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ßFTZ-F1 and Matrix metalloproteinase 2 are required for fat-body remodeling in *Drosophila*

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ABSTRACT

During metamorphosis, holometabolous insects eliminate obsolete larval tissues via programmed cell death. In contrast, tissues required for further development are retained and often remodeled to meet the needs of the adult fly. The larval fat body is involved in fueling metamorphosis, and thus it escapes cell death and is instead remodeled during prepupal development. The molecular mechanisms by which the fat body escapes programmed cell death have not yet been described, but it has been established that fat-body remodeling requires 20-hydroxyecdysone (20E) signaling. We have determined that 20E signaling is required within the fat body for the cell-shape changes and cell detachment that are characteristic of fat-body remodeling. We demonstrate that the nuclear hormone receptor ßFTZ-F1 is a key modulator of 20E hormonal induction of fat body remodeling and Matrix metalloproteinase 2 (MMP2) expression in the fat body. We show that induction of MMP2 expression in the fat body requires 20E signaling, and that MMP2 is necessary and sufficient to induce fat-body remodeling.

Introduction

In holometabolous insects, dramatic changes in animal form occur as the larva undergoes a complete transformation during metamorphosis to give rise to the adult. This transformation is accomplished by the destruction of larval tissues and the proliferation, differentiation, and organogenesis of the adult tissues (Bainbridge and Bownes, 1981; Bodenstein, 1950; Robertson, 1936). Unlike the majority of larval tissues, the larval fat body of *Dipterans*, as exemplified by *Drosophila melanogaster*, is refractory to cell death. Early in metamorphosis the larval fat body is transformed from sheets of attached, polygonal-cells to individual spherical, free-floating cells (Hoshizaki, 2005; Nelliot et al., 2006). These remodeled larval fat cells persist into the adult (Aguila et al., 2007; Butterworth, 1972; Hoshizaki, 2005) and can serve as a nutrient reservoir (Aguila et al., 2007).

Metamorphosis is developmentally regulated by the steroid hormone 20-hydroxyecdysone (20E). 20E binds to the ecdysone receptor, which is a heterodimer composed of two nuclear receptors, EcR and Ultraspiracle (Koelle et al., 1991; Thomas et al., 1993; Yao et al., 1993; see Costantino et al., 2008 for exceptions). The ecdysone receptor mediates gene expression by initiating tissue-specific transcriptional cascades that result in distinct stage-specific developmental changes (Riddiford, 1996; Thummel, 1995). Late in the third-larval instar, an increase in the 20E titer directly induces transcription of a set of primary response genes called the "early" genes, which are necessary for initiation of metamorphosis (i.e., pupariation) and formation of the puparium (Burtis et al., 1990; DiBello et al., 1991; Richards, 1981; Riddiford and Truman, 1993; Segraves and Hogness, 1990;). Pupariation is followed by prepupal development, which in turn is followed by pupal development. The early gene products repress their own expression and induce the subsequent transcription of a set of secondary response genes called the "late" genes in the prepupa (Thummel, 1996). As the ecdysone pulse that initiated pupariation declines, the mid-prepupal genes are induced (Thummel, 1996). One such mid-prepupal gene encodes the competence factor ßFTZ-F1. ßFTZ-F1 is a nuclear receptor and is required to confer competence upon tissues to respond to the prepupal pulse of 20E that occurs approximately 10 h after puparium formation (APF). ßFTZ-F1 and the prepupal pulse of 20E induce transcriptional cascades necessary for the prepupal to pupal transition (Broadus et al., 1999; Fortier et al., 2003; Woodard et al., 1994; Yamada et al., 2000; Reviewed in Pick et al., 2006).

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The temporal control of *ßftz-f1* expression is critical to its function as a competence factor. *ßftz-f1* transcription depends upon the decline in 20E titer that occurs following puparium formation (Lavorgna et al., 1993; Rewitz et al., 2010; Woodard et al., 1994). *ßftz-f1* expression is regulated, in part, by induction via the 20E-induced proteins, DHR3 and DHR4 (Kageyama et al., 1997; King-Jones and Thummel, 2005; Lam et al., 1997, 1999; Ruaud et al., 2010; White et al., 1997) and by the repressive effects of the 20E-induced protein, dBlimp-1, the *Drosophila* homolog of mammalian B lymphocyte-induced maturation protein-1 (Agawa et al., 2007). dBlimp-1 is a rapidly degraded protein that is expressed in response to the late larval (pupariation) pulse of 20E and acts as a transcriptional repressor by direct binding to the *ßftz-f1* promoter (Agawa et al., 2007). The transient nature of the dBlimp-1 protein helps to ensure tight regulation of *ßftz-f1* expression. Thus, the presence of ßFTZ-F1 protein modifies the transcriptional program in response to the prepupal 20E pulse to specify the genetic events characteristic of pupal development, e.g. head eversion, leg and wing extension, and larval salivary gland cell death (Broadus et al., 1999; Fortier et al., 2003; Yamada et al., 2000; Reviewed in Pick et al., 2006).

Many tissue-specific events such as tissue remodeling and cell death are dependent upon 20E signaling (Cherbas et al., 2003; Jiang et al., 2000; Lee et al., 2002, 2003; Oberlander, 1976; Schubiger et al., 1998; Yin and Thummel, 2005). It is likely that aspects of fat-body remodeling might also be developmentally regulated by 20E because fat-body cell detachment characteristic of remodeling occurs at the prepupal to pupal transition (Hoshizaki, 2005; Nelliot et al., 2006) and disruption of 20E signaling in the larval fat body results in aggregates of non-dissociated fat cells (Cherbas et al., 2003). The downstream targets of 20E signaling responsible for specifying the developmental decision to remodel the fat body remain obscure. However, remodeling of the larval fat body presumably requires destruction of the extracellular matrix (ECM) used to maintain tissue integrity. A specialized class of proteases, the matrix metalloproteinases (MMPs), is involved in the degradation of the ECM and is required for remodeling of tissues in mammals and in *D. melanogaster* (Page-McCaw, 2008; Page-McCaw et al., 2003, 2007). Thus, the MMPs are excellent candidate enzymes for fat-body remodeling. In general, MMPs cleave components of the ECM such as collagen and laminin (Page-McCaw, 2007). Cleavage of ECM components can clear space between cells and thus allow cell mobility (Sternlicht and Werb, 2001). MMPs can also cleave signaling molecules residing within the ECM. *D. melanogaster* has two MMPs, MMP1 and MMP2 (Llano et al., 2000, 2002; Page-McCaw et al., 2003). MMP1 is a secreted protein, and MMP2 has a GPI anchor and is membrane associated (Llano et al., 2002; Page-McCaw et al., 2003). The two *D. melanogaster* MMPs have the canonical MMP structure but are not orthologs of any of the 24 mammalian MMPs (Page-McCaw et al., 2003). *MMP1* and *MMP2* are each required for distinct aspects of tissue remodeling and programmed cell-death during metamorphosis (Page-McCaw et al., 2003). *MMP1* expression coincides with the pupariation pulse of 20E in the salivary glands, thus *MMP1* might participate in the 20E-mediated destruction of the salivary glands (Lee et al., 2003; Page-McCaw, 2008).

To gain a better understanding of the genetic control of fat-body remodeling, we carried out a detailed study of fat-body remodeling *in vivo* and in *ex vivo* organ culture and tested the role of *ßftz-f1* as a key regulator of this process. We demonstrated that *ßftz-f1* was sufficient to induce fat-body remodeling in the presence of 20E and fat-body remodeling was a tissue-autonomous process. We identified *MMP2* as a potential downstream target of the ßFTZ-F1-mediated, 20E-signaling cascade and demonstrated that MMP2 was both necessary and sufficient for fat-body remodeling. Finally, we propose a model for the tissue autonomous action of MMP2 and outline the ßFTZ-F1-modulated 20E-signaling cascade required for fat-body remodeling.

Materials and methods

Fly stocks

To visualize fat-body remodeling, a *UAS-GFPgap*; *Lsp2-Gal4* stock was generated. This stock directed expression of the GFPgap marker specifically in the fat body. The *UAS-GFPgap* stock (referred to in the text as *UAS-GFP*) was provided by the *Drosophila* Stock Center, Bloomington IN. The *UAS-EcR-DN* (*UAS-EcR-F645A*), *UAS-ßftz-f1* (LA276), *UAS-dBlimp1* lines (*UAS-dBlimp-1 RB*, *UAS-dBlimp-1 WC*, *UAS-dBlimp-1 XA*), and *FRT2A*, *ftz-f1*^{19}/*TM3*, *Sb* were generously provided by L. Cherbas, J. Merriam, G. Call, and L. Pick respectively. *ßftz-f1* RNAi lines (*UAS-ßftz-f1RNAi #37649*, *UAS-ßftz-f1RNAi #37695*) were provided by the Vienna *Drosophila* RNAi Center with an additional line provided by K. Cho. *UAS-MMP1*, *UAS-MMP2*, *UAS-Timp*, *UAS-MMP1-DN*, *MMP1*^{W439}/*CyO*, *arm-GFP*, *MMP1*^{Q273}/*CyO*, *arm-GFP*, *MMP1-2*/*CyO*, *arm-GFP*, *MMP2*^{W307}/*CyO*, *arm-GFP*, and *MMP2Df*/*CyO*, *arm-GFP* were all generously provided by A. Page-McCaw. Homozygous *MMP1* and *MMP2* mutants were selected by the absence of GFP, as were the mutant progeny from the cross of: and *MMP2*^{W307}/*CyO*, *arm-GFP* crossed to *MMP2Df*/*CyO*, *arm-GFP*.

Microscopy and imaging

Staged animals were collected as white prepupae, placed on wet filter paper in a Petri dish at 25 °C, aged appropriately, then rinsed in deionized water, and mounted on bridged slides in Gel mount (Biomedia). Both fluorescence and confocal imaging were carried out in the University of Nevada, Las Vegas (UNLV) School of Life Sciences Imaging Center using a Zeiss Axioplan 2 microscope. Fluorescence images were captured with the Zeiss Axiocam using the Zeiss Axiovision software. LSM 510 software was used to procure the confocal images. All images were complied in Corel Draw®.

