgene resulted in a delay of βFTZ-F1 expression (Agawa et al., 2007). In the fourth report, Chavoshi et al. (2010) presented additional evidence on the 20E regulating the expression of Blimp-1 during embryonic development (Chavoshi et al., 2010). The fifth and most recent report demonstrated Blimp-1 as a key molecule to the timer system in the fat body to determine pupation timing. In this analysis, stabilized Blimp-1 lead to a delay in pupation timing, which reasoned, as described above, because of Blimp-1 repression of *βftz-f1* (Akagi et al., 2016). Although, the unstable character of Blimp-1 is the key to determine pupation timing, the molecular mechanisms and factors that regulate Blimp-1 turnover around the prepupal period are still unclear.

1.5.2. FTZ-F1

FTZ-F1 was initially found in a biochemical screen for embryonic proteins that bind specifically to the regulatory sequences of the *Drosophila* segmentation gene *fushi tarazu* (*ftz*) (Ueda et al., 1990). Cloning analysis revealed that FTZ-F1 is a transcription factor belongs to the nuclear hormone receptor superfamily (Lavorgna et al., 1991; Ueda et al., 1990). Two isoforms, α FTZ-F1, and βFTZ-F1 are produced from the same gene: they share a common C-terminal region but contain different N-terminal regions (Lavorgna et al., 1993, 1991). The αFTZ-F1 is expressed in early embryos, concomitant with *ftz* expression (Lavorgna et al., 1993; Ueda et al., 1990). The βFTZ-F1 expression is detected during late-stage embryos (primarily

after 16 h of embryogenesis), just before larval ecdysis, mid-to-late prepupal period and before eclosion (Sullivan and Thummel, 2003; Ueda et al., 1990; Woodard et al., 1994; Yamada et al., 2000). The locus containing the *ftz-f1* gene forms a puff during the low ecdysteroid mid-prepupal period (Richards, 1976b), concomitant with βFTZ-F1 expression (Lavorgna et al., 1993; Murata et al., 1996). Likewise in *Drosophila,* the expression of *ftz-f1* after a decline in ecdysone levels has also been reported to occur in several insects (Hiruma and Riddiford, 2001; Li et al., 2000; Sun et al., 1994). In silkworm, BmFTZ-F1, a silkworm ortholog of FTZ-F1, is induced by exposure to and subsequent withdrawal of 20E during the late period of each molting stage (Sun et al., 1994). Thus, generally, insect FTZ-F1 expression periods closely follow declines in ecdysone levels except at early embryonic period.

In *Drosophila*, the first identified function of the FTZ-F1 protein is activating *ftz,* because mutations of the FTZ-F1 binding site result in decreased expression of the *ftz-lacZ* transgene in the early embryos (Ueda et al., 1990). Later, Yamada et al. (2000) showed that the severely affected *ftz-f1* mutants displayed embryonic lethal phenotype, which was rescued by ectopic expression of βFTZ-F1. The resulted larvae were not able to molt but also were rescued by induction of βFTZ-F1 in the appropriate timing. Furthermore, premature expression of βFTZ-F1 at mid-first instar or mid-second instar stages causes defects in the molting process. Ectopic, time-specific expression of βFTZ-F1 can also rescue a hypomorphic *ftz-f1* mutant that arrests in the prepupal stage. These observations suggest that βFTZ-F1 regulates genes associated with ecdysis and metamorphosis and that the exact timing of its action in the ecdysone-induced gene cascade is important for proper development (Yamada et al., 2000). Moreover, another *ftz-f1* mutant showed defects in the prepupa-to-pupa transition, including failure in head eversion and histolysis of salivary glands (Broadus et al., 1999). Importance of βFTZ-F1 was also suggested by the ability of FTZ-F1 to bind to 166 loci in late prepupal salivary gland polytene chromosomes, 51 of which represent ecdysone-regulated puffs (Lavorgna et al., 1993). This observation suggests that βFTZ-F1 plays a role to regulate a wide range of genes. Accordingly, βFTZ-F1 may provide competence for late prepupal genes, *Br-C*, *E74A*, *E75A,* and *E93*, because it has been found that FTZ-F1 is necessary for the expression of these genes to respond to the ecdysteroid peak (Broadus et al., 1999; Woodard et al., 1994). βFTZ-F1 has also been reported to positively regulate the *EDG84A* gene which encodes protein specific to the inside layer of the pupal cuticle during the mid-to-late prepupal period (Murata et al., 1996). Thus, βFTZ-F1 expression and especially its temporally restricted expression are important for late embryogenesis, the normal molting process, and early metamorphosis.

For the temporal regulatory mechanism of *ftz-f1* expression, two transcriptional regulators have been identified and confirmed to regulate *ftz-f1* expression (Kageyama et al., 1997; Lam et al., 1997; White et al., 1997). The previously described early-late gene product DHR3 have been indicated to be a transcriptional activator for the *ftz-f1* gene. Premature expression of DHR3 under the control of a heat shock promoter induces βFTZ-F1 (Lam et al., 1997; White et al., 1997), and a reduction in βFTZ-F1 expression has been reported in the DHR3 mutant (Lam et al., 1999). Also, DHR3 binds to three sites downstream of the transcriptional initiation site of the *ftz-f1* gene (-150, -240, and -300), and mutations in these sites reduce the expression of the *βftz-f1* promoter-*lacZ* fusion transgene (Kageyama et al., 1997). The other transcription factor that is known to regulate the *βftz-f1* gene is the early gene product E75B, which binds directly to DHR3 and inhibits its activator function (White et al., 1997). However, identification of these two factors did not solve the regulatory mechanism of the *βftz-f1* gene completely, and other unknown factors besides DHR3 and E75B are required to regulate the *βftz-f1* gene based on the following observations: first, DHR3 can activate the *βftz-f*1 gene only after ecdysone levels have declined, second, the temporal pattern of the *βftz-f1* gene expression is preserved in DHR3 and E75B mutants (Bialecki et al., 2002; Lam et al., 1999), and third, mutations in the DHR3 binding sites in the *βftz-f1* promoter-*lacZ* fusion gene have no effect on the timing of β-galactosidase expression in transgenic flies (Kageyama et al., 1997). It has also been shown that DHR4 is another transcription factor that is supposed to activate the *βftz-f1* gene (King-Jones et al., 2005), based on the observation that FTZ-F1 expression is reduced in the *DHR4* mutant, but no evidence has been shown to prove that DHR4 directly associate to the *ftz-f1* promoter. As previously described, the transcription factor Blimp-1 has also been identified as one of the factors that bind the *βFTZ-F1* promoter (Kageyama et al., 1997).

1.5.3. Fat body

The larval fat body is a critical structure for *Drosophila*, as it serves the same purpose as liver and fat tissue do in humans: storage of nutrients and metabolizing energy, providing nourishment to the animal as it undergoes metamorphosis (Aguila et al., 2007; Hoshizaki DK., 2005; Liu et al., 2009; Søndergaard, 1993). It also is involved in the fly's metabolism, with sensors measuring the level of available nutrients and signaling the brain to release insulin-like peptides (Géminard et al., 2009). In addition to its role in the fly metabolism and storage of nutrition, fat body was recently specified as the location of the timer system to determine pupation timing. It was demonstrated that βFTZ-F1 determines pupation timing by activating

Shade, which is expressed in the fat body at the late prepupal period and converts released E to 20-E, as previously described (Akagi et al., 2016).

While most of the larval organs and tissues are destroyed during metamorphosis by programmed cell death, the larval fat body undergoes remodeling, providing the metamorphosing animal with energy as transition and into an early adult fly (Aguila et al., 2013). Eventually, the larval fat body cells undergo programmed cell death, but only after the adult has emerged from the pupal casing and has found an alternate source of food (Nelliot et al., 2006). After coming out of the pupal case, it takes a young adult fly roughly one hour before its wings extend and it can fly and find an alternate source of nutrients. The fact that the larval fat body is still present in this young adult fly is crucial to its survival until such a reliable food source is found (Aguila et al., 2007). After the larval fat body cells reabsorbed, they get replaced by adult fat body cells, which still store nutrients but which are not as readily available to the fly as those of the larval fat body (Aguila et al., 2007).

1.6. Factors involved in protein degradation

1.6.1. Ubiquitin Proteasome System

The ubiquitin-proteasome system (UPS) is one of the major post-translational regulation mechanisms of protein turnover. Through a series of concerted enzymatic reactions, target proteins are covalently marked with poly-ubiquitin chain(s) and directed to the 26S proteasome for degradation (Ohanian, 2008; Pickart, 2004). 26S proteasome is involved in the degradation of both normal, short-lived, mutated or damaged ubiquitinated proteins, serving an essential role in the removal of a wide variety of key nuclear and cytosolic proteins (Navon and Ciechanover, 2009; Rock et al., 1994; Ulrich, 2002; von Arnim, 2001; Zwickl et al., 2001). Structurally, 26S proteasome consists of a multi-catalytic core particle 20S bound to two 19S regulatory particles. The 20S proteasome is a multi-catalytic complex composed of 14 different subunits, arranged in four stacked, seven-membered rings (7α, 7β, 7β, 7α) that form the barrel-shaped complex (Groll et al., 1997; Unno et al., 2002). The α subunits restrict substrate entry, while the β subunits contribute to the proteolytic active sites, namely the caspase-like in β1, trypsin-like in β2, and chymotrypsin-like activities in β5 (Kastle and Grune, 2011). The 19S regulatory particle consists of two particular subcomplexes; an ATP-dependent unfoldase referred to as the base and a scaffolding complex known as the lid. These subcomplexes of the 19S work together to facilitate substrate recognition, translocation, unfolding, and recycling of

the polyubiquitin signal before peptide hydrolysis (Glickman et al., 1998; Verma et al., 2002; Yao and Cohen, 2002). For eukaryotic proteasomes, each subunit of the complex is encoded by a unique gene, and most of these genes are essential for both proteasome activity and the survival of the organism (Ghislain et al., 1993; Gordon et al., 1993; Rubin et al., 1998; Saville and Belote, 1993; Smyth and Belote, 1999).

1.6.2. Role of UPS-dependent protein degradation in development

UPS-mediated turnover of proteins have a critical role in protein homeostasis and is involved in various cellular processes such as cell growth, proliferation, differentiation, and apoptosis. Along with its central role in proteostasis, the UPS has roles in transcription, DNA repair, apoptosis, and signal transduction. Thus, changes in the UPS activity could have significant consequences for animal development (Finley, 2009). In mammals, aging of cells is associated with reduced proteasome activity (Chondrogianni et al., 2003). Moreover, the proteasome function was found to decline with age in fibroblast cultures originated from human donors of different ages (Carrard et al., 2002).

In Drosophila, a gradual accumulation of ubiquitinated and carbonylated proteins was reported in the somatic tissue of aging flies, correlated with a ~50% reduction of proteasome expression and catalytic activities (Fredriksson et al., 2012; Tsakiri et al., 2013). Overexpression of *Rpn11*, which encodes a subunit of the 19S RP, suppressed the age-related reduction of the 26S proteasome activity, resulting in increased lifespan with suppression of the age-dependent accumulation of ubiquitinated proteins. On the other hand, the loss of function of *Rpn11* caused early onset of reduced 26S proteasome activity and premature age-dependent accumulation of ubiquitinated proteins (Tonoki et al., 2009), or caused a polyphasic lethality at larval-pupal transition (Szlanka, 2003). In addition, animals with missense mutations of *β2* and *β6* proteasome subunit genes have been reported to develop normally until metamorphosis, but pupae die before eclosion (Covi et al., 1999). Additionally, severe pathophysiological consequences were reported upon partial loss of proteasome function, such as the dose-dependent drop in locomotor performance of young flies exposed to the inhibitor and a dose-dependent reduction in flies' lifespan.

1.6.3. Selective degradation of UPS-substrates

1.6.3.1. F-box proteins as an E3 ligases

In the UPS pathway, ubiquitination is sequentially mediated by three enzymes: ubiquitin-activating enzyme E1, ubiquitin-conjugating enzyme E2 and ubiquitin ligases E3 (Fig. 3). The two main types of E3 ligases, which is in charge of the substrate specificity, are those with RING (also known as HRT1, RBX1 or ROC1) or HECT-domains (Petroski and Deshaies, 2005; Pickart, 2001). In contrast to RING ligases, HECT domain ligases form an essential thioester intermediate with ubiquitin as it is being transferred from the E2 enzyme to the substrate. The SCF (Skp1-Cullin-F-box) complex is a multi-subunit ubiquitin ligase (E3), assembled using the scaffold protein CUL1, whose C-terminus recruits the small RING protein RBX1, which directs the E2 enzyme to the E3 ligase. Then the N-terminus of CUL1 is then bound to SKP1 and F-box protein (Fig. 3) (Petroski and Deshaies, 2005).

