Juvenile hormone and 20-hydroxyecdysone coordinately control the developmental timing of matrix metalloproteinase–induced fat body cell dissociation

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Edited by Ronald C. Wek

Tissue remodeling is a crucial process in animal development and disease progression. Coordinately controlled by the two main insect hormones, juvenile hormone (JH) and 20-hydroxyecdysone (20E), tissues are remodeled context-specifically during insect metamorphosis. We previously discovered that two matrix metalloproteinases (Mmps) cooperatively induce fat body cell dissociation in Drosophila. However, the molecular events involved in this Mmp-mediated dissociation are unclear. Here we report that JH and 20E coordinately and precisely control the developmental timing of Mmp–induced fat body cell dissociation. We found that during the larval–prepupal transition, the anti-metamorphic factor Kr-h1 transduces JH signaling, which directly induced Mmp expression and activated expression of tissue inhibitor of metalloproteinases (timp) and thereby suppressed Mmp–induced fat body cell dissociation. We also noted that upon a decline in the JH titer, a prepupal peak of 20E suppresses Mmp–induced fat body cell dissociation through the 20E primary-response genes, E75 and Blimp-1, which inhibited expression of the nuclear receptor and competence factor βtz-F1. Moreover, upon a decline in the 20E titer, βtz-F1 expression was induced by the 20E early–late response gene DHR3, and then βtz-F1 directly activated Mmp expression and inhibited timp expression, causing Mmp–induced fat body cell dissociation during 6–12 h after puparium formation. In conclusion, coordinated signaling via JH and 20E finely tunes the developmental timing of Mmp–induced fat body cell dissociation. Our findings shed critical light on hormonal regulation of insect metamorphosis.

This work was supported by National Science Foundation of China Grants 31620103917, 31560609, 31330072, and 31572325 and National Key Research and Development Program of China Grant 2016YFD0101900 (to S. Li and D.W.). The authors declare that they have no conflicts of interest with the contents of this article.

This article contains Figs. S1–S4.

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3 The abbreviations used are: APF, after puparium formation; 20E, 20-hydroxyecdysone; JH, juvenile hormone; Mmp, matrix metalloproteinase; TIMP, tissue inhibitor of matrix metalloproteinase; BM, basement membrane; EcR, ecdysone receptor; Met, methoprene-tolerant; JHRR, JH response region; KBS, Kr-h1–binding sites; qPCR, quantitative real-time PCR; IW, initiation of wandering; AIW, after IW; WPP, white prepupal stage; FBS, βtz-F1-binding site.
controls the termination of the 20E signal pulse during the larval–prepupal transition; DHR3 also induces the expression of βtzt-F1, which acts as a competence factor for EcR-ultraspiracle to respond to the subsequent 20E signal pulse during the prepupal–pupal transition. Importantly, E75 prevents DHR3-mediated inhibition of 20E signaling and DHR3-induced βtzt-F1 expression through physical interaction and competition for retinoic acid receptor–related response elements (9–13). Moreover, Blimp-1 acts as a transcriptional repressor to restrict βtzt-F1 expression (14). Therefore, the 20E-induced transcriptional cascade, including E75, Blimp-1, DHR3, and βtzt-F1, governs the two 20E signal pulses during Drosophila metamorphosis (8, 14). The two 20E pulses are likely involved in the regulation of fat body cell dissociation in Dro. This process is controlled in larval–prepupal transition; DHR3 also induces the expression of 20E response genes at least partially accounts for the cross-talk between JH and 20E; thus, Kr-h1 is considered as an anti-metamorphic factor in insects (16, 17). Interestingly, we observed precocious fat body cell dissociation in both JH-deficient animals and Met gce double mutant animals (19, 29). However, nothing is known about whether and how Kr-h1 mediates JH signals to inhibit fat body cell dissociation in Drosophila. During insect metamorphosis, a series of cellular events, including programmed cell death, cell proliferation, cell differentiation, and cell dissociation, occurs in a context-specific manner. Previous investigations indicate all of the events are coordinately controlled by JH and 20E (7, 16, 17, 27). However, our current understanding of how the same two hormones induce different cellular processes at distinct, yet precise, developmental timing is very limited. Here, we discovered that JH and 20E coordinately and precisely control the developmental timing of Mmp–induced fat body cell dissociation in Drosophila at both the mRNA and enzymatic levels. This study provides an example to better understand hormonal regulation of tissue remodeling during insect metamorphosis.