Time-lapse imaging of fat-body remodeling was carried out at Mount Holyoke College. Fluorescent images were captured using a Zeiss Axiocam 2 epifluorescence compound microscope. Animals were placed in a moist chamber, with their dorsal sides closest to the objective lens. The imaging software MetaVue™ was used to capture images of the fat body using a 4× objective. MetaVue™ was also used to record time-lapse movies of fat-body remodeling. Time-lapse movies were captured at a rate of 1 frame per minute over a 6 hour period. Still images were compiled in Corel Draw®.

Ex vivo organ culture

Fat bodies from late third-instar larvae of either the genotype *UAS-GFP*; *Lsp2-Gal4*, or *UAS-GFP*; *Lsp2-Gal4*/*UAS-ßftz-f1* were dissected in 1X DPBS (52 mM l^{-1} NaCl; 40 mM l^{-1} KCl; 10 mM l^{-1} Hepes; 1.2 mM l^{-1} $MgSO_4$; 1.2 mM l^{-1} $MgCl_2$; 2 mM l^{-1} Na_2HPO_4; 0.4 mM l^{-1} KH_2PO_4; 1 mM l^{-1} $CaCl_2$; 45 mM l^{-1} sucrose; 5 mM l^{-1} glucose, pH 7.2) and placed in 200 µl of Schneider Media (Sigma) in tissue-culture chambers at 25 °C. 20-hydroxyecdysone (20E) (Sigma) was diluted to a 10^{-3} M solution in 100% ethanol. A final working solution of 10^{-5} M ecdysone was made by dilution into the Schneider Media. Dissected fat bodies were incubated for 8 h with or without 10^{-5} M 20E and imaged using the methods described above. The experiment was repeated with animals not expressing GFP (*Lsp2-Gal4*/*UAS-ßftz-f1*). Fat body explants from *Lsp2-Gal4*/*UAS-ßftz-f1* animals were stained with Sytox® Green Live/Dead Assay (Invitrogen) according to the manufacturer's instructions and were also imaged as described above.

RNA isolation and cDNA synthesis

To stage 3rd instar larvae, animals were maintained at 25 °C on media containing 0.05% bromophenol blue. Larvae were collected at

two different stages: −8 h APF and −4 h APF, as determined by the intensity of blue color in the gut (Andres and Thummel, 1994). For staging prepupae and pupae, animals were collected at pupariation, placed on moist filter paper in a Petri dish, and aged (8 to 14 h APF) at 25 °C. 4 to 5 aged animals were dissected and the fat bodies were placed in 30 µl of PBS. 300 µl of TriZol® (Life Technology) was then added to the tubes containing fat bodies and PBS and the tissue was homogenized. The sample was then transferred to a 2 ml Phase Lock Gel™ Heavy microfuge tube (Eppendorf/5 Prime), and centrifuged at 12,000 rpm for 10 min at 4 °C. After centrifugation, the aqueous phase was transferred to a new tube and 160 µl of isopropanol was added. RNA was allowed to precipitate overnight at −20 °C. The precipitated sample was centrifuged at 12,000 rpm for 30 min. After centrifugation, the supernatant was removed by pipette. The remaining pellet was washed with 500 µl of 75% ethanol, centrifuged for 10 min and the supernatant was removed by pipette. The pellet was air dried and resuspended in 5 µl of RNase free water. RNA concentration was determined by spectrophotometry. cDNA was synthesized from the RNA samples using the Invitrogen® First-Strand cDNA synthesis kit.

Quantitative RT-PCR

Primers were synthesized by IDT (Integrated DNA Technologies) and designed from sequences from Flybase using the program on the IDT website (www.idtdna.com).

Sequences:

Actin 5C

Forward: 5′-TCTACGAGGGTTATGCCCTT-3′

Reverse: 5′-GCACAGCTTCTCCTTGATGT-3′

MMP2

Forward: 5′-AGCAATCCGGAGTCTCCAGTCTTT-3′

Reverse: 5′-TGGAGCCGATTTCGTGATACAGGT-3′

qRT-PCR was performed using PerfeCta™ SYBR® Green Supermix, ROX (Quanta Biosciences) according to the manufacturer's instructions on an Applied Biosystems cycler using the following program: *MMP2* expression: 95 °C 2 min, 40 cycles of 95 °C for 15 s, 58.2 °C for 30 s, and 72 °C for 30 s, and 1 cycle of standard melt curve at the end of the program. Primer titrations and standard curves were generated by qRT-PCR to test primer efficacy. *MMP2* expression was normalized to *Actin 5C* and the relative expression of *MMP2* in experimental samples (*UAS-EcR-DN/Lsp2-Gal4* or *Lsp2-Gal4/UAS-ßftz-f1*) was compared to the relative expression of *MMP2* in control samples (w^{1118}) of the same stage. Three biological replicates were used from each genotype and stage, and the fold change in expression was calculated using the PFAFFL method (Pfaffl, 2001). Fold change values were log transformed prior to statistical analysis which was conducted using Statistica 7 (Staff Soft, Inc., Tulsa Oklahoma, USA).

MARCM

ftz-f1^{19} is a null allele of *ßftz-f1* (Pick et al., 2006). *Progeny* from *yw hs-flp*; *cg-Gal4*, *UAS-GFP/CyO*; *FRT2A*, *tubulin-gal80/TM6B*, *Tb* females crossed to *yw hs-flp*; *FRT2A*, *ftz-f1*^{19}*/TM6B*, *Tb* males were heat shocked at 37 °C for 30 min 2 to 4 h after egg lay to induce FLP recombinase and generate clones of *ßftz-f1* mutant fat cells expressing GFP. These animals were placed at 25 °C after heat shock. After head eversion, animals clonally expressing GFP were selected and imaged as described above.

Results

Fat-body maturation during pupal development

The remodeling of the larval fat body takes place during early metamorphosis and is divided into three stages: retraction, disaggregation, and detachment (Nelliot et al., 2006). The final step, detachment, is associated with the prepupal to pupal transition and the translocation of individual fat cells into the head capsule and redistribution of cells in the body cavity. To develop a more detailed description of the final steps of fat-body remodeling, we followed larval fat cells marked with green fluorescent protein (GFP) using time-lapse imaging (see supplementary movie). Fat-cell detachment and

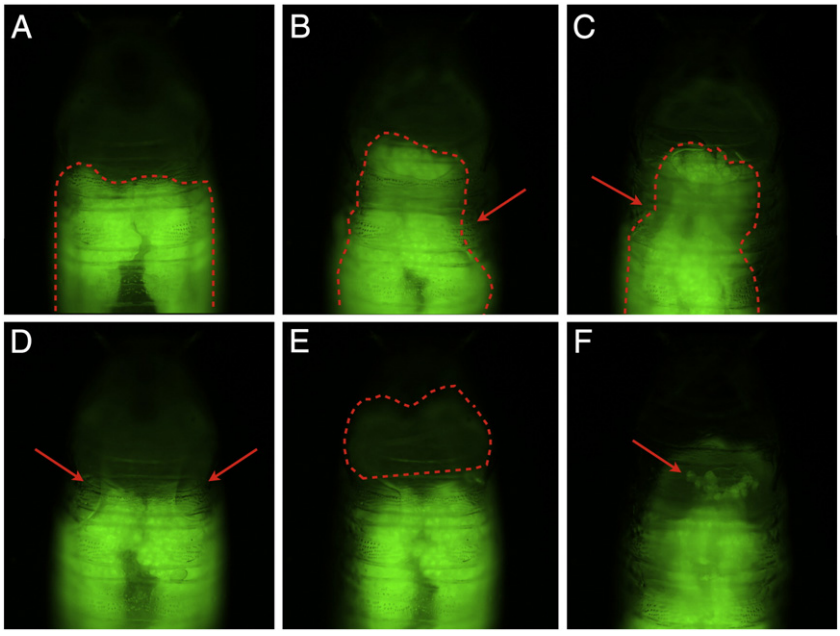

Fig. 1. Still images from time-lapse movie of fat-body remodeling. A whole mount animal expressing GFP in the fat body (*UAS-GFP*; *Lsp2-Gal4*) was collected as a prepupa and imaged during the process of head eversion and subsequent translocation of fat cells into the head capsule. (A) The intact fat body prior to head eversion and remodeling (dotted line outlines the fat body). (B–D). Ecdysis triggered body contractions (B and C, arrow indicates direction of body movement during time-lapse) and translocation of the gas bubble (D, arrows indicate placement of the gas bubble in the animal) (dotted line outlines the fat body). (E) Head eversion occurs shortly after gas bubble translocation (dotted line outlines the head capsule). (F) After the head has everted, the remodeled fat cells are pushed into the head capsule by muscular contractions (detached fat cell indicated by arrow) (see supplementary movie).

translocation into the head capsule occurred during the prepupal to pupal transition (Fig. 1E, F; Nelliot et al., 2006), a time period which is also known as pupal ecdysis (Kim et al., 2006). This transition was characterized by small contractions of the body (Fig. 1B, C) preceding the translocation of a gas bubble (Fig. 1D). Seconds after the gas bubble appeared in the anterior portion of the animal, strong abdominal contractions resulted in head eversion (Fig. 1E). This developmental event marks the beginning of pupal development. Immediately after head eversion, small contractions pushed individual remodeled fat cells into the head capsule (Fig. 1F). Translocation of the fat cells into the head capsule was completed within 43 min of head eversion (+/−2.5 min at 23 °C).