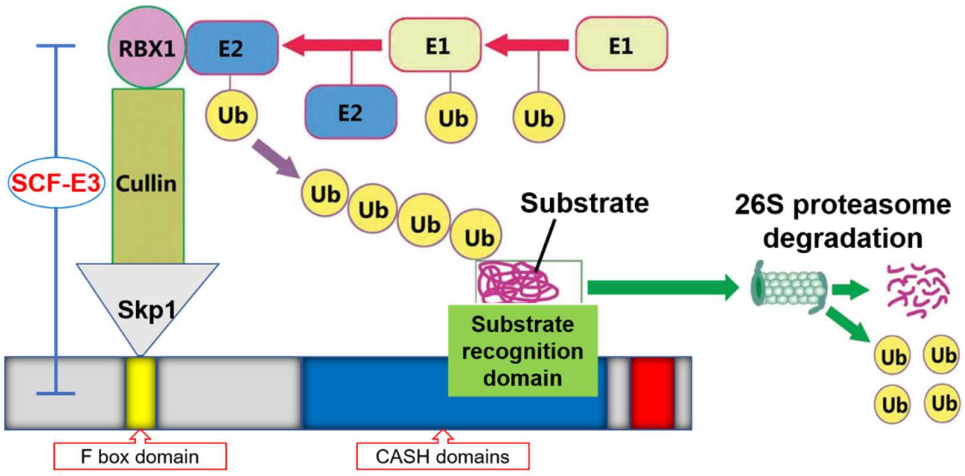


Figure 3. Schematic representation of F-box mediated protein ubiquitination and degradation in proteasome system. The E1 enzyme functions as an activator by creating a high-energy thioester bond between a cysteine of the E1 enzyme and the ubiquitin (Ub) molecule via ATP hydrolysis, which is subsequently transferred to conjugating enzyme (E2). The function of E2 is the transfer of activated Ub to the site of conjugation in the form of an E2-Ub thioester intermediate. Ub is then transferred from E2 to lysine residues in the target through an E3-Ub ligase. Finally, the Ub proteins is recognized and then degraded by the 26S proteasome to several small peptides. Adapted from (Gong et al., 2014).

F-box proteins function as the substrate-recognition components of SCF complexes, thus conferring substrate-specificity to the UPS. Across species, a variety of F-box proteins can bind to the core SCF scaffold, each targeting multiple substrates, enabling SCF complexes to specifically label proteins for subsequent degradation (Jin et al., 2004; Skaar et al., 2009). One of the highly conserved F-box proteins is FBXO11 (Duan et al., 2012). In mammals, FBXO11 is linked to developmental alterations, including facial clefting and otitis media (Hardisty-Hughes et al., 2006). In *C. elegans*, DRE-1, a homolog of human FBXO11, has been shown to regulate the larval-to-adult transition in the epidermis of (Fielenbach et al., 2007). Interestingly, Blmp-1, the *C. elegans* Blimp-1, has been identified as a suppressor for the *dre-1* mutant, which displays heterochronic phenotypes.

1.6.3.2. Pri small ORFs as mediators for selective proteasome-mediated protein processing

The *polished rice* (*pri*) *Drosophila* gene, also referred to as *tarsal-less*, polycistronically encodes four related small peptides (11 or 32 amino acids in length) evolutionarily conserved throughout all arthropods, and are mediating the function of *pri* during development (Fig. 4) (Galindo et al., 2007; Kondo et al., 2007). This unique gene is transcribed as ~1.5 kb intron-less mRNA without obvious long ORFs and thus was initially considered as a non-coding RNA gene (Inagaki et al., 2005; Tupy et al., 2005). However, it has been shown that each small peptide, with no identified protein domain, can provide full Pri function (Galindo et al., 2007; Kondo et al., 2007).

During the embryogenesis of *Drosophila*, *pri* mRNA is first expressed in seven anteroposterior stripes at the blastoderm stage, then it displays fast evolving patterns of expression at different times, and in various tissues, including the spiracles, gut, trachea and epidermal cells (Kondo et al., 2007). *pri* loss of function leads to embryonic lethality, with dramatic defects in the epidermis including the complete absence of trichomes both in the dorsal (hairs) and in the ventral (denticle belts) regions; a phenotype similar to that observed in mutant embryos of the transcription factor Shavenbaby (Svb). Denticle belts are chitin structures that require filamentous F-actin assembly for their generation (Delon and Payre, 2004; Price et al., 2006; Walters et al., 2006). Both the denticle belts and the F-actin assembly are disturbed in the absence of *pri*, which is attributed to the lack of Svb as an activator (Katoh et al., 2010). In addition to its function in trachea and epidermis morphogenesis, Pri also functions in patterning the imaginal leg disc in the late larvae (Galindo et al., 2007; Pueyo and

Couso, 2008), and for the proper development of adult legs during metamorphosis (Pi et al., 2011). In imaginal development, loss of Pri activity induces fused segments in the tarsus region (Pueyo and Couso, 2008). Finally, during wing and external sensory organ development, overexpression of Pri promotes sensory organ precursors and wing specification while suppressing dorsal-ventral boundary formation (Pi et al., 2011).

In *Tribolium castaneum*, knockdown of the *pri* ortholog *mille-pattes* (*mlpt*) induces segmental defects associated with disorganized homeotic gene expression (Savard et al., 2006). Although the developmental events in which Pri is involved seem diverse, this gene may have roles in many aspects of fundamental biological processes, since related genes were found in many other insects and arthropods (Galindo et al., 2007; Savard et al., 2006).

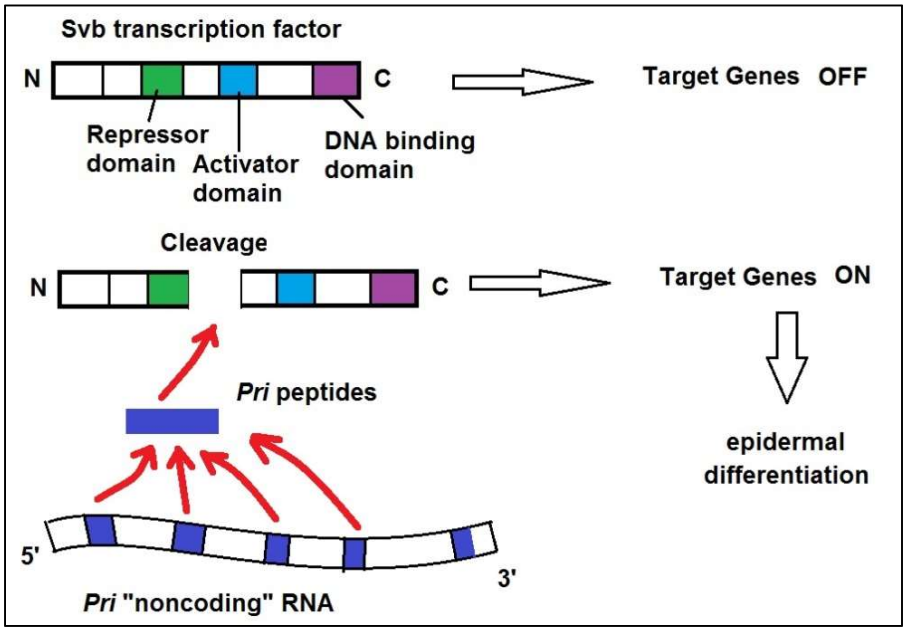


Figure 4. An illustration of "how the *pri* peptides trigger the epidermal differentiation events in insects, and how they direct the proteolytic cleavage of the transcription factor Shavenbaby". (https://blog-biosyn.com /2013/05/07/ what-are-micro-peptides/).

Materials & Methods

2. MATERIALS AND METHODS

2.1 Fly culture and stocks

Flies were raised at 25°C on 10% glucose, 8% cornmeal, 4% yeast extract, and 0.7% agar medium containing propionic acid and butyl-p-hydroxybenzoate as antifungal agents. FBXO11 mutant line *FBXO11*^{EY09314} and proteasome mutant lines *Prosβ3*^{G4206} and *Prosα2*^{G8948} were obtained from Bloomington Stock Center. FBXO11 mutant lines *FBXO11*^{NP2786} and *FBXO11*^{GS10050} and proteasome mutant line *Prosβ6*^{1} were obtained from KYOTO Stock Center. RNAi lines *Prosβ6*$^{NIG.4097R-1}$, *Prosβ6*$^{NIG.4097Rz2}$, and *Prosα4*^{GL00341} were obtained from the National Institute of Genetics in Japan. *ppl-Gal4* was a gift from Dr. Masayuki Miura (University of Tokyo, Tokyo, Japan). *UAS-dicer2*; *Cg-Gal4* was a gift from Dr. Naoki Okamoto (University of California, Riverside, CA, USA). Double homozygotic line *Prosβ6*$^{NIG.4097R-1}$ *Prosβ6*$^{NIG.4097R-2}$ was used to increase RNAi efficiency. Pri mutant lines *(pri*^{4}, *pri*^{F04987}) were a gift from Dr. Yuji Kageyama (Kobe University, Kobe 657-850, Japan). *w* or *yw* was used as a control lines. All mutant fly lines in this work were outcrossed at least seven times into the control background.

2.2. Measurement of the prepupal period

Newly formed white prepupae were collected every 30 min and transferred to a plastic Petri dish kept at 25°C and 80–90% relative humidity, and pupation timing was observed. The Kolmogorov–Smirnov test (KS-test) was used to evaluate differences in pupation timing between test and control animals and to determine *P* values.

2.3. Western blotting

White prepupae of the *hs-Blimp-1* line carrying a heat shock-promoter, Blimp-1 cDNA and FLAG tag sequence fusion gene (Agawa et al., 2007) were collected at 0 h APF, heat shocked at 37°C for 1 h, then homogenized at various time points in a 1.5 mL microtube containing 50 µL of 1× Laemmli sample buffer, chilled immediately in liquid nitrogen and kept at -80°C until use. At each time point, two independent samples of a single animal were used, and western blotting analysis was performed as described previously (Sultan et al., 2014). The efficiency of protein transfer and equal loading of protein were confirmed by staining with Ponceau-S after transfer to the membrane. M2 (FLAG tag) antibody (Sigma, USA) was used at a 1:2000

dilution, and goat anti-mouse IgG HRP (Cayman, USA) at a 1:5000 dilution was used to detect FLAG-tagged Blimp-1.

2.4. MG132 treatment

To block proteasomal activity, white prepupae of the *hs-Blimp-1* line were collected at 0 h APF, heat shocked for 1 h at 37°C, the pupal case was peeled off in Grace insect cell culture medium (GIBCO), and the whole-body organs were untightened. Organs with heat shock-induced Blimp-1 were then cultured in Grace insect cell culture medium with shaking at 25°C with or without 10 μM MG132 (Cayman, USA), which is a 26S proteasome-specific inhibitor, dissolved in DMSO, for the indicated periods. 0.1% DMSO was used as the vehicle control. Culture tubes including the treated organs were centrifuged for 5 min at 5000 rpm, the medium was removed carefully, and the organs were then homogenized and processed for western blotting against FLAG-tagged Blimp-1, as described before.

Results

RESULTS

3.1. Blimp-1 is degraded in a 26S proteasome-dependent manner

To examine whether *Drosophila* Blimp-1 is degraded by the proteasome, I examined whether the degradation speed of Blimp-1 was affected by the proteasome inhibitor MG132. FLAG-tagged Blimp-1 was induced using the *hs-Blimp-1* transgenic fly line under the control of heat shock at 0 h APF. Organs from Blimp-1-induced prepupae were cultured in the presence or absence of MG132 for various periods, and the degradation of Blimp-1 was detected by western blotting. Blimp-1 was clearly detectable until at least 4 h after heat shock when organs were cultured in the presence of MG132, in contrast to almost complete disappearance after 2 h in controls (Fig. 5). This result suggests that Blimp-1 is degraded by the proteasome.

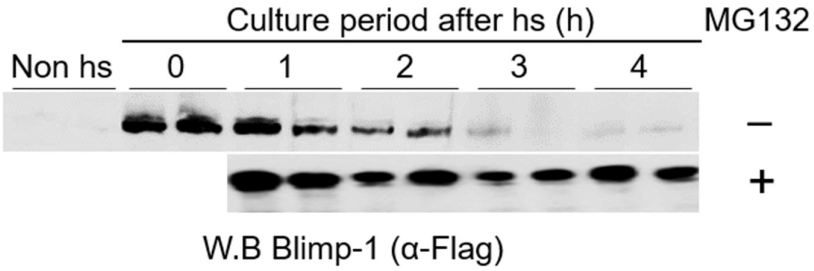
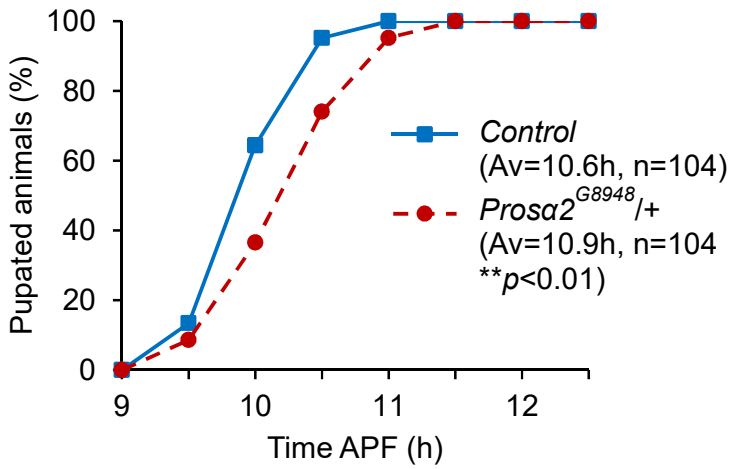


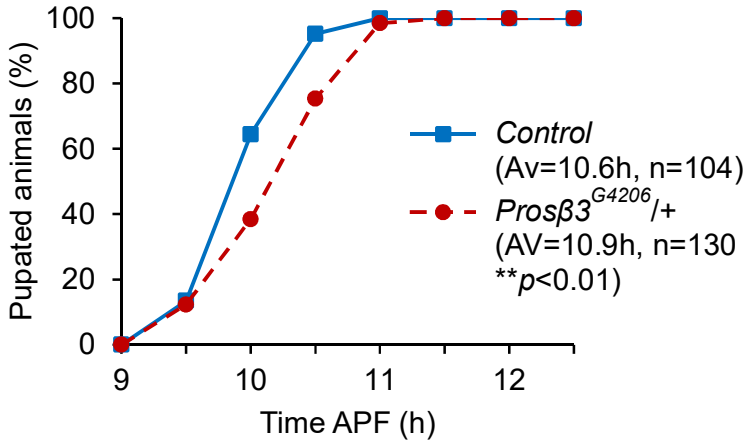
Figure 5. Blimp-1 degradation requires a functional proteasome system. Blimp-1 was induced by 1 h heat shock at 0 h APF in hs-Blimp-1 prepupae and then their organs were cultured for the indicated period in the presence or absence of 10 μM MG132. The expression level of Blimp-1 before and after organ culture was detected by western blotting using anti-FLAG-tag antibody. Two independent samples from a single animal were used for each time point. Upper panel (-); the vehicle control (0.1% DMSO) samples. Lower panel (+); MG132-treated samples. Non-heat shock (Non hs) samples were neither cultured nor treated.