Results

Kr-h1 transduces JH signaling to repress Mmp expression

The developmental timing of fat body cell dissociation is strictly controlled in Drosophila and occurs only within a small window immediately before pupation, from 6 h APF to 12 h APF (1, 2). Consistent with our previous reports (19, 29), enhanced fat body cell dissociation was observed in both JH-deficient animals (Aug21-Gal4 > UAS-grim) and Met gce double mutant (Met27 gce2,5k) at 6 h APF compared with wild-type animals (w1118) (Fig. 1A and Fig. S1). Likewise, the Kr-h1 mutant (Kr-h1k04411) showed enhanced fat body cell dissociation at 6 h APF. By contrast, fat body cell dissociation was inhibited at 12 h APF when Kr-h1 was specifically overexpressed in the fat body (Lsp2-Gal4 > UAS-Kr-h1) (Fig. 1A and Fig. S1).

According to our previous study, Mmp1 and Mmp2 cooperatively induce Drosophila fat body cell dissociation, each assuming a distinct role (2). Therefore, we examined whether JH signaling prevents fat body cell dissociation by regulating Mmp expression. We performed a Western blot analysis (2), and detected increased protein levels for both Mmps at 6 h APF in the fat body of animals lacking JH signaling, including Aug21-Gal4 > UAS-grim, Met27 gce2,5k, and Kr-h1k04411, whereas protein levels decreased at 12 h APF in the fat body of the Kr-h1–overexpressing animals, Lsp2-Gal4 > UAS-Kr-h1 (Fig. 1B).

Using quantitative real-time PCR (qPCR), we also detected up-regulated mRNA levels for both Mmps at 6 h APF in the fat body of animals lacking JH signaling and down-regulated mRNA levels at 12 h APF in the fat body of the Kr-h1–overexpressing animals (Fig. 1, C and D).

After examining the role of Kr-h1 in regulating Mmp expression and fat body cell dissociation, we determined its developmental profile in the fat body. We previously reported that the JHRR-LacZ reporter recapitulates the responsiveness of Kr-h1 to JH and Met/gce (24). Immunohistochemistry indicated that JHRR-LacZ was non-detectable in the fat body of the feeding larvae, emerged upon the initiation of wandering (IW), peaked at 3 h after IW (AIW), gradually decreased from 6 h AIW to the white prepupal stage (WPP), and became barely detectable at 3 h APF (Fig. 1E). These results were consistent with previous reports describing developmental profiles for JH titers (21, 30).

Interestingly, we found that the developmental profile of Kr-h1 expression was several hours delayed when compared with that of JHRR-LacZ. Kr-h1 mRNA levels in the fat body of w1118 animals gradually increased upon IW to 3 h APF but dramatically decreased thereafter (Fig. 1F). The developmental profile for Kr-h1 mRNA expression corroborates previous findings (22, 31), indicating that it should be activated by both JH and 20E with overlapping effects. The detailed molecular mechanisms by which JH and 20E coordinate Kr-h1 expression warrant further investigation. Nevertheless, the experimental data show that the anti-metamorphic factor Kr-h1 transduces JH signaling to repress Mmp expression and prevent fat body cell dissociation during the larval–prepupal transition in Drosophila.

Identification of KBS in Mmp1 promoter

We next examined whether Kr-h1 overexpression in Drosophila Kc cells represses Mmp expression to the same extent as that in the fat body. Although Mmp2 expression was not significantly repressed, the down-regulation of Mmp1 expression was significant (Fig. 2A). Thus, we focused on investigating how Kr-h1 represses Mmp1 expression in Kc cells.
Figure 1. Kr-h1 transduces JH signaling to repress Mmp expression. A, compared with w1118 animals, fat body cell dissociation in JH signaling-deficient animals at 6 h APF (left) and Kr-h1–overexpressing animals at 12 h APF (right). B, compared with w1118 animals, Mmps protein levels in the fat body of JH signaling-deficient animals at 6 h APF (left) and in the fat body of Kr-h1–overexpressing animals at 12 h APF (right). C and D, compared with w1118 animals, Mmp mRNA levels in the fat body of JH signaling-deficient animals at 6 h APF (C) and in the fat body of Kr-h1–overexpressing animals at 12 h APF (D). E and F, developmental profiles of JHRR-LacZ (E), JH levels, Kr-h1 mRNA levels in fat body, and fat body cell dissociation (F) of w1118 animals at 3-h intervals. In F, the JH titer is depicted according to Dubovsky 2005 (30); the columns show mRNA level of Kr-h1, and the curve shows the degree of fat body dissociation.
To identify the Kr-h1 response region in the Mmp1 promoter, we employed a dual luciferase assay system and Kc cells. A 3-kb promoter region of Mmp1 (−3000 to 0 upstream of the transcriptional start site) was cloned into the pGL3-Basic vector. Upon Kr-h1 overexpression, this promoter region exhibited ~25% of the luciferase activity achieved with EGFP overexpression (Fig. 2B). Five truncated regions were also cloned into the pGL3-Basic vector, and Kr-h1 overexpression inhibited three truncated regions (−3000 to −1000, −2000 to 0, and −2000 to −1000) the same degree as the 3-kb promoter region (Fig. 2B). Considering some preliminary ChIP and qPCR results, we deleted two small fragments (−1770 to −1530 and −1473 to −1308) from the −2000 to −1000 region in the pGL3-Basic vector. Although the former construct still exhib-
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iterated similar inhibitory responses, the latter showed no response to Kr-h1 overexpression (Fig. 2B). The −1473 to −1308 fragment was then cloned into the pGL3-promoter vector, which responded to Kr-h1 inhibition (Fig. 2C).