The role of 20E signaling in fat-body remodeling

Blocking 20E signaling in the fat body results in clumps of non-dissociated cells in the pupa (Cherbas et al., 2003), thus 20E signaling is required for some aspect of fat-body remodeling. To understand the role of 20E signaling in specific stages of fat-body remodeling, a dominant-negative form of *EcR*, (EcR^{F645A}) was expressed in larval fat body by expression of *UAS-*EcR^{F645A} (herein referred to as *UAS-EcR-DN*) driven by *Lsp2-Gal4*. We did a detailed analysis of fat-body remodeling by following the changes in gross fat-body morphology and fat-cell shape during the three stages of remodeling (Fig. 2).

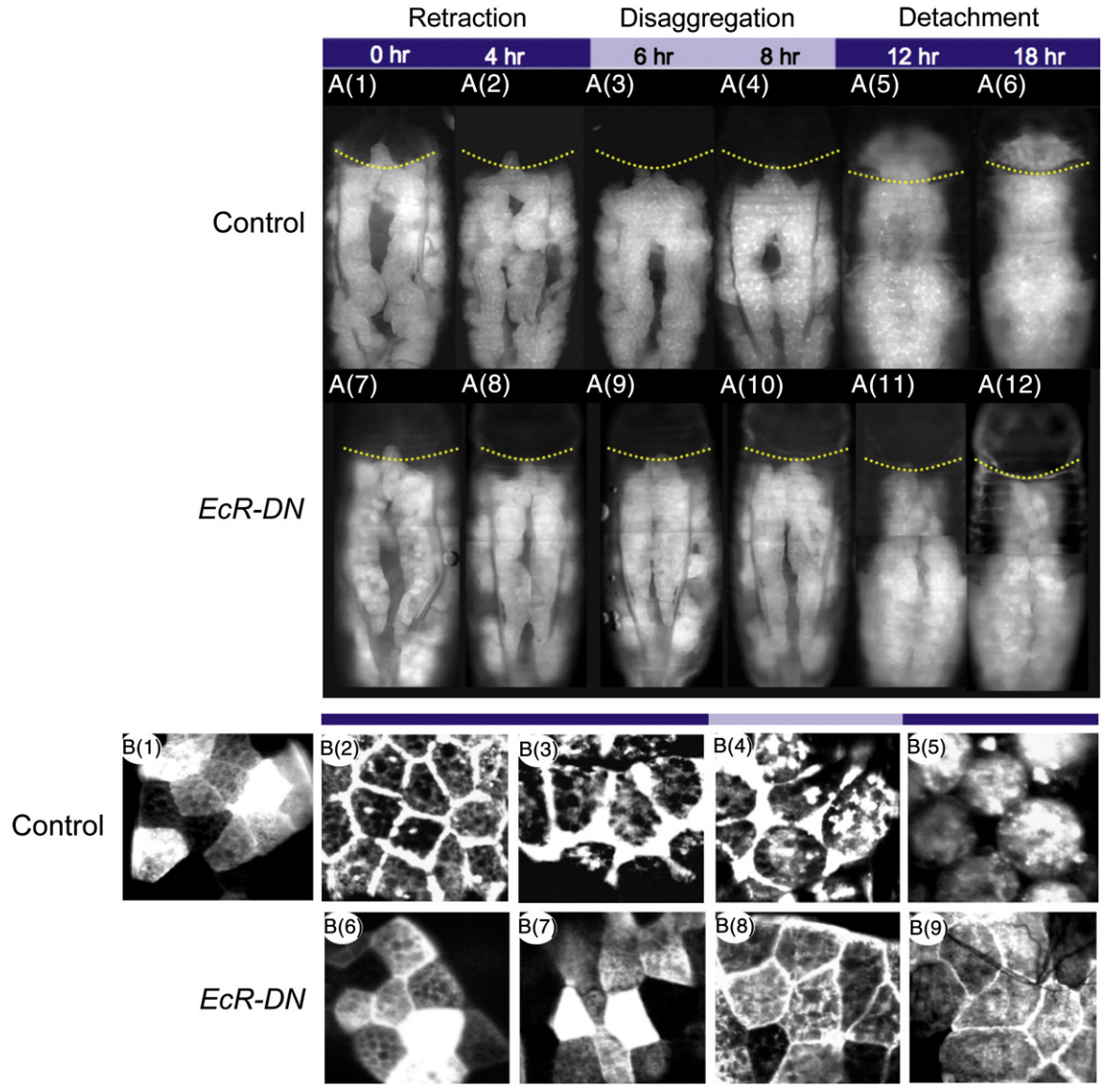

Fig. 2. Fat-body disaggregation and detachment requires 20E signaling. (A). Fluorescent images of whole mount animals expressing GFP in the fat body, dotted line marks the operculum. A(1–6): Control animals, fat body is marked by GFP (*UAS-GFP*; *Lsp2-Gal4*). A(7–12): Experimental animals, fat body is marked by GFP and 20E signaling is disrupted by expression of a dominant-negative (DN) form of EcR (*UAS-EcR-DN*, *UAS-GFP*; *Lsp2-Gal4*). A(1–2) and A(7–8): The retraction of the fat body to the level of the operculum is not disrupted when 20E signaling is disrupted in the fat body. A(3–4) and A(9–10): In the disaggregation phase, fat-body cells do not change shape when 20E signaling is disrupted in the fat body (also see Fig. 2 B8). A(5–6) and A(11–12): In the detachment phase, the fat cells do not separate and translocate into the head capsule when 20E signaling is disrupted in the fat body. Developmental time points marked in hours after pupariation. (B). Confocal images of fat-body cells expressing GFP. B(1–5): Fat body from control animals, fat body is marked by GFP (*UAS-GFP*; *Lsp2-Gal4*). B(6–9): Fat body from experimental animals, fat body is marked by GFP and 20E signaling is disrupted by expression of a dominant-negative (DN) form of EcR (*UAS-EcR-DN*,*UAS-GFP*; *Lsp2-Gal4*). B(1): Fat body cells from a third instar larva are attached, flattened and irregularly shaped. GFP expression is variable. B(2–3) and B(6–7): In the retraction phase, the fat cells do not change shape. GFP accumulates at the membrane in control animals while GFP expression remains variable when 20E signaling is disrupted in the fat body. B(4) and B(8): In the disaggregation phase, fat-body cells do not round up when 20E signaling is disrupted in the fat body. B(5) and B(9): In the detachment phase the fat cells do not detach and become spherical when 20E signaling is disrupted in the fat body.

As described, fat body remodeling begins with the retraction of the fat body followed by disaggregation (Nelliot et al., 2006). We found that disruption of 20E signaling did not affect fat-body retraction (compare Fig. 2A(2) to 2A(8)), but did inhibit fat-body disaggregation (compare Fig. 2B(4) to 2B(8)). The fat cells expressing EcR-DN did not disaggregate. They retained their flattened shape and did not round up (Fig. 2A(9–10), B(7–8)). Finally, at 12 h APF, when remodeled fat cells become free-floating and are translocated into the head capsule (Fig. 2A(5–6), B(5)), we found that disruption of 20E signaling resulted in persistence of flattened, attached fat cells (Fig. 2B(9)) that did not enter into the head capsule (Fig. 2A(11–12)). We conclude that 20E signaling within the fat body is required for the disaggregation and detachment phases of fat-body remodeling.

ßftz-f1, in concert with 20E signaling, is sufficient to induce fat-body remodeling

ßFTZ-F1 is a key transcription factor involved in the modulation of the transcriptional response to 20E signaling necessary to initiate the prepupal to pupal transition (Broadus et al., 1999; Fortier et al., 2003; Yamada et al., 2000). The final steps of fat-body remodeling, detachment and fat cell translocation into the head capsule, also occur at this time. We find that *ßftz-f1* is expressed in the fat body just prior to the prepupal to pupal transition (data not shown). As a first step toward testing the role of *ßftz-f1* in fat-body remodeling, we expressed *ßftz-f1* prematurely in the larval fat body using the *Lsp2-Gal4* driver. Ectopic *ßftz-f1* transcripts were readily detected in the larval fat body 8 h before pupariation (data not shown) and fat-body remodeling occurred prematurely in the prepupa (Fig. 3B–C). While normal fat-body disaggregation occurs at 6 to 8 h APF (Fig. 2A(3–4) and Nelliot et al., 2006), premature expression of *ßftz-f1* in the larval fat body resulted in premature disaggregation at 4 h APF (Fig. 3A(2)). This was followed by premature fat-cell detachment by 6 h APF (Fig. 3A(3)), where normal detachment occurs at 12 h APF (see Fig. 2A(5) and Nelliot et al., 2006). Thus, premature expression of *ßftz-f1* in the fat body of the third-instar larva induced premature fat-body remodeling in the prepupa.