Next, I analyzed the pupation timing in mutants of 26S proteasome subunit components, because retardation in Blimp-1 degradation showed a delay in pupation timing (Akagi et al., 2016). Pupation timing was significantly delayed in heterozygous mutants of 20S proteolytic core subunits compared with that in control animals; pupation was 0.3 h slower on average in mutants of α2, one of the structural subunits (Fig. 6A), and of β3 (Fig. 6B) or β6 (Fig. 6C), which are endopeptidase active subunits. Taken together, these results suggest that Blimp-1 is degraded by the 26S proteasome in the UPS, and that proteasome functionality at these stages is required for the accurate completion of the developmental program.

A


100
80
60
40
20
0
Pupated animals (%)
9
10
11
12
Time APF (h)
Control
(Av=10.6h, n=104)
Prosα2G8948/+
(Av=10.9h, n=104
**p<0.01)

B


100
80
60
40
20
0
Pupated animals (%)
9
10
11
12
Time APF (h)
Control
(Av=10.6h, n=104)
Prosβ3G4206/+
(AV=10.9h, n=130
**p<0.01)

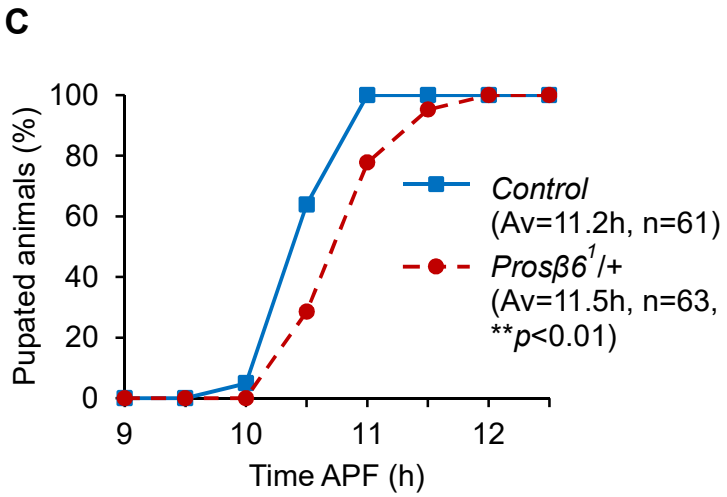
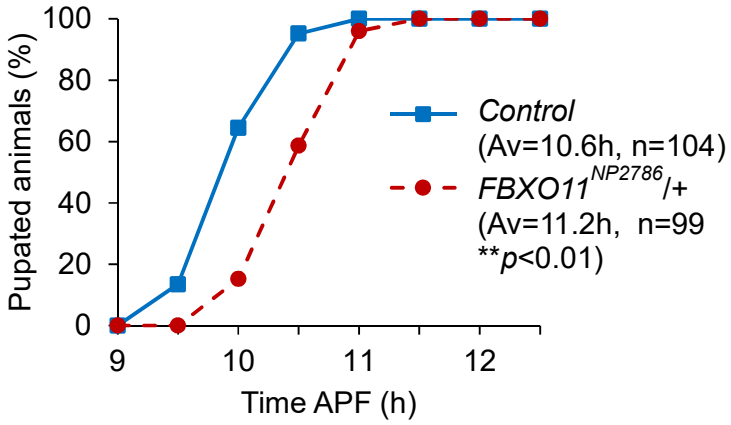


Figure 6. 26S proteasome mutants exhibited a delay in pupation timing. Heterozygous mutants of 26S proteasome subunits and control (*yw*) animals were cultured at 25°C, and pupation timing was observed every 30 min. The Kolmogorov–Smirnov test was used to evaluate the differences between pupation timing and to determine *P* values. *Prosα2*^{G8948} line (A) was used for the α subunit mutant, *Prosβ3*^{G4206} (B) and *Prosβ6*^{1} (C) were used for β subunit mutants. Av; average of pupation timing, n; number of animals examined.

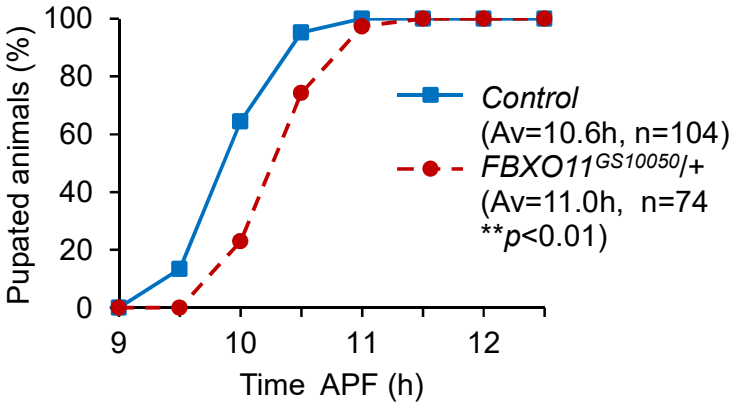
3.2. FBXO11 contributes to Blimp-1 degradation as an E3-ligase

The results on the role of UPS in the degradation of *Drosophila* Blimp-1 led us to investigate whether Blimp-1 is recruited to the 26S proteasome by the same conserved E3-ligase FBXO11 as identified previously in *C. elegans* and human (Horn et al., 2014). To examine this possibility, I examined the pupation timing in several hypomorphic loss-of-function mutations in *FBXO11*. Heterozygous *FBXO11* mutants *FBXO11*^{NP2786} (Fig. 7A) and *FBXO11*^{GS10050} (Fig. 7B) showed significant delays in pupation timing by 0.6 and 0.4 h, respectively. Of note, the homozygous viable mutant *FBXO11*^{EY09314} (Fig. 7C) showed an obvious dose-dependent delay by 0.5 h compared with 0.3 h in heterozygotes. These results suggest that the degradation speed of Blimp-1 was slowed down in *FBXO11* mutants, and FBXO11 acted as an E3-ligase for Blimp-1 recognition to the SCF^{FBXO11} complex.

A


Pupated animals (%)
100
80
60
40
20
0
9
10
11
12
Time APF (h)
Control
(Av=10.6h, n=104)
FBXO11NP2786/+
(Av=11.2h, n=99
**p<0.01)

B


Pupated animals (%)
100
80
60
40
20
0
9
10
11
12
Time APF (h)
Control
(Av=10.6h, n=104)
FBXO11GS10050/+
(Av=11.0h, n=74
**p<0.01)

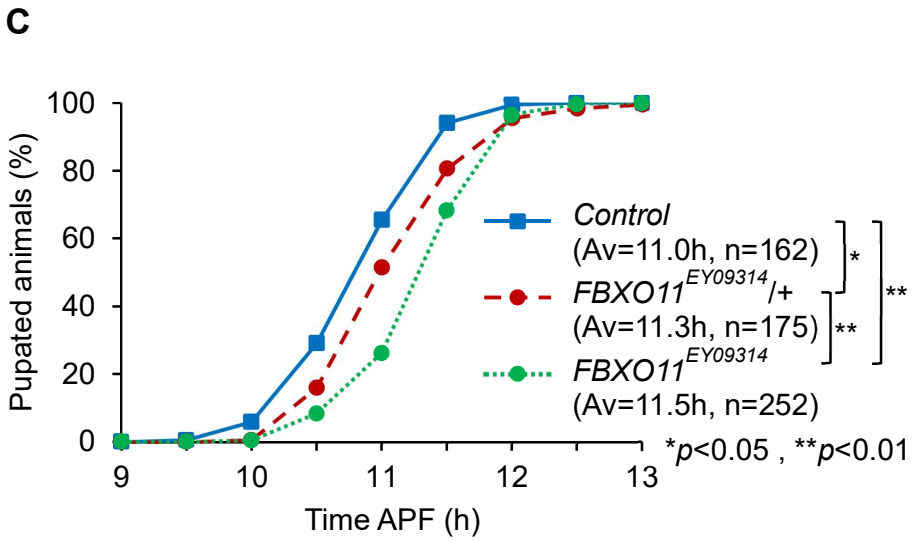


Figure 7. FBXO11 contributes to the correct determination of pupation timing. *FBXO11* mutants and control (*yw*) animals were cultured at 25°C, and pupation timing was observed every 30 min. The Kolmogorov–Smirnov test was used to evaluate differences between pupation timing and to determine *P* values. Heterozygous lines $FBXO11^{NP2786}$ (A), $FBXO11^{GS10050}$ (B), and homozygous line $FBXO11^{EY09314}$ (C) were used. Av; average of pupation timing, n; number of animals examined.

3.3. Degradation speed of Blimp-1 is affected by proteasome subunit and *FBXO11* gene doses

To confirm that the degradation speed of Blimp-1 is dependent on the expression level of proteasome subunits or FBXO11, I examined the degradation speed of Blimp-1 in these mutant backgrounds. I induced FLAG-tagged Blimp-1 from the *hs-Blimp-1* transgene at 0 h APF, and degradation speed of Blimp-1 was observed by western blotting. In the control animals, Blimp-1 was clearly detected until 2 h after heat shock. In contrast, in the heterozygous mutant of both subunits of the 26S proteasome and *FBXO11*, Blimp-1 was detected clearly until 2.5 and 3 h after heat shock (Fig.8). This result further supports the idea that Blimp-1 is degraded by the 26S proteasome, and this process is mediated by FBXO11 as the E3 ubiquitin ligase.

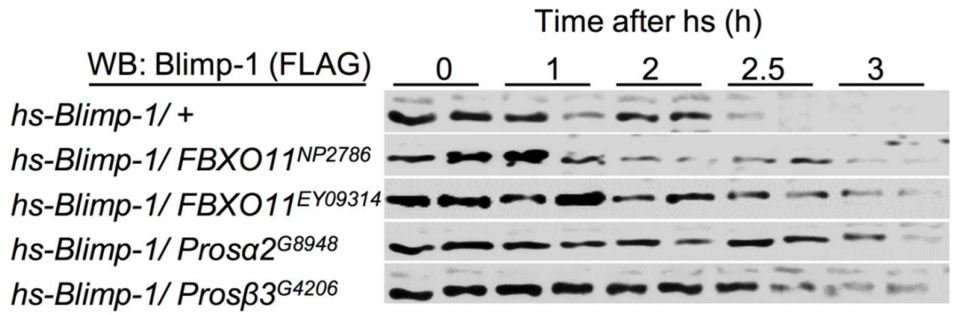
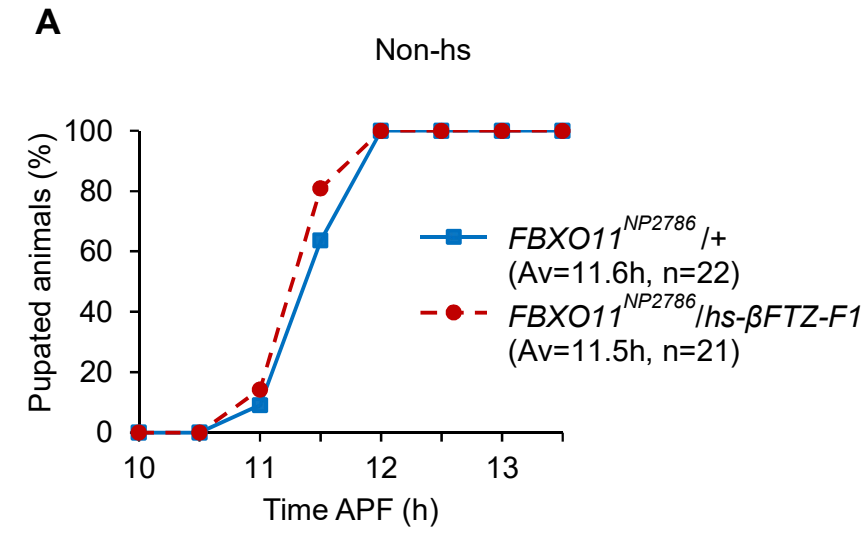
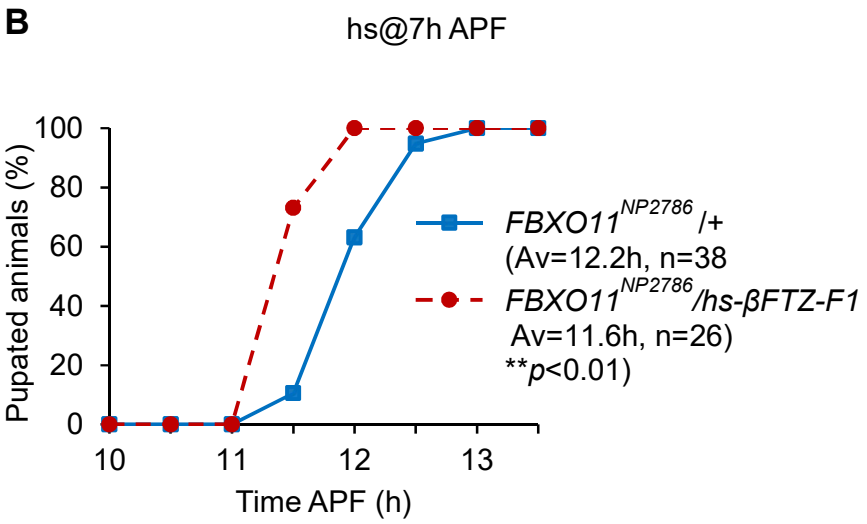


Figure 8. Blimp-1 stability increases in the mutants of either proteasome subunits or FBXO11. Flag-tagged Blimp-1 was induced by 1 h heat shock at 0 h APF in heterozygotes between *hs-Blimp-1* and the mutants of proteasome subunit or FBXO11 and was detected after indicated time by Western blotting using Flag antibody. Two independent samples of a single animal were used at each time point.

3.4. Pupation delay in FBXO11 mutant is caused by the identified timer system

It has shown that prolonged Blimp-1 expression causes a delay in pupation timing due to the delayed expression of βFTZ-F1 (Agawa et al., 2007; Akagi et al., 2016). To investigate whether the delayed pupation timing in *FBXO11* mutant animals is caused by altered βFTZ-F1 expression, I examined whether temporal induction of βFTZ-F1 at the endogenous expression timing is sufficient to rescue the delay in pupation timing in an *FBXO11* mutant. βFTZ-F1 was induced from the *hs-βFTZ-F1* transgene (Murata et al., 1996) by heat shock in a heterozygous $FBXO11^{NP2786}$ mutant, and pupation timing was observed. Although a difference in pupation timing was not observed whether or not animals carried the *hs-βFTZ-F1* gene without heat shock in a *FBXO11* heterozygous mutant (Fig. 9A,C), advancement of pupation timing was observed by the induction of βFTZ-F1 at 7 h APF, which is the time when endogenous *βFTZ-F1* is expressed (Fig. 9B,D). This result means that delayed pupation in the *FBXO11* mutant was advanced by the induction of βFTZ-F1, and thus it is clear that delaying effect from the *FBXO11* mutation is caused by affecting the timer system to determine pupation timing.


A
Non-hs
Pupated animals (%)
100
80
60
40
20
0
10
11
12
13
Time APF (h)
FBXO11NP2786/+
(Av=11.6h, n=22)
FBXO11NP2786/hs-βFTZ-F1
(Av=11.5h, n=21)


B
hs@7h APF
Pupated animals (%)
100
80
60
40
20
0
10
11
12
13
Time APF (h)
FBXO11NP2786/+
(Av=12.2h, n=38
FBXO11NP2786/hs-βFTZ-F1
Av=11.6h, n=26)
**p<0.01)

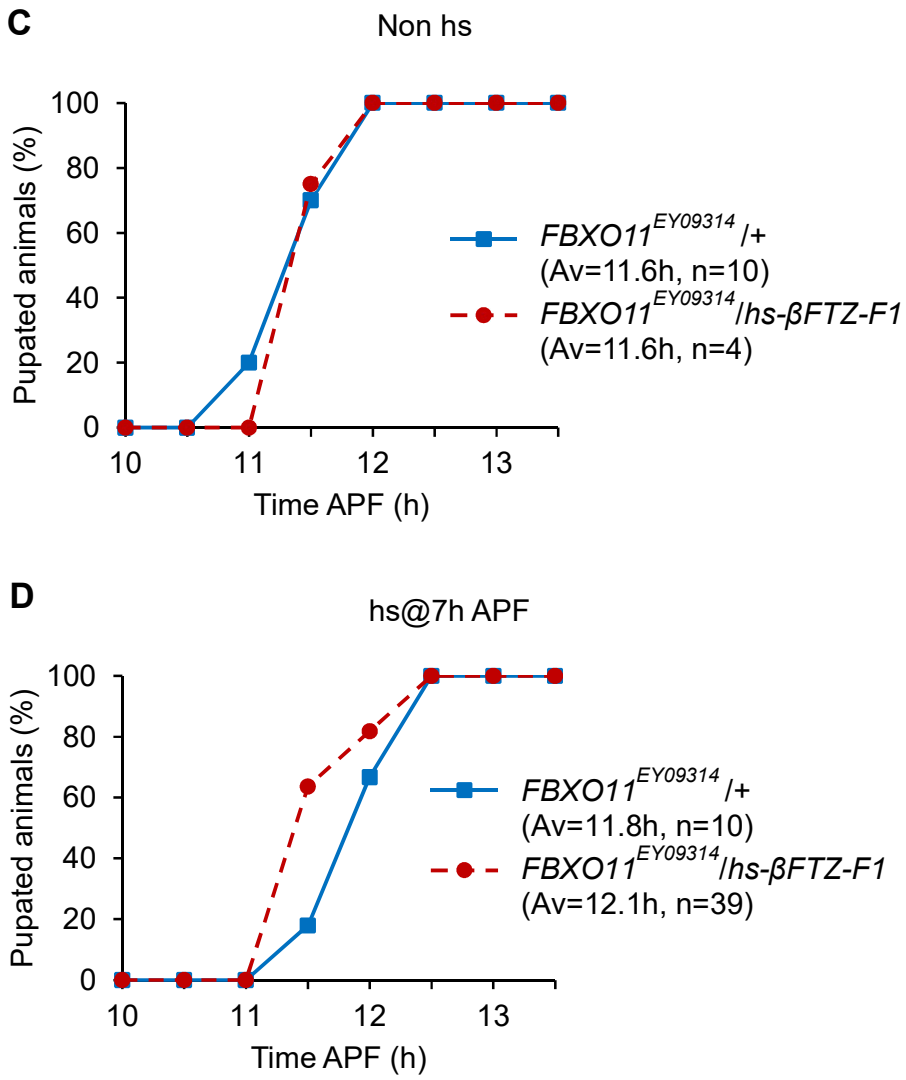
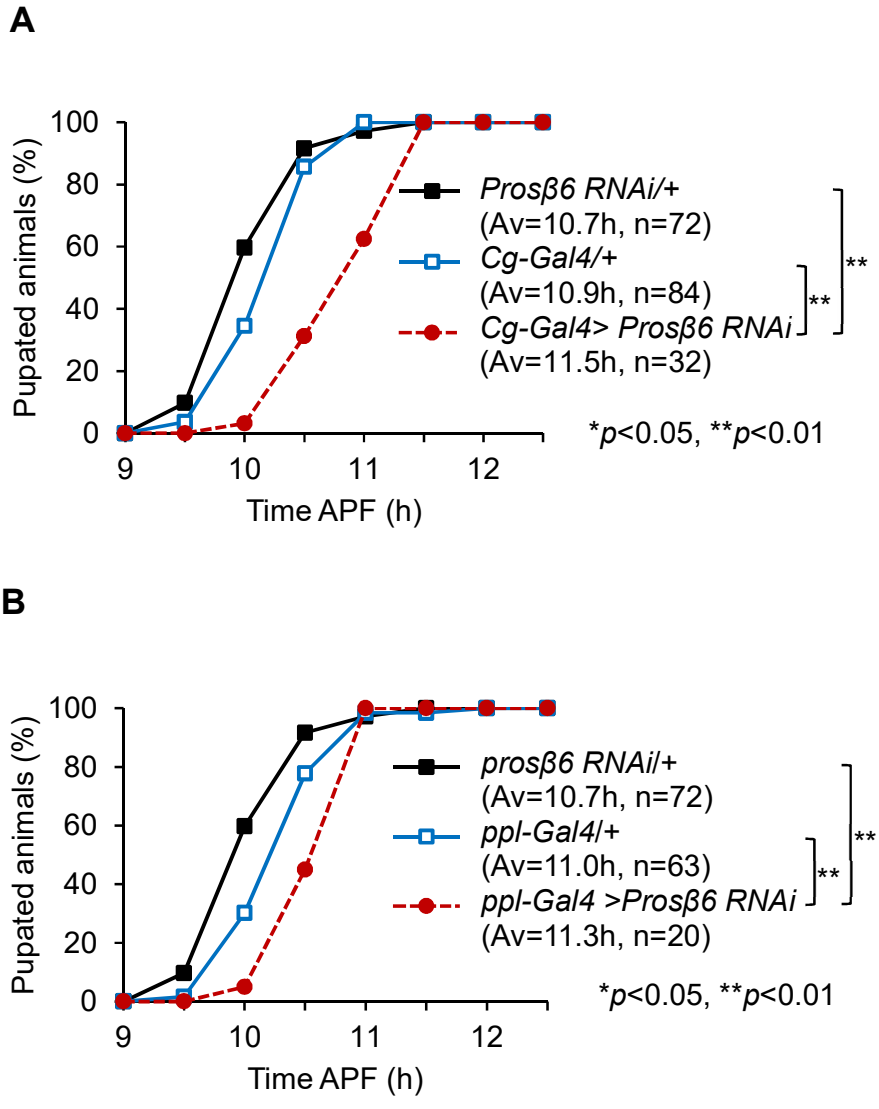


Figure 9. Rescue of pupation timing delay in FBXO11 mutant by induction of βFTZ-F1. Pupation timing of *FBXO11* mutant animals with/without *hs-βFTZ-F1* transgene was observed every 30 min. Non-heat shocked (A) and heat shocked (B) animals. Heat shock was given at 34°C for 1 h at 7 h APF. The Kolmogorov–Smirnov test was used to evaluate differences in pupation timing between test and control animals and to determine *P* values. Av; average of pupation timing, n; number of animals examined.

3.5. The proteasome system in the fat body affects pupation timing

Because it have been identified that the timer system for pupation timing is located in the fat body (Akagi et al., 2016), I knocked down proteasome subunit genes in the fat body using fat body-specific GAL4 drivers. Pupation timing was obviously delayed by 0.8 and 0.6 h upon knockdown of β6 subunit gene using *Cg-GAL4* compared with GAL4 and UAS control lines, respectively (Fig. 10A). Furthermore, a slightly shorter, but still significant, delay in pupation timing was observed when either β6 (Fig. 10B) or α4 (Fig. 10C) subunit genes were knocked down using a *ppl-GAL4* driver. These results indicate that the observed delay in pupation is caused by affecting the proteasome system in the fat body.



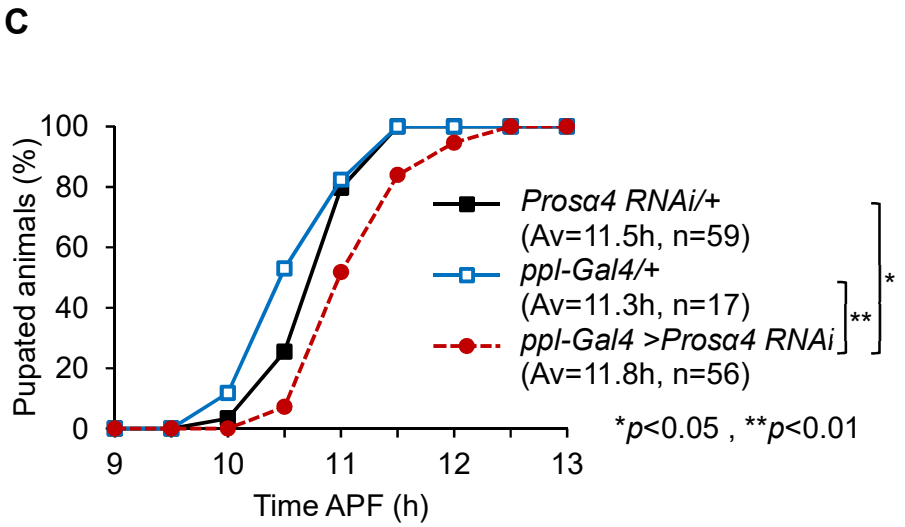


Figure 10. The fat body is responsible for the control of pupation timing by proteasome activity. Pupation timing was observed every 30 min in animals with knockdown of 20S components β6 (A, B) and α4 (C) in the fat body. The Kolmogorov–Smirnov test was used to evaluate differences between pupation timing and to determine *P* values versus the *RNA*i/+ (A, B, C), *Cg-Gal4*/+ (A) or *ppl-Gal4*/+ (B, C) as controls. Av; average of pupation timing, n; number of animals examined.

3.6. Pri is a temporal regulator of pupation.

Given the crucial role of ecdysone in developmental timing, *pri* has been recently shown to have a role in mediating the action of ecdysone for the temporal control of morphogenesis (Chanut-Delalande et al., 2014), I wonder whether Pri peptides also provide temporal control of the pupation timing. Pupation timing analyzed the in two independent *pri* mutants. Slight but significant delay in pupation timing was observed by 0.2 h in the heterozygote pri^{4} mutant (Fig. 11A), while a more significant delay by 0.4 h was observed in the homozygote viable pri^{F04987} mutant (Fig. 11B). These results indicate that Pri peptides may contribute to the time determination mechanism of pupation.