The timing of premature remodeling suggests that *ßftz-f1* might modify the transcriptional response of the fat body to the 20E pulse

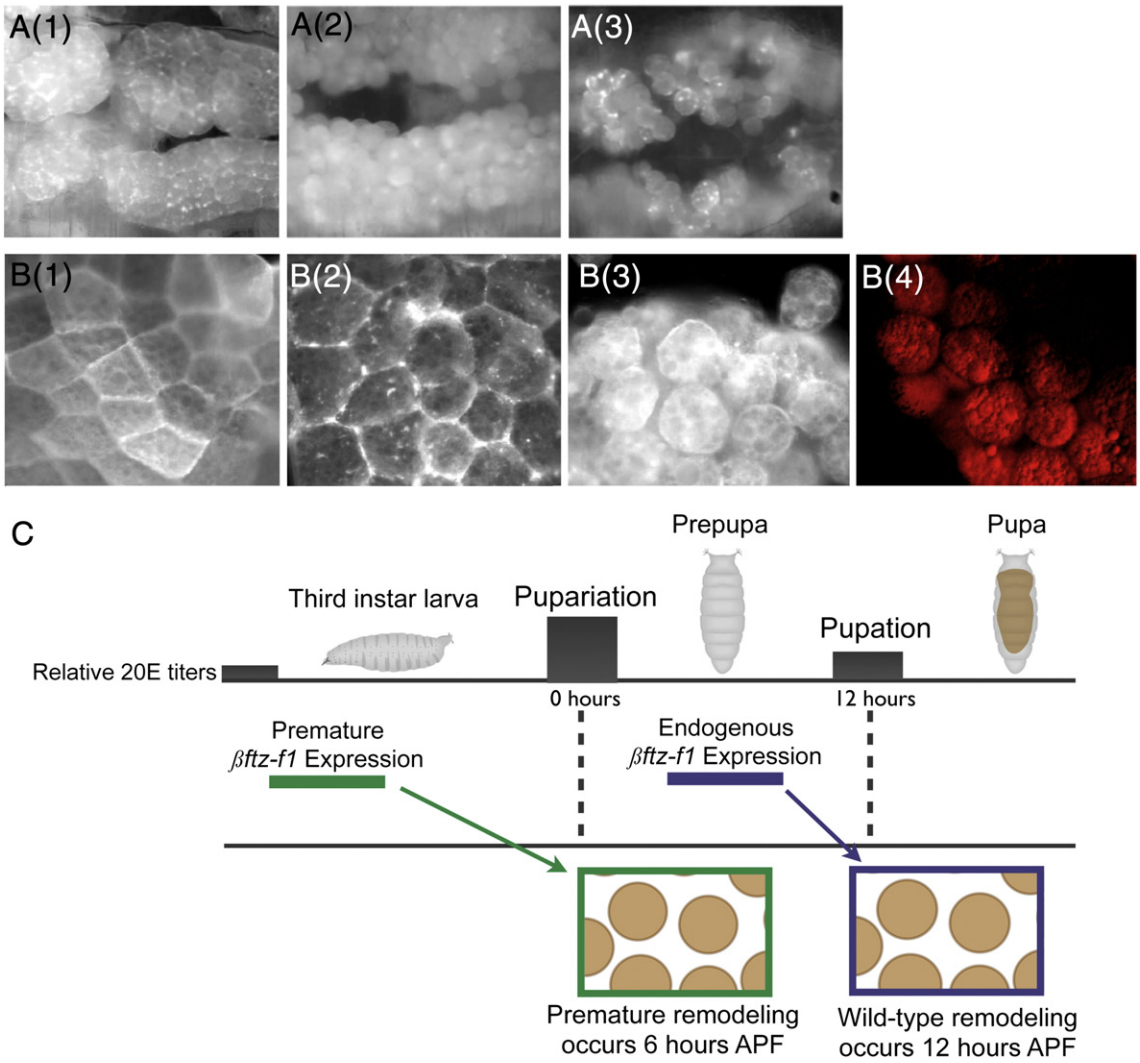

Fig. 3. Expression of *ßftz-f1* induces premature fat-body remodeling in the presence of 20E. (A). Fluorescent images of whole mount prepupae expressing GFP in the fat body. A(1): Fat body from control pupa expressing GFP (*UAS-GFP*; *Lsp2-Gal4*). A(2–3): Fat body from experimental pupa, marked by GFP, prematurely expressing *ßftz-f1* in the fat body (*UAS-GFP*; *Lsp2-Gal4*/*UAS-ßftz-f1*). A(2): At 4 h APF, over expression of *ßftz-f1* resulted in premature disaggregation (compare A(1) to A(2)). A(3): At 6 h APF, overexpression of *ßftz-f1* resulted premature fat-cell detachment (compare A(1) to A(3)). (B). Fluorescent images of wandering third-instar larvae fat bodies in which *ßftz-f1* is prematurely expressed (*UAS-GFP*; *Lsp2-Gal4*/*UAS-ßftz-f1*). B(1): *In vivo* fat body; fat cells are attached, flattened and retain a polygonal shape. B(2): Fat body explant cultured in the absence of 20E retains its larval fat body morphology. B(3): Fat body explant cultured with 10^{-5} M 20E for 8 h undergoes fat-body remodeling. B(4): Fat body explant stained with SYTOX® after 8 hour incubation with 20E. The red staining indicates that cells are not necrotic. (C). Schematic summarizing fat-body remodeling in response to *ßftz-f1* expression followed by exposure to 20E. The process of fat-body remodeling in a wild-type animal is completed after the pupation pulse of 20E (endogenous expression of *ßftz-f1* is shown in purple). Expression of *ßftz-f1* during the third-larval instar (in green) results in premature fat-body remodeling occurring after the pupariation pulse of 20E.

occurring at pupariation, thus setting in motion the prepupal to pupal response to 20E (Fig. 3C). To test this idea, we expressed *UAS-ßftz-f1* in the fat body while blocking 20E signaling by co-expression of *UAS-EcR-DN*. Even in the presence of ectopic *ßftz-f1*, blocking 20E signaling in the fat body prevented fat-body remodeling (Fig. 4C). Head eversion occurred but fat cells were not translocated into the head capsule (Fig. 4C). To further test the idea that ßFTZ-F1 works in concert with 20E signaling to program the final steps in the remodeling of the fat body, we used a fat-body *ex vivo* organ culture assay. Fat-body explants from control larvae and larvae in which *ßftz-f1* is prematurely expressed in the larval fat body (*Lsp2-Gal4*; *UAS-ßftz-f1*) were co-cultured with and without 20E. In the absence of 20E, wild-type fat bodies did not exhibit the cell-shape changes associated with remodeling (data not shown). Likewise, cultured fat bodies ectopically expressing *ßftz-f1* did not remodel in the absence of 20E (Fig. 3B(2)). Addition of 20E in the presence of *ßftz-f1* expression however, was sufficient to induce fat-body remodeling and individual detached cells were detected within 8 h (Fig. 3B(3)). The remodeled fat cells were viable (Fig. 3B(4)), and therefore it is unlikely that cell detachment in the *ex vivo* organ culture was due to degeneration of the tissue. These data taken together suggest that, in the fat body, *ßftz-f1* is required to modulate the transcriptional response to 20E in order to achieve fat-body remodeling at the prepupal to pupal transition.

ßftz-f1 is necessary for fat-body remodeling

To determine whether *ßftz-f1* is necessary for fat-body remodeling, we carried out a series of loss-of-function experiments. Because *ßftz-f1* null mutant animals die during embryonic development (Broadus et al., 1999; Ruaud et al., 2010; Yamada et al., 2000), we first chose to examine *ßftz-f1* hypomorphs (*ftz-f1*^{17}/*Df(3L)Cat*^{DH104}), wherein a small percentage of the mutants complete the prepupal to pupal transition (Broadus et al., 1999). Unfortunately, the mutant phenotype of the *ßftz-f1* hypomorph was extremely variable and thus, it was impossible to determine whether *ßftz-f1* is necessary for fat-body remodeling (data not shown). We attempted to knock down *ßftz-f1* expression by fat-body specific RNA interference using the *Lsp2-Gal4* driver and three different UAS-*ßftz-f1*-RNAi lines. However, we determined by qRT-PCR analysis that this approach failed to reduce levels of *ßftz-f1* transcripts in the fat body (data not shown).

As an alternative approach, we took advantage of the known repressor of *ßftz-f1*, the *Drosophila* homolog of the mammalian B lymphocyte-induced maturation protein-1 (dBlimp-1) (Agawa et al., 2007). We drove *UAS-dBlimp-1 XA* (herein referred to as *UAS-dBlimp-1*) in the fat body using the fat-cell driver, *cg-Gal4*, which is expressed in the fat body throughout larval and pupal development. Misexpression of *UAS-dBlimp-1* in the fat body resulted in lethality at various stages of development, however, some animals proceeded through the prepupal to pupal transition. In these animals, fat-cell detachment and the migration of fat cells into the head capsule did not occur (Fig. 4D). Similar results were also achieved using the fat body driver *Lsp2-Gal4* (data not shown).

As an additional strategy, we chose to use Mosaic Analysis with a Repressible Cell Marker (MARCM) (Lee and Luo, 1999) to assess the loss of *ßftz-f1* function in individual fat cells. Based on our previous results we predicted that individual cells mutant for *ßftz-f1* would not undergo fat-body remodeling. Fat bodies from the MARCM progeny were examined after head eversion. A variable number of *ßftz-f1* null mutant (*ftz-f1*^{19}) fat body cells were observed in each animal, but an average of 6 *ßftz-f1* mutant fat cell clusters were identified per pupa. Some clusters contained a single *ßftz-f1* mutant cell that was completely surrounded by non-mutant cells (Fig. 5A, A″) while other clusters contained either two or more mutant cells clustered together (Fig. 5B, B", C, C″, D, D″). The number of mutant cells per cluster was associated with distinct fat cell shapes. The majority (91%) of mutant cells residing in clusters of 3 or more cells did not undergo disaggregation and retained a more polygonal shape (Fig. 5I). In contrast, 74% of the single mutant cells that were not surrounded by other mutant cells were spherical and