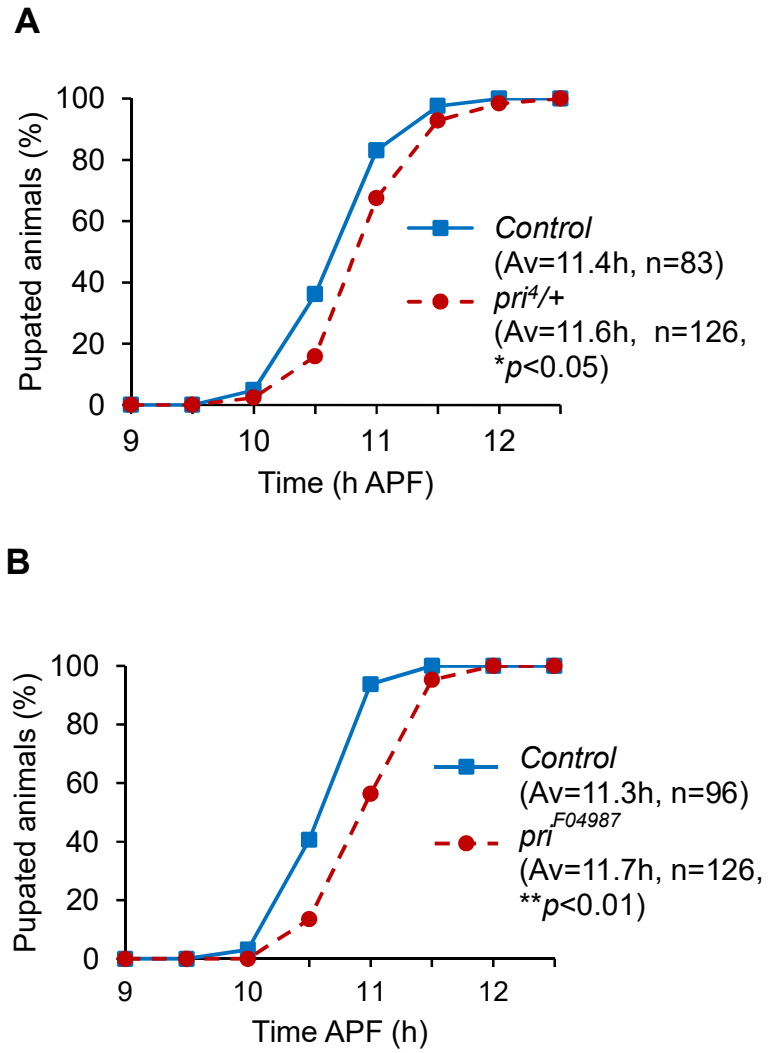


Figure 11. Pri peptides might contribute to the control of pupation timing by activating Blimp-1 processing in proteasome. Heterozygote (A) and homozygote (B) mutant of *pri* and control animals were cultured in 25°C, and pupation timing was observed every 30 min. The Kolmogorov-Smirnov test was used to evaluate differences of pupation timing between test and control animals and to determine *P* values. Av; average of pupation timing, n; number of animals examined.

Discussion

4. DISCUSSION

Here, I showed that *Drosophila* Blimp-1 is degraded by the 26S proteasome system and is recruited by FBXO11 as the substrate-recognition component of the SCF complex. Furthermore, I showed the importance of proteasome activity in the fat body to determine pupation timing. My results are correlated with previously described results that the biological timer system for pupation is located in the fat body (Akagi et al., 2016).

4.1. FBXO11-mediated proteasome degradation of Blimp-1 determine pupation timing

I observed a delay in pupation timing in all of the examined heterozygous mutants of 26S proteasome components. These results suggest that gene dosage effects due to loss-of-function mutations of these 26S proteasome components. In addition, a heterozygous mutant of recruiter FBXO11 also exhibited the same level of delay in pupation timing. These results indicated that the expression level of these components is an important factor to determine pupation timing; therefore, pupation timing can be controlled by the expression level of these components. Thus, I assumed that the UPS contributes to determine pupation timing as one of the components in the biological timer during the early prepupal period. Of note, a sudden increase in the concentration of the 26S proteasome at 0 to 4 h APF has been reported (Szlanka et al., 2003), suggesting the importance of protein degradation in developmental control. Furthermore, RNA-Seq data in the modENCODE developmental transcriptome of *D. melanogaster* (Graveley et al., 2011) showed that the expression of the FBXO11 increases gradually from the 3rd instar larval stage (L3) to a moderately high level at pupation and then starts to decrease again 24 h later. These developmental changes may allow control of the degradation speed of specific targets, including Blimp-1, among many UPS target proteins that must be degraded at appropriate time points.

I have shown that both the *Blimp-1* and *βftz-f1* are induced by 20E and are temporally expressed in almost all organs (Akagi et al., 2016; Yamada et al., 2000), but the identified target genes are still limited in number. βFTZ-F1 has multiple functions in each organ during the mid to late prepupal period. For instance, βFTZ-F1 regulates two pupal cuticle genes that are expressed in slightly different parts of the epidermis (Kawasaki et al., 2002; Murata et al., 1996), and it also regulates a protease that is expressed in the fat body and contributes to its morphological change (Bond et al., 2011). Furthermore, the expression of βFTZ-F1 in the inka cells is essential for releasing the ecdysis triggering hormone ETH, which induces pupation in the late prepupal period, and also βFTZ-F1 expression in muscles is necessary to determine the

timing of muscle apoptosis during metamorphosis (Fortier et al., 2003; Zirin et al., 2013). Moreover, βFTZ-F1 is a master regulator of late prepupal gene expression, which is essential for histolysis of the salivary gland cells during the early pupal period (Broadus et al., 1999; Yamada et al., 2000). In addition, the expression timing of βFTZ-F1 is not completely the same among different organs (unpublished results). In a large transcriptional profiling platform, involving 29 dissected tissues from larval, pupal, and adult stages of *Drosophila* (Brown et al., 2014), FBXO11 appeared to be expressed in many tissues and/or during development with specific upregulation in the fat body from L3 up to pupation. I deduced that the expression levels of the 26S proteasome and FBXO11 may differ depending on tissue and contribute to the determination of timing of tissue-specific developmental events through control of the degradation speed of Blimp-1.

In *C. elegans*, *Blmp-1* was previously identified using RNAi-based suppressor screening to suppress *dre-1* heterochronic phenotypes (Horn et al., 2014). A *dre-1* mutant showed retarded migration of the gonad, whereas a *Blmp-1* mutant showed precocious gonadal migration during L2 to L3 larva and was able to suppress the retarded phenotype of *dre-1*. In addition, precocious fusion and differentiation of epidermal stem cells, called seam cells, was partially suppressed by the *Blmp-1* mutant in *C. elegans*. Moreover, similar genetic interactions were observed between DRE-1 and Blmp-1 for dauer formation (Horn et al., 2014). These observations suggest a conserved role of Blimp-1 degradation for the determination of developmental timing across taxa.

4.2. Pri and Blimp-1 together contribute to determine pupation timing

Here, I report a bit more analysis to the *pri* functions in the control of *Drosophila* development. My result raises the possibility that the Pri peptides might be involved in the accurate determination of pupation timing by regulating Blimp-1 degradation. A set of evidence supports my idea; first, the delayed pupation timing in the two independent *pri* mutants indicating a probable function of Pri in the time determination of this stage (Fig. 11). Secondly, the timing of both *pri* and *Blimp-1* expression is directly regulated by ecdysone, indicating the crucial role of these factors for the proper developmental timing. Thirdly, Pri has found to induce a partial degradation of Svb protein by binding to the E3-ubiquitin ligase Ubr3. Pri induces a conformational change to Ubr3 and triggers proteasome-dependent maturation of Svb which leads to a shorter activator (Kondo et al., 2010; Zanet et al., 2015), when Pri works for the formation of epidermal trichomes (Chanut-Delalande et al., 2014; Stern and Franke,

2013). This mode of Pri acting as an E3-ligase modifier may hint the prediction of a Pri dependent modification of the E3-ligase FBXO11 along the way to Blimp-1 ubiquitination and degradation. In addition, the expression peak of *pri* is between 2-4 h APF (Chanut-Delalande et al., 2014), compared to 0-2 h APF of Blimp-1 (Agawa et al., 2007). This shift in the expression peak of *pri* does fit to a possible function of Pri in Blimp-1 processing. Furthermore, data obtained from transcriptome analysis (Brown et al., 2014) showed a striking increase of *pri* expression in the fat body at 0 h APF compared to that in the whole body, indicating that Pri might provide a more contribution to the rapid degradation of Blimp-1 in the fat body as a part of time measuring system.

A characteristic feature of *pri* during *Drosophila* embryogenesis is its specific expression in a restricted set of developing tissues, and also the highly dynamic changes between developmental stages (Inagaki et al., 2005; Tupy et al., 2005). In *Tribolium*, the highly specific expression of *mlpt* in the posterior growth zone was the first hint of its function in the segmentation of the beetle embryo (Savard et al., 2006). In *Drosophila*, tissues which display altered differentiation in *pri* mutant embryos were initially anticipated by dynamics of *pri* mRNA. *pri* displays a transient wave of expression in the embryonic epidermis, and *pri* mutants display severe defects in epidermal differentiation (Chanut-Delalande et al., 2014; Galindo et al., 2007; Kondo et al., 2010, 2007). *pri* is also well expressed in the developing tracheal system from stages 9 to 15 (Kondo et al., 2007), and the lack of *pri* function leads to various defects in its initial formation (Galindo et al., 2007) and later differentiation (Katoh et al., 2010; Öztürk-Çolak et al., 2016). Hence the remarkable dynamics of *pri* expression is suggestive of functions for Pri peptides in a wide variety of tissues. Also, the spatiotemporal wave of *pri* expression seen around puparium formation may also indicate its implication in the control of the developmental timing of this stage.

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

It has shown recently that the pupation timer is composed of Blimp-1, βFTZ-F1 and Shade, however, the delays in pupation by heat induction of *Blimp-1* or knockdown of either *βftz-f1* or *Shade* were different (Akagi et al., 2016). Fat body is expected to incorporate the nutritional status of the animal and sends a cue for the final decision of pupation dependent on the comprised timer system. Akagi et al. (2016) has 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). Here I showed a further evidence to support the previous demonstration about fat body's role in pupation timing. First, the observation that fat body specific RNAi depletion of proteasome components lead to delay in pupation. Second, fat-body specific increase of either *FBXO11* or *Pri* mRNA levels upon pupariation (Brown et al., 2014), together pointing to the importance of fat body in time regulation of this period.

Summary

5. SUMMARY

Developmental timing can be adapted to the environmental changes such as nutrition and temperature, but this timing is well controlled and many developmental events occur after specific period of another event in organisms. During development of fruit fly *Drosophila melanogaster*, a drastic change from larval shape to pupal shape occurs at the end of larval stage, called puparium formation. Subsequently, pupation occurs about 11 hours after puparium formation at standard rearing condition at 25°C. This means that they have a system to determine pupation timing. The insect hormone ecdysteroids play an important role for these transitions. Recently, our lab shown that two ecdysone-inducible transcription factors, Blimp-1 and βFTZ-F1, play a crucial role for the determination of pupation timing. The first trigger of these events is the increase of 20E level at the end of larval period, which induces puparium formation and *Blimp-1* expression. After 2 or 3 h of puparium formation, 20E level decreases. Subsequently, production of Blimp-1 stops because of the termination of *Blimp-1* mRNA production and rapid degradation of the mRNA. Blimp-1 works as a repressor for the *βftz-f1* which encodes transcriptional activator. Thus, βFTZ-F1 is induced after Blimp-1 disappearance at around 6 h APF and activates *Shade* which encodes ecdysone to 20E conversion enzyme, ecdysone-20-monooxygenase, in fat body. Hence, pupation timing is determined by a biological timer in fat body comprising Blimp-1, βFTZ-F1 and Shade. Although Blimp-1 is unstable, and its rapid loss is required for the proper timing of *βftz-f1* expression and pupation, the mechanism underlying its temporal control is poorly understood.

To understand the degradation mechanism of *Drosophila* Blimp-1 and its contribution to time measuring system for pupation, I first examined whether the degradation speed of *Drosophila* Blimp-1 is affected by the proteasome inhibitor MG132, using organ culture system. Blimp-1 was clearly detectable until at least 4 h after heat shock, when organs were cultured in the presence of MG132, in contrast to almost complete disappearance after 2 h in controls, suggesting that Blimp-1 is degraded by proteasome. I next analyzed the pupation timing in mutants of 26S proteasome subunit components. Pupation timing was significantly delayed in the heterozygote mutants of 20S proteolytic core subunits compared to that in the host control animals, suggesting that proteasome functionality is required for the accurate completion of the developmental program at this stage.

Proteasomes are found within the cytoplasm and nucleus of all eukaryotic cells and function to degrade normal, short-lived and mutated or damaged intracellular proteins that have been modified with polyubiquitin chains. Targeting and processing substrates of the 26S

proteasome require a covalent linkage to ubiquitin, a process that is controlled by a three-step enzymatic cascade comprising an E1, E2 and E3 ubiquitin ligase. In *C. elegans,* Blmp-1 has been shown to be regulated by the $SCF^{DRE-1/FBXO11}$ complex via proteasome-dependent degradation and the system is conserved in human, suggesting that *Drosophila* Blimp-1 may be degraded by the same system that contributes to the correct time determination for pupation.