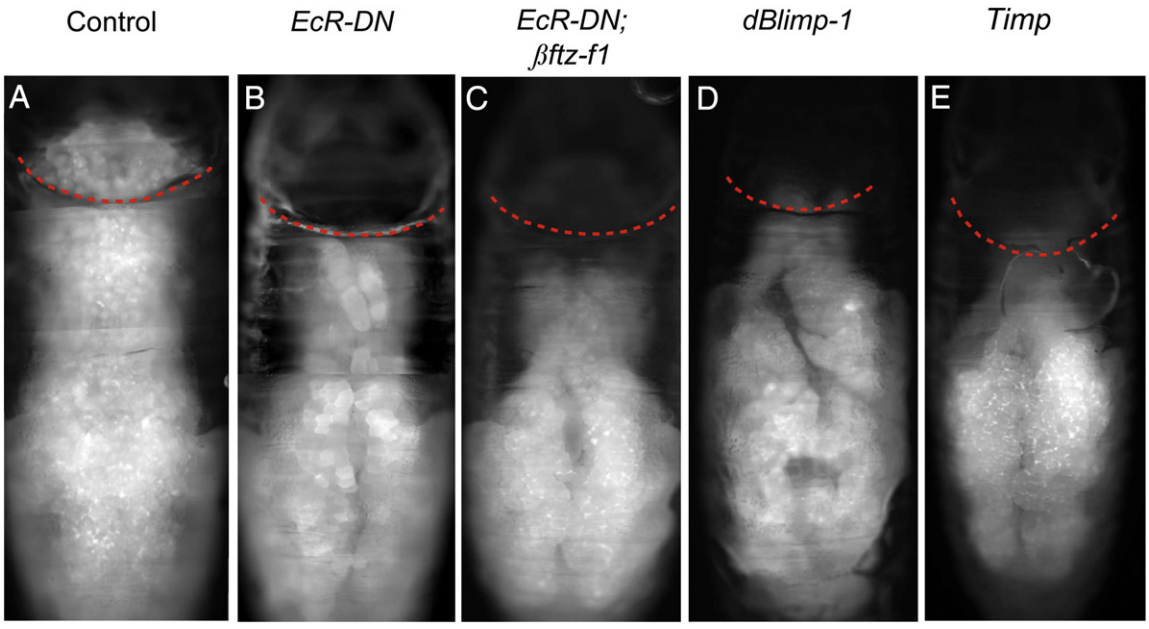

Fig. 4. Fat-body remodeling requires *ßftz-f1* expression, 20E signaling, and Matrix metalloproteinase activity. (A–E) Fluorescent images of animals expressing GFP in the fat body (animals imaged at post-head eversion stage, 12–14 h APF, dotted line marks operculum). (A) Fat-body remodeling in the control pupa expressing *UAS-GFP*; *Lsp2-Gal4*. Remodeled fat cells have entered the head capsule. (B) Fat-body remodeling is disrupted when *EcR-DN* is expressed in the fat body (*UAS-GFP*, *UAS-EcR-DN*; *Lsp2-Gal4*). Fat body remains intact and fat cells do not enter the head capsule. (C) Fat-body remodeling is also disrupted when *ßftz-f1* and *EcR-DN* are coexpressed in the fat body (*UAS-GFP*, *UAS-EcR-DN*; *Lsp2-Gal4*/*UAS-ßftz-f1*). (D) Fat-body remodeling is disrupted in animal expressing *dBlimp-1* in the fat body (*cg-Gal4*, *UAS-GFP*; *UAS-dBlimp-1*). (E) Fat-body remodeling is disrupted in animals expressing *Timp* specifically in the fat body (*Lsp2-Gal4*, *UAS-GFP*; *UAS-Timp*).

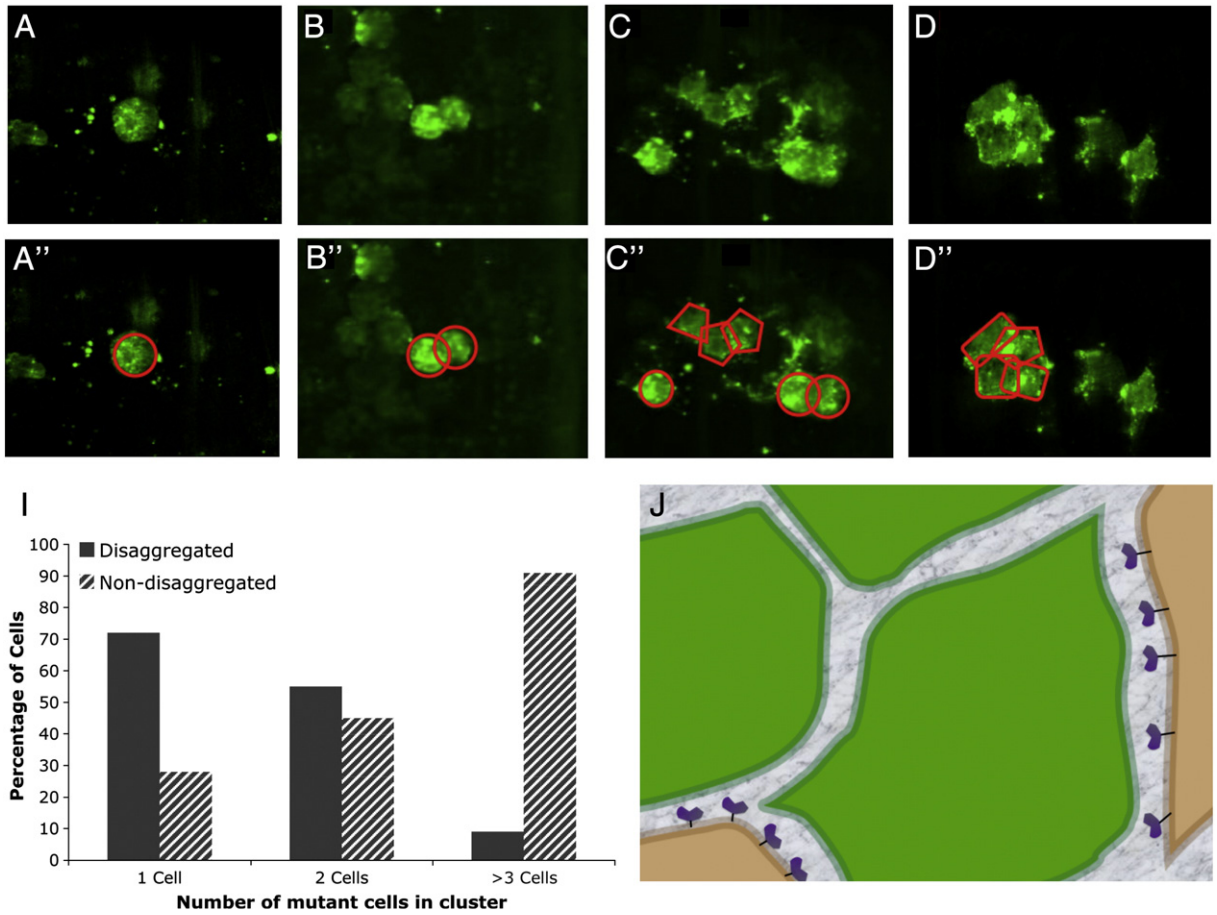

Fig. 5. Mosaic analysis with a repressible cell marker. (A–D) Progeny from *yw hs-flp*; *cg-Gal4*, *UAS-GFP/CyO*; *FRT2A*, *tubulin-gal80/TM6B*, *Tb* females crossed to *yw hs-flp*; *FRT2A*, *ftz-f1*^{19}*/TM6B*, *Tb* males were heat shocked at 37 °C for 30 min 2 to 4 h after egg lay to induce FLP recombinase and generate clones of *ßftz-f1* null mutant (*ftz-f1*^{19}) fat cells expressing GFP. Cells were imaged *in vivo* after head eversion using confocal microscopy. (A″–D″) GFP expressing clones are outlined in red for emphasis. (A, A″) Single *ßftz-f1* mutant cell, cell is spherical and detached from adjacent cells. (B, B″) Two adjacent *ßftz-f1* mutant cells, cells are disaggregated and spherical but attached to each other. (C, C″) Three adjacent *ßftz-f1* mutant cells, cells are attached and not disaggregated; two adjacent *ßftz-f1* mutant cells which are disaggregated, spherical and attached to each other and a single *ßftz-f1* mutant cell which is spherical and detached. (D, D″) Cluster of four *ßftz-f1* mutant cells, all cells are attached and not disaggregated. (I) Percentage of disaggregation that occurs *ßftz-f1* mutant cells. *ßftz-f1* mutant cells (GFP expressing cells) were categorized based on the number of adjacent mutant cells (number of mutant cells in cluster) and how many of those cells were disaggregated. Spherical cells were considered disaggregated and flat cells with polygonal shape were scored as not having undergone remodeling. (J) Diagram illustrating that fat body-remodeling is a tissue-autonomous process. *ßftz-f1* mutant cells are depicted in green, wild-type cells in beige. Wild-type cells are capable of expressing *MMP2* (proteinase shown in purple) which degrades ECM while the *ßftz-f1* mutant cells are not. MMP2 is present at the membrane of the wild-type cells and degrades ECM that the wild-type cell shares with the *ßftz-f1* mutant cells. Thus, the *ßftz-f1* mutant cell's ECM is remodeled by an adjacent wild-type cell's MMP2.

detached from the other fat cells (Fig. 5I). Due to the lack of complete remodeling in *ßftz-f1* mutant MARCM cells, we conclude that *ßftz-f1* is required for fat-body remodeling.

Misexpression of MMP2 is sufficient to induce fat-body remodeling

The process of fat-cell detachment presumably involves proteases that can cleave substrates present in the ECM that hold the cells together. The detachment phase of fat-body remodeling occurs concurrently with the expression of *MMP1* and *MMP2* during the prepupal to pupal transition (Nelliot et al., 2006; Page-McCaw et al., 2003). Previous reports have shown that the MMPs are required for midgut and tracheal remodeling (Llano et al., 2002; Page-McCaw et al., 2003). Moreover, there is evidence that the expression of MMPs and their inhibitor TIMP (tissue inhibitor of metalloproteases) might be regulated by 20E signaling (Lee et al., 2003). Thus, these proteases are likely candidates for involvement in fat-body remodeling and are possible target genes of the ßFTZ-F1-mediated 20E-signaling cascade. Therefore, we set out to test the role of MMPs in fat-body tissue remodeling.

We first tested whether *Drosophila* MMPs were sufficient to promote fat-body remodeling by misexpression of *MMP1* and *MMP2* in the fat body. *UAS-MMP1* and *UAS-MMP2* were individually expressed specifically in fat body using the *Lsp2-Gal4* driver (Cherbas et al., 2003). Misexpression of *MMP2* resulted in lethality and premature fat-body remodeling in mid-third instar larvae. Dissected fat cells from third-instar larvae were free floating, spherical and resembled wild-type remodeled fat cells (Fig. 6C). We do not believe that this is a generalized phenomenon of ectopic expression of metalloproteases because, although misexpression of *MMP1* in the fat body also caused larval lethality, it did not induce fat-body remodeling. The fat cells maintained their associations with their neighboring cells and did not display cell shape changes associated fat-body remodeling (Fig. 6B).