To know how Blimp-1 was recognized by proteasome, pupation timing was analyzed in some mutants of FBXO11. The results revealed that the pupation timing in the mutated animals is delayed compared to that in host control animals. These results strongly suggest that Blimp-1 is targeted to a ubiquitin proteasome-dependent proteolysis system mediated by FBXO11 and the system is conserved among wide range of species including *Drosophila*. To confirm the Blimp-1 degradation of is dependent on the expression level of proteasome subunits or FBXO11, the degradation speed of Blimp-1 was examined in these mutant background. Induced Blimp-1 from the *hs-Blimp-1* transgene could obviously be detected until 2 h after heat shock, compared to 2.5 and 3 h in mutants of both *FBXO11* and 26S proteasome subunits. This result further supports the idea that Blimp-1 is degraded by 26S proteasome mediated by FBXO11 as E3 ubiquitin ligase, and that pupation timing is determined by the degradation speed of Blimp-1. If Blimp-1 works as timer molecule by repressing *βftz-f1*, I wander whether the delayed pupation timing in *FBXO11* mutant animals is caused by delayed βFTZ-F1 expression. For that, βFTZ-F1 was induced from *hs-βFTZ-F1* transgene in *FBXO11* mutant and pupation timing was observed. Results revealed that pupation timing was advanced by induction of βFTZ-F1 at the time endogenous *βFTZ-F1* expression. This result means that delayed pupation in *FBXO11* mutant was suppressed by induction of βFTZ-F1, and thus it is clear that delay effect by *FBXO11* mutation is caused by affecting the identified timer system. Since this timer system for pupation timing is located in fat body, I thought to knock down proteasome subunit genes in fat body using fat body-specific GAL4 drivers. As expected, pupation timing was obviously delayed for 0.8 and 0.6 h in *β6* subunit gene suing *Cg-GAL4*, and a slightly less but significant delay was observed when either *β6* or *α4* using *ppl-GAL4* driver. These results indicate that observed delay in pupation is caused by affecting proteasome system in fat body.

I also analyzed mutants on the micropeptides encoded by *polished rice* (*pri*), which are known as proteasome mediators for protein processing, and found that Pri might be a temporal regulator for Blimp-1. All these results support the idea that pupation timing is determined by the degradation speed of Blimp-1.

6. CONCLUSION

My study is mainly aimed to uncover the molecular mechanisms underlying a biological timer to ensure that a sequence of developmental events appropriately occur at prepupal period (Fig 12). I demonstrate that the ubiquitin proteasome system is involved in the rapid degradation of a labile transcriptional factor Blimp-1 that determines the onset of pupation by repression of the gene encoding transcriptional factor βFTZ-F1 in *Drosophila* (Fig 12). My study provides insight into the evolutionarily conservation of proteasome-dependent degradation of Blimp-1 in the regulation of developmental timing. In addition, the result adds more evidence to support the previously identified timer system in fat body, shedding light for molecular complication of its role in development. Furthermore, a small part of my results is welcoming the contribution of "Pri" small peptides, The new comer to *Drosophila* development in regulation of pupation timing.

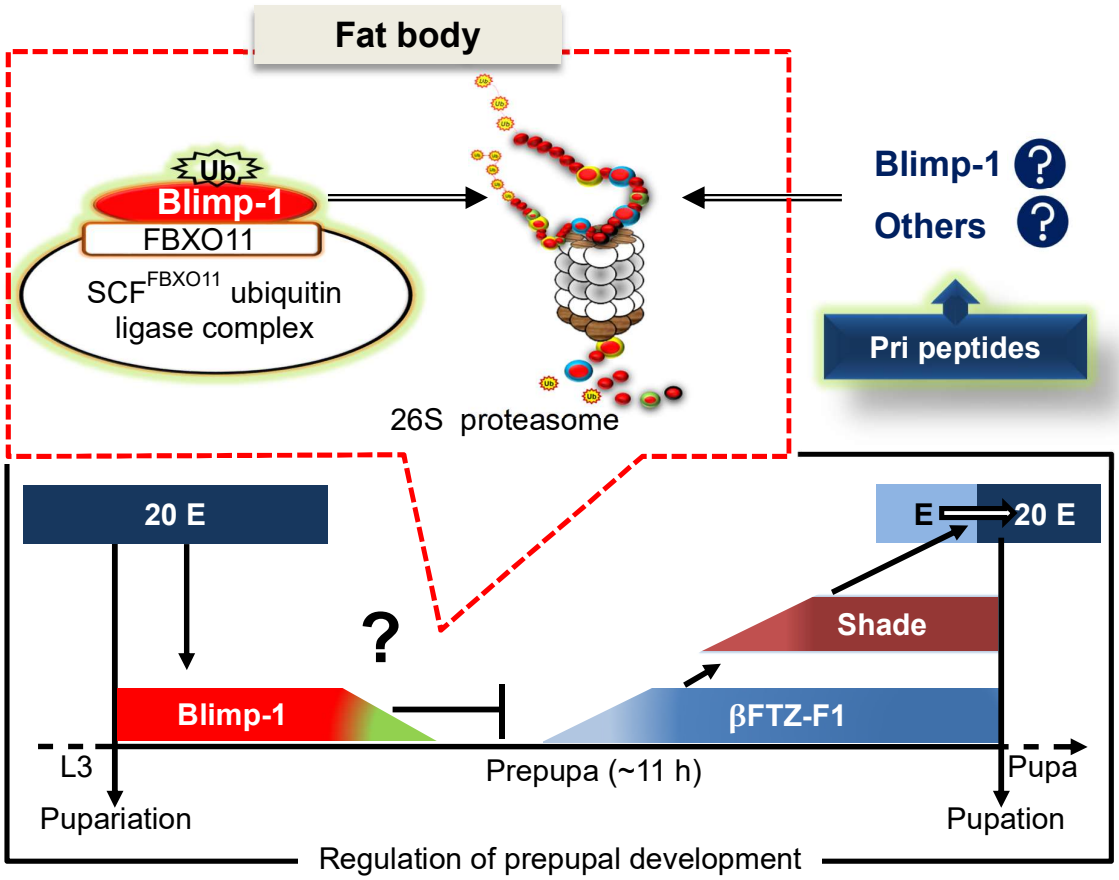


Figure 12. Schematic representation of the suggested proteasomal regulation of Blimp-1 processes by controlling its turnover in fat body. Proteasome function to degrade Blimp-1 in fat body specifically recognized by recruiter FBXO11, permitting the next developmental events. After a decline of the level of 20E, Blimp-1 degrade rapidly (Agawa et al., 2007; Akagi and Ueda, 2011) allowing expression of *βftz-f1*, which induces *Shade* in the fat body in the late prepupal period. Then, Shade converts released E to 20E, which induces pupation. Pri peptides somehow contribute to determine pupation timing but the exact protein targets in this period is still unclear.

References

7. REFERENCES

Agawa, Y., Sarhan, M., Kageyama, Y., Akagi, K., Takai, M., Hashiyama, K., Wada, T., Handa, H., Iwamatsu, A., Hirose, S., Ueda, H., 2007. *Drosophila* Blimp-1 Is a Transient Transcriptional Repressor That Controls Timing of the Ecdysone-Induced Developmental Pathway. Mol. Cell. Biol. 27, 8739–8747.

Aguila, J.R., Hoshizaki, D.K., Gibbs, A.G., 2013. Contribution of larval nutrition to adult reproduction in *Drosophila melanogaster*. J. Exp. Biol. 216, 399–406.

Aguila, J.R., Suszko, J., Gibbs, A.G., Hoshizaki, D.K., 2007. The role of larval fat cells in adult *Drosophila melanogaster*. J. Exp. Biol. 210, 956–963.

Akagi, K., Sarhan, M., Sultan, A.-R.S., Nishida, H., Koie, A., Nakayama, T., Ueda, H., 2016. A biological timer in the fat body comprising Blimp-1, βFtz-f1 and Shade regulates pupation timing in *Drosophila melanogaster*. Development 143, 2410–2416.

Akagi, K., Ueda, H., 2011. Regulatory mechanisms of ecdysone-inducible Blimp-1 encoding a transcriptional repressor that is important for the prepupal development in *Drosophila*. Dev. Growth Differ. 53, 697–703.

Ancelin, K., Lange, U.C., Hajkova, P., Schneider, R., Bannister, A.J., Kouzarides, T., Surani, M.A., 2006. Blimp1 associates with Prmt5 and directs histone arginine methylation in mouse germ cells. Nat. Cell Biol. 8, 623–630.

Andres, A.J., Fletcher, J.C., Karim, F.D., Thummel, C.S., 1993. Molecular analysis of the initiation of insect metamorphosis: A comparative study of *Drosophila* ecdysteroid-regulated transcription. Dev. Biol. 160, 388–404.

Andres, A.J., Thummel, C.S., 1992. Hormones, puffs and flies: the molecular control of metamorphosis by ecdysone. Trends Genet.

Ashburner, M., 1974. Sequential gene activation by ecdysone in polytene chromosomes of *Drosophila melanogaster*. II. The effects of inhibitors of protein synthesis. Dev. Biol.

Ayer, S., Walker, N., Mosammaparast, M., Nelson, J.P., Shilo, B. zion, Benyajati, C., 1993. Activation and repression of *Drosophila* alcohol dehydrogenase distal transcription by two steroid hormone receptor superfamily members binding to a common response element. Nucleic Acids Res. 21, 1619–1927.

Baxendale, S., Davison, C., Muxworthy, C., Wolff, C., Ingham, P.W., Roy, S., 2004. The B-cell maturation factor Blimp-1 specifies vertebrate slow-twitch muscle fiber identity in response to Hedgehog signaling. Nat. Genet. 36, 88–93.

Bialecki, M., Shilton, A., Fichtenberg, C., Segraves, W.A., Thummel, C.S., 2002. Loss of the ecdysteroid-inducible E75A orphan nuclear receptor uncouples molting from metamorphosis in *Drosophila*. Dev. Cell.

Bond, N.D., Nelliot, A., Bernardo, M.K., Ayerh, M.A., Gorski, K.A., Hoshizaki, D.K., Woodard, C.T., 2011. ßFTZ-F1 and Matrix metalloproteinase 2 are required for fat-body remodeling in *Drosophila*. Dev. Biol. 360, 286–296.

Broadus, J., McCabe, J.R., Endrizzi, B., Thummel, C.S., Woodard, C.T., 1999. The *Drosophila* βFTZ-F1 orphan nuclear receptor provides competence for stage-specific responses to the steroid hormone ecdysone. Mol. Cell 3, 143–149.

Brown, J.B., Boley, N., Eisman, R., May, G.E., Stoiber, M.H., Duff, M.O., Booth, B.W., Wen, J., Park, S., Suzuki, A.M., Wan, K.H., Yu, C., Zhang, D., Carlson, J.W., Cherbas, L., Eads,

B.D., Miller, D., Mockaitis, K., Roberts, J., Davis, C.A., Frise, E., Hammonds, A.S., Olson, S., Shenker, S., Sturgill, D., Samsonova, A.A., Weiszmann, R., Robinson, G., Hernandez, J., Andrews, J., Bickel, P.J., Carninci, P., Cherbas, P., Gingeras, T.R., Hoskins, R.A., Kaufman, T.C., Lai, E.C., Oliver, B., Perrimon, N., Graveley, B.R., Celniker, S.E., Supplementary, 2014. Diversity and dynamics of the *Drosophila* transcriptome. Nature 512, 393–399.

Carrard, G., Bulteau, A.L., Petropoulos, I., Friguet, B., 2002. Impairment of proteasome structure and function in aging. Int. J. Biochem. Cell Biol. 34, 1461–1474.

Chang, D.H., Angelin-Duclos, C., Calame, K., 2000. BLIMP-I: Trigger for differentiation of myeloid lineage. Nat. Immunol. 1, 169–176.

Chanut-Delalande, H., Hashimoto, Y., Pelissier-Monier, A., Spokony, R., Dib, A., Kondo, T., Bohère, J., Niimi, K., Latapie, Y., Inagaki, S., Dubois, L., Valenti, P., Polesello, C., Kobayashi, S., Moussian, B., White, K.P., Plaza, S., Kageyama, Y., Payre, F., 2014. Pri peptides are mediators of ecdysone for the temporal control of development. Nat. Cell Biol. 16, 1035–1044.

Chavoshi, T.M., Moussian, B., Uv, A., 2010. Tissue-autonomous EcR functions are required for concurrent organ morphogenesis in the *Drosophila* embryo. Mech. Dev.

Chondrogianni, N., Stratford, F.L.L., Trougakos, I.P., Friguet, B., Rivett, A.J., Gonos, E.S., 2003. Central role of the proteasome in senescence and survival of human fibroblasts. J. Biol. Chem. 278, 28026–28037.

Colombani, J., Raisin, S., Pantalacci, S., Radimerski, T., Montagne, J., Léopold, P., 2003. A nutrient sensor mechanism controls *Drosophila* growth. Cell.

Covi, J.A., Belote, J.M., Mykles, D.L., 1999. Subunit compositions and catalytic properties of proteasomes from developmental temperature-sensitive mutants of *Drosophila melanogaster*. Arch. Biochem. Biophys. 368, 85–97.

De Souza, F.S.J., Gawantka, V., Gómez, A.P., Delius, H., Ang, S.L., Niehrs, C., 1999. The zinc finger gene Xblimp1 controls anterior endomesodermal cell fate in Spemann's organizer. EMBO J.

Delon, I., Payre, F., 2004. Evolution of larval morphology in flies: Get in shape with shavenbaby. Trends Genet.