Misexpression of Timp in the fat body inhibits remodeling

TIMP inhibits the catalytic activity of MMPs by occupying the active site of the proteinase (Gomis-Rüth et al., 1997). There is one *Timp* gene in the *Drosophila* genome (as compared to four in vertebrates) (Wei et al., 2003). To further explore the role of MMP2 in fat-body remodeling, we ectopically expressed *UAS-Timp* in the fat body using the *Lsp2-Gal4* driver. If MMP2 activity is required for fat-body remodeling, we expected to observe a block in remodeling by

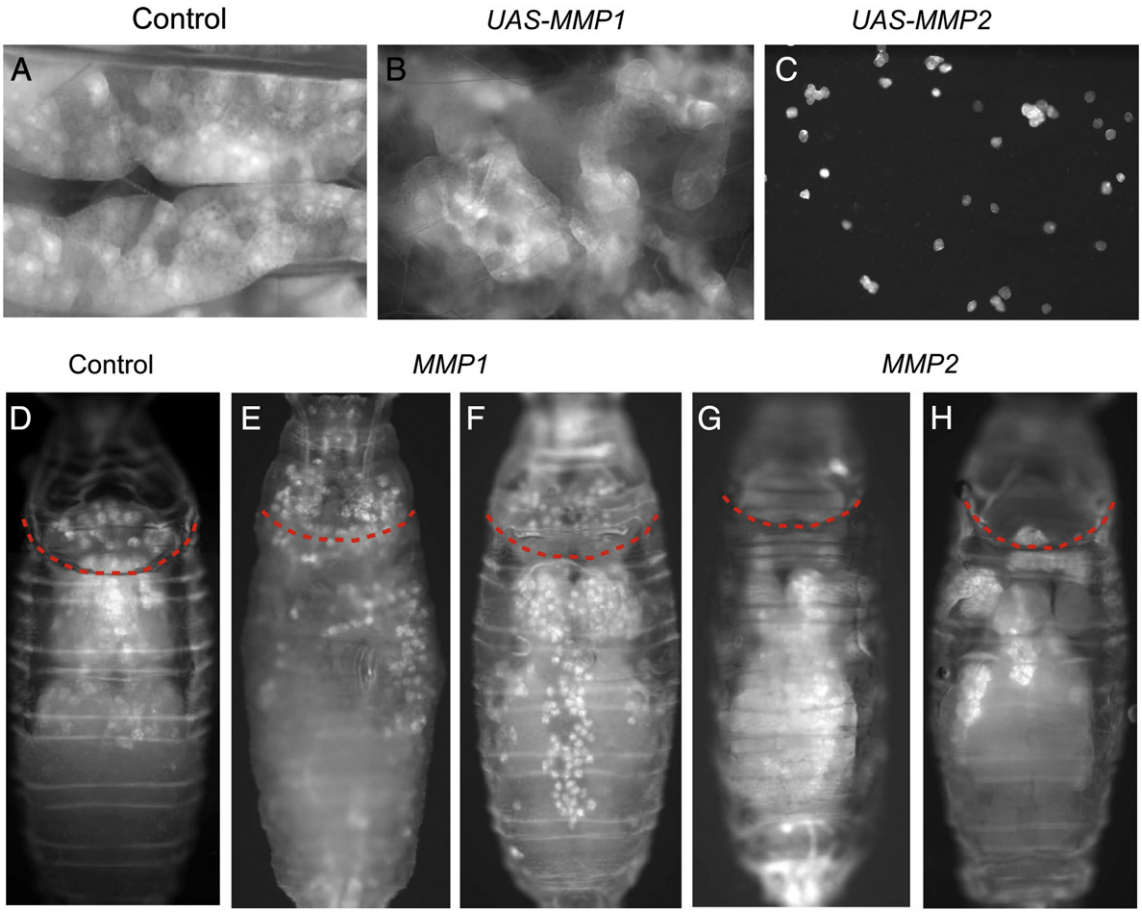

Fig. 6. MMP2 is necessary and sufficient to induce fat-body remodeling. (A–B) Fat body imaged *in vivo* during the third-larval instar by fluorescent microscopy. (A) Intact fat body tissue in control larva expressing *UAS-GFP*; *Lsp2-Gal4*. (B) Intact fat body in larva in which *MMP1* is prematurely expressed (*UAS-GFP*; *Lsp2-Gal4*/*UAS-MMP1*). (C) Dissected fat cells from animal prematurely expressing *MMP2* (*UAS-GFP*; *Lsp2-Gal4*/*UAS-MMP2*). Fat body has remodeled and fat cells are spherical and completely detached. (D–H) *MMP* mutant analysis. Animals staged to 14 h APF, fat cells detected by autofluorescence, (dotted line marks operculum). (D) Fat-body remodeling occurred in the control pupae (*Lsp2-Gal4*). (E–F) Fat-body remodeling occurs in *MMP1* mutant pupae and fat cells are detected within the head capsule. (E) $MMP1^{W439}$ (F) $MMP1^{Q273}$. (G–H) Fat-body remodeling is blocked in *MMP2* mutant animals and the fat body remains intact; fat cells are not detected in the head capsule. (B) $MMP2^{W309}$ (C) $MMP2^{W309}/MMP2^{Df}$.

misexpressing *Timp* in the fat body. Indeed, when *UAS-Timp* was expressed in the fat body, the fat body did not appear to undergo any remodeling (Fig. 4E). However, animals misexpressing *Timp* in the fat body were developmentally arrested before head eversion occurred. Thus, it is difficult to distinguish between a defect in the fat-cell detachment stage of remodeling and disruption of detachment due to the premature death of the animal. Some aspects of fat-body remodeling (disaggregation, for example) occur during the prepupal stage of development, before animals misexpressing *Timp* in the fat body die. Misexpression of *Timp* in the fat body caused a failure in the disaggregation step of fat-body remodeling (Fig. 4E). The fat cells maintained their larval morphology until the time of animal death, just prior to pupation. These data suggest that fat-body remodeling requires the action of MMPs.

MMP2 is necessary for fat-body remodeling

Although *MMP* mutants infrequently pupariate, a small percentage of mutants with the weaker alleles of both *MMP1* and *MMP2* do complete the prepupal to pupal transition and evert their heads (Page-McCaw et al., 2003). We took advantage of these weaker alleles and conducted a mutant analysis. As expected, all *MMP1* mutant animals that progressed through the prepupal to pupal transition completed the fat-body remodeling program, resulting in detached free-floating cells in the pupa (Fig. 6E, F). *MMP2* mutants that completed the prepupal to pupal transition, however, did not remodel their fat bodies. *MMP2* mutant animals retained larval fat-body morphology and the fat cells did not translocate into the head capsule (Fig. 6G, H). These data suggest that MMP2 is required for fat-body remodeling.

Expression of EcR-DN results in down-regulation of MMP2 expression

To test whether *MMP2* is a potential downstream target gene of ßFTZ-F1-mediated 20E signaling, we first tested whether *MMP2* expression in the fat body is dependent on 20E signaling. We blocked 20E signaling in the fat body and determined levels of *MMP2* expression by qRT-PCR in fat bodies during metamorphosis. *MMP2* transcripts in control animals peaked during the prepupal to pupal transition period (data not shown) while disruption of 20E signaling prevented the induction of fat-body specific *MMP2* expression. *MMP2* expression was down-regulated from 8 h APF through 14 h APF in fat bodies expressing *EcR-DN* (Fig. 7A). These data are consistent with our hypothesis that *MMP2* expression is regulated by 20E signaling in the fat body.

Premature expression of ßftz-f1 results in early induction of MMP2 expression

Our results suggest that *MMP2* expression is induced in a ßFTZ-F1-mediated, 20E dependent manner in order to achieve fat-body remodeling. We have demonstrated that *ßftz-f1* and 20E signaling are

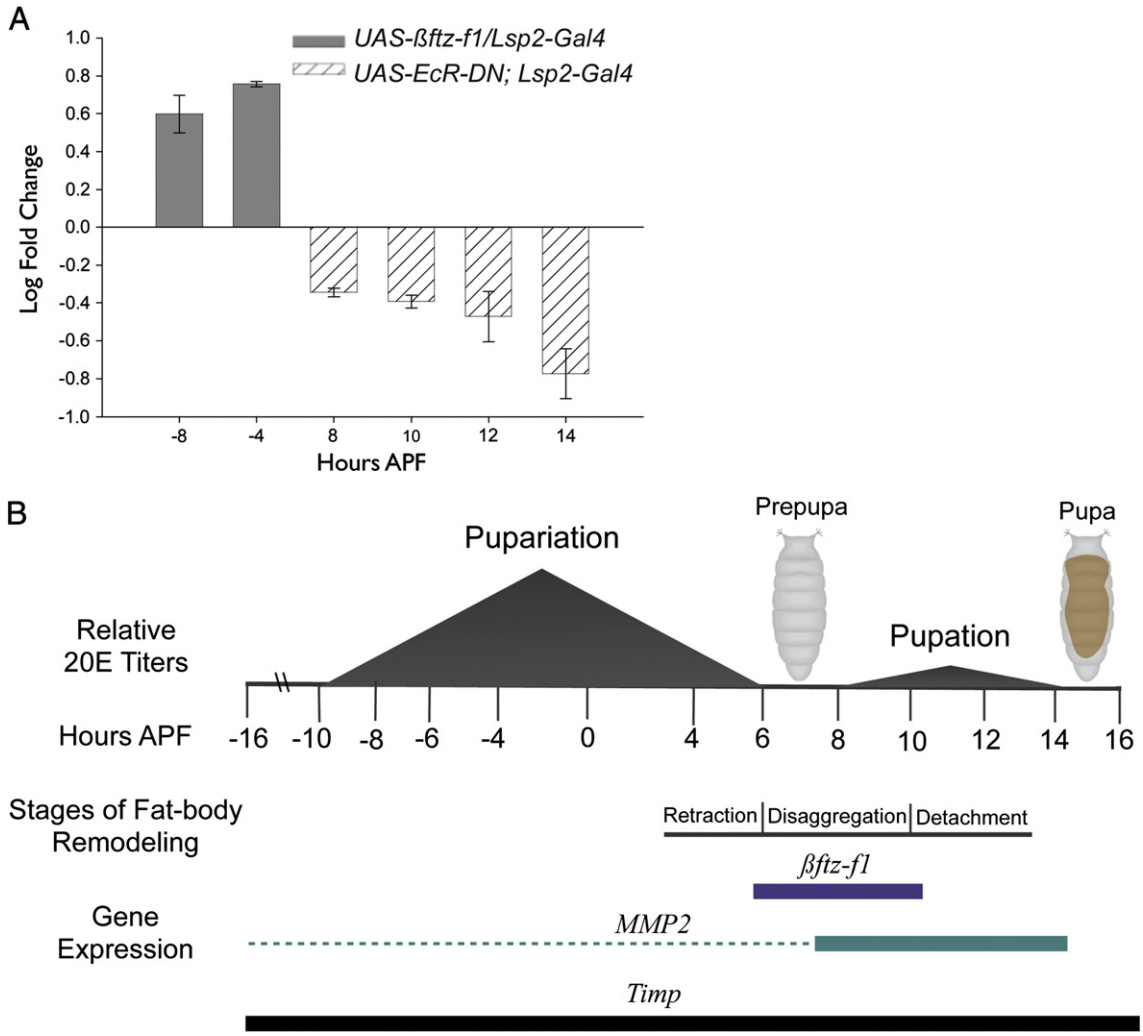

Fig. 7. 20E signaling and ßftz-f1 are involved in induction of *MMP2* expression in the fat body. (A). Relative expression of *MMP2* in dissected fat bodies from at several time points. Relative expression of *MMP2* in dissected fat bodies expressing *UAS-EcR-DN*, *UAS-GFP*; *Lsp2-Gal4* (hatched bars) or *UAS-GFP*; *Lsp2-Gal4*/*UAS-ßftz-f1* (gray bars) was compared to control animal (w^{1118}) expression levels at several time points. *MMP2* transcripts were normalized to *Actin 5C* and expressed as relative fold change compared to control animals (w^{1118}). Note the logarithmic y-axis where values >1 indicate up-regulation and values <1 indicate down-regulation. Mean and +/− SEM represented, n = 3 in all cases. A student *T*-test was performed and no difference was found between stages (p = 0.19) for animals expressing *UAS-GFP*; *Lsp2-Gal4*/*UAS-ßftz-f1* (gray bars). A Tukey post-hoc test revealed that there was no significant difference in fold change expression between −8 and −4 h APF. A one-way ANOVA was performed and a statistically significant difference in fold change expression was found between stages (p = 0.049) for animals expressing *UAS-EcR-DN*, *UAS-GFP*; *Lsp2-Gal4* (hatched bars). However a Tukey post-hoc test revealed that there was no significant difference in fold change expression between 8, 10, 12 or 14 h APF. (B). Model for the fat-body remodeling signaling cascade. Relative 20E titers are depicted above corresponding hours APF, stages of fat-body remodeling and expression profile of genes involved in the remodeling process. *ßftz-f1* is expressed from 6 to 10 h APF. Expression of *ßftz-f1* and 20E signaling (through EcR) is required for fat-body remodeling. The pupation pulse of 20E initiates signaling through EcR and this signaling, in combination with prior expression of *ßftz-f1*, is required for induction of *MMP2* expression. MMP2 then reshapes the extracellular matrix and degrades the basement membrane of the fat body, resulting in a remodeled fat body. Basal levels of *MMP2* (dotted line) are expressed in the fat body throughout development. *TIMP* is also expressed throughout development (Page-McCaw et al., 2003). We propose large quantities of MMP2 protein are able to overcome TIMP inhibition, thus allowing fat-body remodeling to occur.

sufficient to induce premature fat-body remodeling (Fig. 3). We speculated that premature remodeling was achieved through early induction of *MMP2* expression by ßFTZ-F1 and 20E signaling. To test whether premature expression of *ßftz-f1* results in early induction of *MMP2* expression, we examined *MMP2* expression via qRT-PCR in larvae prematurely expressing *ßftz-f1* in the fat body. For larvae in which *ßftz-f1* is prematurely expressed in the fat body, we observe an increase in levels of *MMP2* transcripts during the wandering (−8 h APF) and late (−4 h APF) stages of the third-larval instar (Fig. 7A). These data suggest that misexpression of *ßftz-f1* induces premature fat-body remodeling by early induction of *MMP2* gene transcription. Furthermore, these data support our hypothesis that *MMP2* expression is regulated by the ßFTZ-F1-mediated 20E signaling cascade necessary for fat-body remodeling.

Discussion

The idea that fat-body remodeling might require fat-cell-specific 20E signaling has been put forth by Cherbas et al., (2003) and has been further investigated here. We found that only certain aspects of fat-body remodeling, namely changes in cell shape (disaggregation) and the generation of individual fat cells (detachment), required a functional EcR in the fat body. We have observed the pulsating abdominal movements of pupal ecdysis, and with each contraction of the abdomen individual fat cells are propelled into the head capsule. It may appear as though the abdominal contractions themselves might be sufficient to promote fat cell detachment, but in the absence of fat-body remodeling, fat-cell translocation did not occur. Disruption of 20E signaling in the fat body by expression of

EcR-DN did not affect the peristaltic abdominal contractions, but did prevent fat-body remodeling and translocation of the fat cells into the head capsule. Thus, the abdominal movements do not provide shearing forces sufficient to detach cells from the fat-body tissue mass. The ecdysial abdominal contractions appear to be involved in the translocation of the *remodeled* fat cells into the head capsule and redistribution of the cells with the abdomen of the animal and not the process of detachment itself.

We are intrigued by our observation that tissue-specific misexpression *of MMP2* in the larval fat body results in animal lethality in addition to premature fat-body remodeling. Likewise, blocking 20E signaling in the fat body (and thus down-regulating *MMP2* expression in the pupa) results in animal death (Bond et al., 2010; Cherbas et al., 2003), as does blocking MMP2 function by misexpression of *Timp*. It is known that MMPs are involved in cleaving signaling factors in the ECM of mammalian cells (Sternlicht and Werb, 2001). It is possible that MMPs, in addition to remodeling the fat body, are involved in cleavage of signaling molecules in *Drosophila* that have global developmental effects necessary for normal animal development.

Due to the fact that *ßftz-f1* null mutants do not survive embryogenesis, we were not able to directly observe the effect of a *ßftz-f1* null mutation in the fat body at the prepupal to pupal transition. RNAi experiments were unsuccessful and hypomorphic mutants were unreliable, so we employed the Mosaic Analysis with a Repressible Cell Marker (MARCM) technique (Lee and Luo, 2001) to examine *ßftz-f1* null mutant cells. Interestingly, the degree of fat-body remodeling observed seemed to coincide with the number of adjacent *ßftz-f1* null mutant cells. *ßftz-f1* null mutant cells that were found in clusters (3 or more adjacent mutant cells) did not undergo any remodeling, while mutant cells found in clusters of two achieved disaggregation but not detachment, and single mutant cells (completely surrounded by non-mutant cells) were disaggregated and detached. The clusters of 3 or more *ßftz-f1* null mutant cells maintained their larval morphology, suggesting that, in these cells, ßFTZ-F1 is required for fat-body remodeling. Moreover, these results suggest that the process of fat-body remodeling is not a cell autonomous process. Instead, perhaps fat-body remodeling is a *tissue* autonomous process in which adjacent fat cells can participate in the remodeling of neighboring mutant cells. We have shown that the remodeling of the fat body requires expression of *MMP2*, presumably to break down the ECM holding the tissue together. Because MMP2 is attached to the membrane of cells by a GPI anchor (Page-McCaw et al., 2003), its localization supports our model for tissue-autonomous fat-body remodeling. A membrane bound MMP2 would not only have the potential to remodel the cell that it is directly bound to but it could also potentially have an effect on other cells in the immediate vicinity (Fig. 5J).

In this paper, we take advantage of the wealth of knowledge about the 20E-signaling cascade, the expression patterns of *MMP2* and *Timp*, and our data presented here to propose a model for the ßFTZ-F1-mediated, 20E-signaling cascade in the fat body (Fig. 7B). The cell-shape changes and cell detachment phases of fat-body remodeling occur at the pre-pupal to pupal transition. We propose that ßFTZ-F1 makes the fat-body cells competent to respond to the 20E pulse released at the prepupal to pupal transition resulting in an increase in *MMP2* expression, and that MMP2 functions to degrade fat body ECM in a tissue-autonomous manner. Although *TIMP* is expressed throughout development (Page-McCaw et al., 2003), we propose that the large quantities of MMP2 resulting from up-regulation of the gene are able to overcome TIMP inhibition, allowing fat-body remodeling to occur. According to our results, up-regulation of *MMP2* can be achieved by premature expression of *ßftz-f1* in the fat body. Although it is highly suggestive, our results implicate a role for ßFTZ-F1 in the regulation of *MMP2* expression. ßFTZ-F1 protein is indeed present at the time of *MMP2* expression (White et al., 1997; Yamada et al., 2000). It remains to be determined whether ßFTZ-F1 is capable of activating transcription of *MMP2* by directly binding to the *MMP2* promoter. In the mosquito, ßFTZ-F1 and the ecdysone receptor bind to the promoter of the vitellogenin (*Vg*) gene. ßFTZ-F1 also binds to FISC, a p160 coactivator of the ecdysone receptor. The binding of ßFTZ-F1 to FISC is required in order to achieve 20E-induction of *Vg* transcription (Zhu et al., 2006). Further experiments are needed to determine whether ßFTZ-F1 acts as a direct regulator of *MMP2* transcription in *Drosophila*, or if the effect of ßFTZ-F1 on *MMP2* expression is indirect.