Duan, S., Cermak, L., Pagan, J.K., Rossi, M., Martinengo, C., Di Celle, P.F., Chapuy, B., Shipp, M., Chiarle, R., Pagano, M., 2012. FBXO11 targets BCL6 for degradation and is inactivated in diffuse large B-cell lymphomas. Nature 481, 90–94.

Fielenbach, N., Guardavaccaro, D., Neubert, K., Chan, T., Li, D., Feng, Q., Hutter, H., Pagano, M., Antebi, A., 2007. DRE-1: An Evolutionarily Conserved F Box Protein that Regulates C. elegans Developmental Age. Dev. Cell 12, 443–455.

Finley, D., 2009. Recognition and Processing of Ubiquitin-Protein Conjugates by the Proteasome. Annu. Rev. Biochem. 78, 477–513.

Fletcher, J.C., Thummel, C.S., 1995. The ecdysone-inducible *Broad-Complex* and *E74* early genes interact to regulate target gene transcription and *Drosophila* metamorphosis. Genetics 141, 1025–1035.

Fletcher, J.C., Thummel, C.S., 1995. The \textit{*Drosophila*} E74 gene is required for the proper stage-and tissue-specific transcription of ecdysone-regulated genes at the onset of metamorphosis. Development 121, 1411–1421.

Fortier, T.M., Vasa, P.P., Woodard, C.T., 2003. Orphan nuclear receptor ^{2}FTZ-F1 is required for muscle-driven morphogenetic events at the prepupal-pupal transition in *Drosophila melanogaster*. Dev. Biol. 257, 153–165.

Fredriksson, Å., Johansson Krogh, E., Hernebring, M., Pettersson, E., Javadi, A., Almstedt, A., Nyström, T., 2012. Effects of aging and reproduction on protein quality control in soma and gametes of *Drosophila melanogaster*. Aging Cell 11, 634–643.

Galindo, M.I., Pueyo, J.I., Fouix, S., Bishop, S.A., Couso, J.P., 2007. Peptides encoded by short ORFs control development and define a new eukaryotic gene family. PLoS Biol. 5, 1052–1062.

Géminard, C., Rulifson, E.J., Léopold, P., 2009. Remote Control of Insulin Secretion by Fat Cells in *Drosophila*. Cell Metab. 10, 199–207.

Gene, A.B.E., Intermolt, R., Guay, P.S., Guild, G.M., 1991. The Ecdysone-Induced Puffing Cascade in *Drosophila* Salivary Glands: A. Genetics.

Ghislain, M., Udvardy, A., Mann, C., 1993. S. cerevisiae 26S protease mutants arrest cell division in G2/metaphase. Nature 366, 358–362.

Glickman, M.H., Rubin, D.M., Coux, O., Wefes, I., Pfeifer, G., Cjeka, Z., Baumeister, W., Fried, V.A., Finley, D., 1998. A subcomplex of the proteasome regulatory particle required for ubiquitin-conjugate degradation and related to the COP9-signalosome and elF3. Cell.

Gong, J., Lv, L., Huo, J., 2014. Roles of F-box proteins in human digestive system tumors (review). Int. J. Oncol.

Gordon, C., McGurk, G., Dillon, P., Rosen, C., Hastie, N.D., 1993. Defective mitosis due to a mutation in the gene for a fission yeast 26S protease subunit. Nature 366, 355–357.

Graveley, B.R., Brooks, A.N., Carlson, J.W., Duff, M.O., Landolin, J.M., Yang, L., Artieri, C.G., van Baren, M.J., Boley, N., Booth, B.W., Brown, J.B., Cherbas, L., Davis, C.A., Dobin, A., Li, R., Lin, W., Malone, J.H., Mattiuzzo, N.R., Miller, D., Sturgill, D., Tuch, B.B., Zaleski, C., Zhang, D., Blanchette, M., Dudoit, S., Eads, B., Green, R.E., Hammonds, A., Jiang, L., Kapranov, P., Langton, L., Perrimon, N., Sandler, J.E., Wan, K.H., Willingham, A., Zhang, Y., Zou, Y., Andrews, J., Bickel, P.J., Brenner, S.E., Brent, M.R., Cherbas, P., Gingeras, T.R., Hoskins, R.A., Kaufman, T.C., Oliver, B., Celniker, S.E., 2011. The developmental transcriptome of \textit{*Drosophila*} *melanogaster*. Nature 471, 473–479.

Groll, M., Ditzel, L., Löwe, J., Stock, D., Bochtler, M., Bartunik, H.D., Huber, R., 1997. Structure of 20S proteasome from yeast at 2.4 Å resolution. Nature 386, 463–471.

Gyory, I., Wu, J., Fejér, G., Seto, E., Wright, K.L., 2004. PRDI-BF1 recruits the histone H3 methyltransferase G9a in transcriptional silencing. Nat. Immunol. 5, 299–308.

Hardisty-Hughes, R.E., Tateossian, H., Morse, S.A., Romero, M.R., Middleton, A., Tymowska-Lalanne, Z., Hunter, A.J., Cheeseman, M., Brown, S.D.M., 2006. A mutation in the F-box gene, Fbxo11, causes otitis media in the Jeff mouse. Hum. Mol. Genet. 15, 3273–3279.

Hernandez-Lagunas, L., Choi, I.F., Kaji, T., Simpson, P., Hershey, C., Zhou, Y., Zon, L., Mercola, M., Artinger, K.B., 2005. Zebrafish narrowminded disrupts the transcription factor prdm1 and is required for neural crest and sensory neuron specification. Dev. Biol.

Hiruma, K., Riddiford, L.M., 2001. Regulation of transcription factors MHR4 and betaFTZ-

F1 by 20-hydroxyecdysone during a larval molt in the tobacco hornworm, Manduca sexta. Dev. Biol.

Hochstrasser, M., 2004. Ubiquitin signalling: what's in a chain? Nat. Cell Biol. 6, 571–572.

Horn, M., Geisen, C., Cermak, L., Becker, B., Nakamura, S., Klein, C., Pagano, M., Antebi, A., 2014. DRE-1/FBXO11-dependent degradation of BLMP-1/BLIMP-1 governs C. elegans developmental timing and maturation. Dev. Cell 28, 697–710.

Horner, M.A., Thummel, C.S., 1997. Mutations in the DHR39 orphan receptor gene have no effect on viability. . D. I. S. 80, 35–37.

Horner MA, Chen T, T.C.S., 1995. Ecdysteroid regulation and DNA binding properties of *Drosophila* nuclear hormone receptor superfamily members. Dev. Biol. 168, 490–502.

Hoshizaki DK., 2005. Fat-cell development. In: Gilbert LI, Iatrou K, Gill S, editors. Comprehesive Molecular Insect Science. Oxford, Engl. Elsevier 2, 315–345.

Hosie, A.M., Wilkins, M.E., Da Silva, H.M.A., Smart, T.G., 2006. Endogenous neurosteroids regulate GABAAreceptors through two discrete transmembrane sites. Nature.

Inagaki, S., Numata, K., Kondo, T., Tomita, M., Yasuda, K., Kanai, A., Kageyama, Y., 2005. Identification and expression analysis of putative mRNA-like non-coding RNA in *Drosophila*. Genes to Cells 10, 1163–1173.

Jin, J., Cardozo, T., Lovering, R.C., Elledge, S.J., Pagano, M., Harper, J.W., 2004. Systematic analysis and nomenclature of mammalian F-box proteins. Genes Dev. 18, 2573–2580.

Kageyama, Y., Masuda, S., Hirose, S., Ueda, H., 1997. Temporal regulation of the mid-prepupal gene FTZ-F1: DHR3 early late gene product is one of the plural positive regulators. Genes Cells 2, 559–69.

Karim, F.D., Guild, G.M., Thummel, C.S., Thummel, C.S., 1993. The *Drosophila* Broad-Complex plays a key role in controlling ecdysone-regulated gene expression at the onset of metamorphosis. Development 118, 977–988.

Kastle, M., Grune, T., 2011. Protein Oxidative Modification in the Aging Organism and the Role of the Ubiquitin Proteasomal System. Curr. Pharm. Des. 17, 4007–4022.

Katoh, K., Omori, Y., Onishi, A., Sato, S., Kondo, M., Furukawa, T., 2010. Blimp1 Suppresses Chx10 Expression in Differentiating Retinal Photoreceptor Precursors to Ensure Proper Photoreceptor Development. J. Neurosci.

Kawasaki, H., Hirose, S., Ueda, H., 2002. βFTZ-F1 dependent and independent activation of Edg78E, a pupal cuticle gene, during the early metamorphic period in *Drosophila melanogaster*. Dev. Growth Differ.

Keller, a D., Maniatis, T., 1991. Identification and characterization of a novel repressor of beta-interferon gene expression. Genes Dev. 5, 868–879.

King-Jones, K., Charles, J.P., Lam, G., Thummel, C.S., 2005. The ecdysone-induced DHR4 orphan nuclear receptor coordinates growth and maturation in *Drosophila*. Cell.

Kiss, I., Beaton, A.H., Tardiff, J., Fristrom, D., Fristrom, J.W., 1988. Interactions and developmental effects of mutations in the Broad-Complex of *Drosophila melanogaster*. Genetics 118, 247–59.

Koelle, M.R., Talbot, W.S., Segraves, W. a, Bender, M.T., Cherbas, P., Hogness, D.S., 1991. The *Drosophila* EcR gene encodes an ecdysone receptor, a new member of the steroid receptor superfamily. Cell.

Kondo, T., Hashimoto, Y., Kato, K., Inagaki, S., Hayashi, S., Kageyama, Y., 2007. Small

peptide regulators of actin-based cell morphogenesis encoded by a polycistronic mRNA. Nat. Cell Biol. 9, 660–665.

Kondo, T., Plaza, S., Zanet, J., Benrabah, E., Valenti, P., Hashimoto, Y., Kobayashi, S., Payre, F., Kageyama, Y., 2010. Small peptides switch the transcriptional activity of shavenbaby during *Drosophila* embryogenesis. Science (80-.). 329, 336–339.

Kouzarides, T., 2002. Histone methylation in transcriptional control. Curr. Opin. Genet. Dev. 12, 198–209.

Kozlova, T., Thummel, C.S., 2003. Essential roles for ecdysone signaling during *Drosophila* mid-embryonic development. Science (80-.).

Lachaise, D., Cariou, M.-L., David, J.R., Lemeunier, F., Tsacas, L., Ashburner, M., 1988. Historical Biogeography of the *Drosophila melanogaster* Species Subgroup, Evolutionary Biology.

Lam, G., Hall, B.L., Bender, M., Thummel, C.S., 1999. DHR3 is required for the prepupal-pupal transition and differentiation of adult structures during *Drosophila* metamorphosis. Dev. Biol.

Lam, G.T., Jiang, C., Thummel, C.S., 1997. Coordination of larval and prepupal gene expression by the DHR3 orphan receptor during *Drosophila* metamorphosis. Development.

Lavorgna, G., Karim, F.D., Thummel, C.S., Wu, C., 1993. Potential role for a FTZ-F1 steroid receptor superfamily member in the control of *Drosophila* metamorphosis. Proc. Natl. Acad. Sci. U. S. A. 90, 3004–8.

Lavorgna, G., Ueda, H., Clos, J., Wu, C., 1991. FTZ-F1, a steroid hormone receptor-like protein implicated in the activation of fushi tarazu. Science (80-.). 252, 848–851.

Li, C., Kapitskaya, M.Z., Zhu, J., Miura, K., Segraves, W., Raikhel, A.S., 2000. Conserved molecular mechanism for the stage specificity of the mosquito vitellogenic response to ecdysone. Dev. Biol.

Liu, Y., Liu, H., Liu, S., Wang, S., Jiang, R.J., Li, S., 2009. Hormonal and nutritional regulation of insect fat body development and function. In: Archives of Insect Biochemistry and Physiology. pp. 16–30.

Marmorstein, R., 2003. Transcription initiation at its most basic level. Cell 115, 370–2.

Martins, G. a, Cimmino, L., Liao, J., Magnusdottir, E., Calame, K., 2008. Blimp-1 directly represses Il2 and the Il2 activator Fos, attenuating T cell proliferation and survival. J. Exp. Med.

Murata, T., Kageyama, Y., Hirose, S., Ueda, H., 1996. Regulation of the EDG84A gene by FTZ-F1 during metamorphosis in *Drosophila melanogaster*. Mol. Cell. Biol. 16, 6509–6515.

Navon, A., Ciechanover, A., 2009. The 26 S proteasome: From basic mechanisms to drug targeting. J. Biol. Chem. 284, 33713–33718.

Nelliot, A., Bond, N., Hoshizaki, D.K., 2006. Fat-body remodeling in *Drosophila melanogaster*. Genesis 44, 396–400.

Ng, T., Yu, F., Roy, S., 2006. A homologue of the vertebrate SET domain and zinc finger protein Blimp-1 regulates terminal differentiation of the tracheal system in the *Drosophila* embryo. Dev. Genes Evol.

Ohanian, L.E., 2008. Back to the Future with Keynes *. Elsevier 10–17.

Ohinata, Y., Payer, B., O'Carroll, D., Ancelin, K., Ono, Y., Sano, M., Barton, S.C., Obukhanych, T., Nussenzweig, M., Tarakhovsky, A., Saitou, M., Surani, M.A., 2005. Blimp1 is a critical determinant of the germ cell lineage in mice. Nature 436, 207–213.

Ohno, C.K., Petkovich, M., 1993. FTZ-F1 beta, a novel member of the *Drosophila* nuclear receptor family. Mech. Dev. 40, 13–24.

Öztürk-Çolak, A., Moussian, B., Araújo, S.J., 2016. *Drosophila* chitinous aECM and its cellular interactions during tracheal development. Dev. Dyn.

Petroski, M.D., Deshaies, R.J., 2005. Function and Regulation of Cullin-Ring Ubiquitin Ligases. Nat. Rev. | Mol. Cell Biol. 6.

Pi, H., Huang, Y.C., Chen, I.C., Lin, C. De, Yeh, H.F., Pai, L.M., 2011. Identification of 11-amino acid peptides that disrupt Notch-mediated processes in *Drosophila*. J. Biomed. Sci. 18.

Pickart, C.M., 2001. Mechanisms Underlying Ubiquitination. Annu. Rev. Biochem. 70, 503–533.

Pickart, C.M., 2004. Back to the Future with Ubiquitin. Cell 116, 181–190.

Price, M.H., Roberts, D.M., McCartney, B.M., Jezuit, E., Peifer, M., 2006. Cytoskeletal dynamics and cell signaling during planar polarity establishment in the Drosophila embryonic denticle. J. Cell Sci.

Pueyo, J.I., Couso, J.P., 2008. The 11-aminoacid long Tarsal-less peptides trigger a cell signal in *Drosophila* leg development. Dev. Biol. 324, 192–201.

Ren, B., Chee, K.J., Kim, T.H., Maniatis, T., 1999. PRDI-BF1/Blimp-1 repression is mediated by corepressors of the Groucho family of proteins. Genes Dev. 13, 125–137.

Rewitz, K.F., Yamanaka, N., O'Connor, M.B., 2013. Developmental Checkpoints and Feedback Circuits Time Insect Maturation. Curr. Top. Dev. Biol.

Richards, 1976. The in vitro induction of puffing in salivary glands of the mutant l(2)gl of *Drosophila melanogaster* by ecdysone. Roux Arch. dev. Biol. 179, 339–348.

Richards, G.P., Richards, 1976. The control of prepupal puffing patterns in vitro; Implications for prepupal ecdysone titres in Drosophilia *melanogaster*. Dev. Biol. 48, 191–195.

Riddiford, L.M., Cherbas, P., Truman, J.W., 2000. Ecdysone Receptors and Their Biological Actions. Vitam. Horm.

Riddiford, L.M., Truman, J.W., 1993. Hormone receptors and the regulation of insect metamorphosis. Integr. Comp. Biol. 33, 340–347.

Robertson, C.W., 1936. The metamorphosis of *Drosophila melanogaster*, including an accurately timed account of the principal morphological changes. J. Morphol. 59, 351–399.

Rock, K.L., Gramm, C., Rothstein, L., Clark, K., Stein, R., Dick, L., Hwang, D., Goldberg, A.L., 1994. Inhibitors of the proteasome block the degradation of most cell proteins and the generation of peptides presented on MHC class I molecules. Cell 78, 761–771.

Rubin, D.M., Glickman, M.H., Larsen, C.N., Dhruvakumar, S., Finley, D., 1998. Active site mutants in the six regulatory particle ATPases reveal multiple roles for ATP in the proteasome. EMBO J. 17, 4909–4919.

Russell, S.R.H., Heimbeck, G., Goddard, C.M., Carpenter, A.T.C., Ashburner, M., 1996. The *Drosophila* Eip78C gene is not vital but has a role in regulating chromosome puffs. Genetics 144, 159–170.

Savard, J., Marques-Souza, H., Aranda, M., Tautz, D., 2006. A Segmentation Gene in Tribolium Produces a Polycistronic mRNA that Codes for Multiple Conserved Peptides. Cell 126, 559–569.

Saville, K.J., Belote, J.M., 1993. Identification of an essential gene, l(3)73Ai, with a dominant temperature-sensitive lethal allele, encoding a *Drosophila* proteasome subunit. Pnas 90, 8842–8846.

Savitsky, D., Calame, K., 2006. B-1 B lymphocytes require Blimp-1 for immunoglobulin secretion. J. Exp. Med.

Sciammas, R., Davis, M.M., 2005. Blimp-1; immunoglobulin secretion and the switch to plasma cells. Curr. Top. Microbiol. Immunol. 290, 201–24.

Sciammas, R., Shaffer, A.L., Schatz, J.H., Zhao, H., Staudt, L.M., Singh, H., 2006. Graded Expression of Interferon Regulatory Factor-4 Coordinates Isotype Switching with Plasma Cell Differentiation. Immunity.

Segraves, W.A., Hogness, D.S., 1990. The E75 ecdysone-inducible gene responsible for the 75B early puff in *Drosophila* encodes two new members of the steroid receptor superfamily. Genes Dev.

Shaffer, A.L., Lin, K.I., Kuo, T.C., Yu, X., Hurt, E.M., Rosenwald, A., Giltnane, J.M., Yang, L., Zhao, H., Calame, K., Staudt, L.M., 2002. Blimp-1 orchestrates plasma cell differentiation by extinguishing the mature B cell gene expression program. Immunity 17, 51–62.

Shi, Z., Goulle, V., Thummel, R.P., 1996. An aza-analogue of TTF1: 1,1′;3,3′-bistrimethylene-2,2′-diimidazolinylidine. Tetrahedron Lett.

Skaar, J.R., D'Angiolella, V., Pagan, J.K., Pagano, M., 2009. SnapShot: F Box Proteins II. Cell 137.

Smyth, K.A., Belote, J.M., 1999. The dominant temperature-sensitive lethal DTS7 of *Drosophila melanogaster* encodes an altered 20S proteasome ??-type subunit. Genetics.

Søndergaard, L., 1993. Homology between the mammalian liver and the *Drosophila* fat body. Trends Genet. 9, 193.

Stern, D.L., Franke, N., 2013. The structure and evolution of cisregulatory regions: The shavenbaby story. Philos. Trans. R. Soc. B Biol. Sci. 368.

Su, S.-T., Ying, H.-Y., Chiu, Y.-K., Lin, F.-R., Chen, M.-Y., Lin, K.-I., 2009. Involvement of Histone Demethylase LSD1 in Blimp-1-Mediated Gene Repression during Plasma Cell Differentiation. Mol. Cell. Biol. 29, 1421–1431.

Sullivan, A. a, Thummel, C.S., 2003. Temporal profiles of nuclear receptor gene expression reveal coordinate transcriptional responses during *Drosophila* development. Mol. Endocrinol.

Sultan, A.R.S., Oish, Y., Ueda, H., 2014. Function of the nuclear receptor FTZ-F1 during the pupal stage in *Drosophila melanogaster*. Dev. Growth Differ. 56, 245–253.

Sun, G.C., Hirose, S., Ueda, H., 1994. Intermittent expression of BmFTZ-F1, a member of the nuclear hormone receptor superfamily during development of the silkworm Bombyx mori. Dev. Biol. 162, 426–437.

Szlanka, T., 2003. Deletion of proteasomal subunit S5a/Rpn10/p54 causes lethality, multiple mitotic defects and overexpression of proteasomal genes in *Drosophila melanogaster*. J. Cell Sci. 116, 1023–1033.

Thompson, J.N., Ashburner, M., Woodruff, R.C., 1977. Presumptive control mutation for alcohol dehydrogenase in *Drosophila melanogaster*. Nature 270, 363.

Thummel, C.S., 1996. Flies on steroids - *Drosophila* metamorphosis and the mechanisms of steroid hormone action. Trends Genet. 12, 306–310.

Thummel, C.S., Burtis, K.C., Hogness, D.S., 1990. Spatial and temporal patterns of E74 transcription during *Drosophila* development. Cell.

Tonoki, A., Kuranaga, E., Tomioka, T., Hamazaki, J., Murata, S., Tanaka, K., Miura, M., 2009. Genetic Evidence Linking Age-Dependent Attenuation of the 26S Proteasome with the Aging Process. Mol. Cell. Biol. 29, 1095–1106.

Tsakiri, E.N., Sykiotis, G.P., Papassideri, I.S., Gorgoulis, V.G., Bohmann, D., Trougakos, I.P., 2013. Differential regulation of proteasome functionality in reproductive vs. somatic tissues of *Drosophila* during aging or oxidative stress. FASEB J. 27, 2407–2420.

Tupy, J.L., Bailey, A.M., Dailey, G., Evans-Holm, M., Siebel, C.W., Misra, S., Celniker, S.E., Rubin, G.M., 2005. Identification of putative noncoding polyadenylated transcripts in *Drosophila melanogaster*. Proc. Natl. Acad. Sci. 102, 5495–5500.

Turner, C.A., Mack, D.H., Davis, M.M., 1994. Blimp-1, a novel zinc finger-containing protein that can drive the maturation of B lymphocytes into immunoglobulin-secreting cells. Cell.

Ueda, H., Sonoda, S., Brown, J.L., Scott, M.P., Wu, C., 1990. A sequence-specific DNA-binding protein that activates fushi tarazu segmentation gene expression. Genes Dev. 4, 624–635.

Ulrich, H.D., 2002. Natural substrates of the proteasome and their recognition by the ubiquitin system. Curr. Top. Microbiol. Immunol. 268, 137–74.

Unno, M., Mizushima, T., Morimoto, Y., Tomisugi, Y., Tanaka, K., Yasuoka, N., Tsukihara, T., 2002. The structure of the mammalian 20S proteasome at 2.75 Å resolution. Structure 10, 609–618.

Verma, R., Aravind, L., Oania, R., McDonald, W.H., Yates, J.R., Koonin, E. V., Deshaies, R.J., 2002. Role of Rpn11 metalloprotease in deubiquitination and degradation by the 26S proteasome. Science (80-.). 298, 611–615.

Vincent, S.D., Dunn, N.R., Sciammas, R., Shapiro-Shalef, M., Davis, M.M., Calame, K., Bikoff, E.K., Robertson, E.J., 2005. The zinc finger transcriptional repressor Blimp1/Prdm1 is dispensable for early axis formation but is required for specification of primordial germ cells in the mouse. Development 132, 1315–1325.

von Arnim, A.G., 2001. A Hitchhiker's Guide to the Proteasome. Sci. Signal. 2001, pe2-pe2.

Walters, J.W., Dilks, S.A., DiNardo, S., 2006. Planar polarization of the denticle field in the Drosophila embryo: Roles for Myosin II (Zipper) and Fringe. Dev. Biol.

Warren, J.T., Yerushalmi, Y., Shimell, M.J., O'Connor, M.B., Restifo, L.L., Gilbert, L.I., 2006. Discrete pulses of molting hormone, 20-hydroxyecdysone, during late larval development of *Drosophila melanogaster*: Correlations with changes in gene activity. Dev. Dyn.

White, K.P., Hurban, P., Watanabe, T., Hogness, D.S., 1997. Coordination of *Drosophila* metamorphosis by two ecdysone-induced nuclear receptors. Science (80-.).

Wilm, T.P., Solnica-Krezel, L., 2005. Essential roles of a zebrafish prdm1/blimp1 homolog in embryo patterning and organogenesis. Development 132, 393–404.

Woodard, C.T., Baehrecke, E.H., Thummel, C.S., 1994. A molecular mechanism for the stage specificity of the *Drosophila* prepupal genetic response to ecdysone. Cell.

Wright, L.G., Chen, T., Thummel, C.S., Guild, G.M., 1996. Molecular characterization of the 71E late puff in *Drosophila melanogaster* reveals a family of novel genes. J. Mol. Biol. 255, 387–400.

Yamada, M., Murata, T., Hirose, S., Lavorgna, G., Suzuki, E., Ueda, H., 2000. Temporally restricted expression of transcription factor βFTZ-F1: significance for embryogenesis, molting and metamorphosis in *Drosophila melanogaster*. Development 127, 5083–92.

Yan, J., Jiang, J., Lim, C.A., Wu, Q., Ng, H.-H., Chin, K.-C., 2007. BLIMP1 regulates cell growth through repression of p53 transcription. Proc. Natl. Acad. Sci. U. S. A.

Yao, T., Cohen, R.E., 2002. A cryptic protease couples deubiquitination and degradation by the proteasome. Nature 419, 403–407.

Yao, T.P., Forman, B.M., Jiang, Z., Cherbas, L., Chen, J.D., McKeown, M., Cherbas, P., Evans, R.M., 1993. Functional ecdysone receptor is the product of EcR and Ultraspiracle genes. Nature.

Yu, J., Angelin-Duclos, C., Greenwood, J., Liao, J., Calame, K., 2000. Transcriptional Repression by Blimp-1 (PRDI-BF1) Involves Recruitment of Histone Deacetylase. Mol. Cell. Biol. 20, 2592–2603.

Zanet, J., Benrabah, E., Li, T., Pélissier-Monier, A., Chanut-Delalande, H., Ronsin, B., Bellen, H.J., Payre, F., Plaza, S., 2015. Pri sORF peptides induce selective proteasome-mediated protein processing. Science (80-.).

Zirin, J., Cheng, D., Dhanyasi, N., Cho, J., Dura, J.M., VijayRaghavan, K., Perrimon, N., 2013. Ecdysone signaling at metamorphosis triggers apoptosis of *Drosophila* abdominal muscles. Dev. Biol.

Zwickl, P., Voges, D., Baumeister, W., 1999. The proteasome: A macromolecular assembly designed for controlled proteolysis, Philosophical Transactions of the Royal Society B: Biological Sciences.