Determining the genetic cascade required for cell detachment in *Drosophila* fat-body remodeling might pave the way for development of a tractable model system for understanding mammalian processes such as metastasis and wound healing. The larval fat body also provides a good model system in which to analyze the genetic and molecular control of programmed cell death. Unlike other larval tissues such as the salivary gland and midgut, the fat body is spared from programmed cell death during metamorphosis. We are currently examining the role of anti-cell death genes, such as *diap1*, in the regulation of cell death in the larval fat body. Understanding the molecular mechanisms responsible for sparing the larval fat body from apoptosis may provide insight for potential ways to prevent cell death.

Supplementary materials related to this article can be found online at doi:10.1016/j.ydbio.2011.09.015.

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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., 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.

Andres, A.J., Thummel, C.S., 1994. Methods for quantitative analysis of transcription in larvae and prepupae. In: Goldstein, L.S.B., Fyrberg, E.A. (Eds.), Methods in Cell Biology, Volume 44 — *Drosophila melanogaster*: Practical Uses in Cell and Molecular Biology. Academic Press, New York, pp. 565–573.

Bainbridge, S.P., Bownes, M., 1981. Staging the metamorphosis of *Drosophila melanogaster*. J. Embryol. Exp. Morphol. 66, 57–80.

Bodenstein, D., 1950. The postembryonic development of *Drosophila*. In: Demerec, M. (Ed.), Biology of *Drosophila*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, pp. 275–375.

Bond, N.D., Hoshizaki, D.K., Gibbs, A.G., 2010. The role of 20-hydroxyecdysone signaling in *Drosophila* pupal metabolism. Comp. Biochem. Physiol. Part A Mol. Integr. Physiol. 157, 398–404.

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

Burtis, K.C., Thummel, C.S., Jones, C.W., Karim, F.D., Hogness, D.S., 1990. The *Drosophila* 74EF early puff contains *E74*, a complex ecdysone-inducible gene that encodes two ets-related proteins. Cell 61, 85–99.

Butterworth, F.M., 1972. Adipose tissue of *Drosophila melanogaster*. V. Genetic and experimental studies of an extrinsic influence on rate of cell death in larval fat-body. Dev. Biol. 28, 311–325.

Cherbas, L., Hu, X., Zhimulev, I., Belyaeva, E., Cherbas, P., 2003. EcR isoforms in *Drosophila*: testing tissue-specific requirements by targeted blockade and rescue. Development 130, 271–284.

Costantino, B.F.B., Bricker, D.K., Alexandre, K., Shen, K., Merriam, J.R., Antoniewski, C., Callender, J.L., Henrich, V.C., Presente, A., Andres, A.J., 2008. A novel ecdysone receptor mediates steroid-regulated developmental events during the mid-third instar of *Drosophila*. Plos Genet. e1000102.

DiBello, P.R., Withers, D.A., Bayer, C.A., Fristrom, J.W., Guild, G.M., 1991. The *Drosophila Broad-Complex* encodes a family of related proteins containing zinc fingers. Genetics 129, 385–397.

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

Gomis-Rüth, F.-X., Maskos, K., Betz, M., Bergner, A., Huber, R., Suzuki, K., Yoshida, N., Nagase, H., Brew, K., Bourenkov, G.P., Bartunik, H., Bode, W., 1997. Mechanism of inhibition of the human matrix metalloproteinase stromelysin-1 by TIMP-1. Nature 389, 77–81.

Hoshizaki, D.K., 2005. Fat-cell development. In: Gilbert, L.I., Iatrou, K., Gill, S.S. (Eds.), Comprehensive Molecular Insect Science. Elsevier, Oxford, pp. 315–345.

Jiang, C., Lamblin, A.-F.J., Steller, H., Thummel, C.S., 2000. A steroid-triggered transcriptional hierarchy controls salivary gland cell death during *Drosophila* metamorphosis. Mol. Cell. 5, 445–455.

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, 559–569.

Kim, Y.-J., Zitnan, D., Galizia, C.G., Cho, K.-H., Adams, M.E., 2006. A command chemical triggers an innate behavior by sequential activation of multiple peptidergic ensembles. Curr. Biol. 16, 1395–1407.

King-Jones, K., Thummel, C.S., 2005. Less steroids make bigger flies. Science 310, 630–631.

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 67, 59–77.

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 124, 1757–1769.

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. 212, 204–216.

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, 3004–3008.

Lee, T., Luo, L., 1999. Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis. Neuron 22, 451–461.

Lee, T., Luo, L., 2001. Mosaic analysis with a repressible cell marker (MARCM) for *Drosophila* neural development. Trends Neurosci. 24, 251–254.

Lee, C.-Y., Cooksey, B.A.K., Baehrecke, E.H., 2002. Steroid regulation of midgut cell death during *Drosophila* development. Dev. Biol. 250, 101–111.

Lee, C.-Y., Clough, E.A., Yellon, P., Teslovich, T.M., Stephan, D.A., Baehrecke, E.H., 2003. Genome-wide analyses of steroid- and radiation-triggered programmed cell death in *Drosophila*. Curr. Biol. 13, 350–357.

Llano, E., Pendas, A.M., Aza-Blanc, P., Kornberg, T.B., Lopez-Otin, C., 2000. Dm1-MMP, a matrix metalloproteinase from *Drosophila* with a potential role in extracellular matrix remodeling during neural development. J. Biol. Chem. 275, 35978–35985.

Llano, E., Adam, G., Pendas, A.M., Quesada, V., Sanchez, L.M., Santamaria, I., Noselli, S., Lopez-Otin, C., 2002. Structural and enzymatic characterization of *Drosophila* Dm2-MMP, a membrane-bound matrix metalloproteinase with tissue-specific expression. J. Biol. Chem. 277, 23321–23329.

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

Oberlander, H., 1976. Hormonal control of growth and differentiation of insect tissues cultured *in vitro*. In Vitro Cell Dev. Biol. 12, 225–235.

Page-McCaw, A., 2008. Remodeling the model organism: Matrix metalloproteinase functions in invertebrates. Semin. Cell Dev. Biol. 19, 14–23.

Page-McCaw, A., Serano, J., Sante, J.M., Rubin, G.M., 2003. *Drosophila* matrix metalloproteinases are required for tissue remodeling, but not embryonic development. Dev. Cell 4, 95–106.

Page-McCaw, A., Ewald, A.J., Werb, Z., 2007. Matrix metalloproteinases and the regulation of tissue remodeling. Nat. Rev. Mol. Cell Biol. 8, 221–233.

Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29, e45.

Pick, L., Anderson, W.R., Shulz, J., Woodard, C.T., 2006. The Ftz-F1 family: orphan nuclear receptors regulated by novel protein-protein interactions. In: Reshma Taneja, R. (Ed.), Advances in Developmental Biology Series. : Nuclear Receptors in Development, vol. 16. Elsevier, New York, pp. 255–296.

Rewitz, K.F., Yamanaka, N., O'Connor, M.B., 2010. Steroid hormone inactivation is required during the juvenile-adult transition in *Drosophila*. Dev. Cell 19, 895–902.

Richards, G., 1981. The radioimmunoassay of ecdysteroid titers in *Drosophila melanogaster*. Mol. Cell. Endocrinol. 21, 181–197.

Riddiford, L.M., 1996. Juvenile hormone: the status of its "status quo" action. Arch. Insect Biochem. 32, 271–286.

Riddiford, L.M., Truman, J.W., 1993. Hormone receptors and the regulation of insect metamorphosis. Am. Zool. 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.

Ruaud, A.-F., Lam, G., Thummel, C.S., 2010. The *Drosophila* nuclear receptors DHR3 and ßFTZ-F1 control overlapping developmental responses in late embryos. Development 137, 123–131.

Schubiger, M., Wade, A.A., Carney, G.E., Truman, J.W., Bender, M., 1998. *Drosophila* EcR-B ecdysone receptor isoforms are required for larval molting and for neuron remodeling during metamorphosis. Development 125, 2053–2062.

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. 4, 204–219.

Sternlicht, M.D., Werb, Z., 2001. How matrix metalloproteinases regulate cell behavior. Annu. Rev. Cell Dev. Biol. 17, 463–516.

Thomas, H.E., Stunnenberg, H.G., Stewart, A.F., 1993. Heterodimerization of the *Drosophila* ecdysone receptor with retinoid-x receptor and ultraspiracle. Nature 362, 471–475.

Thummel, C.S., 1995. From embryogenesis to metamorphosis — the regulation and function of *Drosophila* nuclear receptor superfamily members. Cell 83, 871–877.

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

Wei, S., Xie, Z., Filenova, E., Brew, K., 2003. *Drosophila* TIMP is a potent inhibitor of MMPs and TACE: similarities in structure and function to TIMP-3. Biochemistry 42, 12200–12207.

White, K.P., Hurban, P., Watanabe, T., Hogness, D.S., 1997. Coordination of *Drosophila* metamorphosis by two ecdysone-induced nuclear receptors. Science 276, 114–117.

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 79, 607–615.

Yamada, M., Murata, T., Hirose, S., Lavorgna, G., Suzuki, E., Ueda, H., 2000. Temporally restricted expression of transcription factor ßFTZ-F1: significance for embryogenesis, molting and metamorphosis in *Drosophila melanogaster*. Development 127, 5083–5092.

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 366, 476–479.

Yin, V.P., Thummel, C.S., 2005. Mechanisms of steroid-triggered programmed cell death in *Drosophila*. Semin. Cell. Dev. Biol. 16, 237–243.

Zhu, J., Chen, L., Sun, G., Raikhel, A.S., 2006. The competence factor ßFtz-F1 potentiates ecdysone receptor activity via recruiting a p160/SRC coactivator. Mol. Cell. Biol. 26, 9402–9412.