KBs were previously identified in the promoter regions of Br-C and E93 in the silkworm (27, 28), and we searched to determine whether the consensus sequence TGACCTNNNYAC was also conserved in the −1473 to −1308 region of the Drosophila Mmp1 promoter. We found a sequence, CTCAATAACCTATGCCACAT (−1407 to −1427), that was similar to the consensus sequence above (Fig. 2D). After the core consensus sequence TACCTA was deleted from the −2000 to −1000 fragment containing pGL3-Basic vector, the promoter region did not respond to Kr-h1 inhibition any longer (Fig. 2B). Then either one copy or four copies of CTCAATAACCTATGCCACAT were individually cloned into the pGL3-promoter vector. One copy of the sequence produced an inhibitory response similar to the −1473 to −1308 fragment, whereas four copies of the sequence resulted in luciferase activity that was reduced by half compared with that with EGFP overexpression (Fig. 2C), suggesting the sequence CTCAATAACCTATGCCACAT is a true KBS in the Mmp1 promoter.

To validate this hypothesis, we performed EMSA with three tandem repeats of CTCAATAACCTATGCCACAT and His tag–purified Kr-h1 protein. Importantly, Kr-h1 and CTCAATAACCTATGCCACAT formed a protein–DNA complex. In competition assays, the specific band disappeared upon the addition of 100-fold molar excess of an unlabeled probe but not a nonspecific competitor (Fig. 2E). Thus, our experimental data identified a true KBS in the Mmp1 promoter. In conclusion, Kr-h1 transduces JH signaling to repress fat body cell dissociation through the direct inhibition of Mmp1 expression during the larval–prepupal transition.

E75, DHR3, and Blimp-1: desirable regulation of b†tz-F1 and Mmps

Previous reports indicate the 20E primary-response gene E75 prevents DHR3 transactivation of b†tz-F1 expression (9–13). Therefore, we verified whether the 20E-triggered transcriptional cascade, involving E75, DHR3, and b†tz-F1, was conserved in the regulation of fat body cell dissociation. Although fat body dissociation visually appeared to be reduced upon E75A overexpression or DHR3 depletion alone (Fig. S3), only the combination of both treatments inhibited the tissue dissociation significantly when quantified (Fig. 4A). By contrast, depletion of E75 or overexpression of DHR3 in the fat body led to increased tissue dissociation at 6 h APF, and combined manipulation of both genes had an additive effect (Fig. 4A and S3).

We then investigated the role of E75 and DHR3 in regulating the expression of E75, DHR3, b†tz-F1, and Mmps in the fat body. At 12 h APF, E75A overexpression had no effect on DHR3 expression but significantly repressed the expression of b†tz-F1 and Mmps; DHR3 RNAi had no effect on E75 expression but significantly repressed the expression of b†tz-F1 and Mmps; and simultaneous E75A overexpression and DHR3 RNAi exerted additive effects (Fig. 4B). By contrast, at 6 h APF, E75 RNAi had no effect on DHR3 expression but significantly induced the expression of b†tz-F1 and Mmps; DHR3 overexpression had no effect on E75 expression but significantly induced the expression of b†tz-F1 and Mmps, and simultaneous E75 RNAi and DHR3 overexpression exerted additive effects (Fig. 4C).

The 20E primary-response gene Blimp-1 acts as a transcriptional repressor for b†tz-F1 (14). When Blimp-1 expression was inhibited by RNAi (Lsp2-Gal4>UAS-Blimp-1–dsRNA) or Blimp-1 was overexpressed (Lsp2-Gal4>UAS-Blimp-1), fat body cell dissociation was inhibited at 12 h APF, respectively (Fig. 4A and S4). Blimp-1 RNAi increased the expression of b†tz-F1 and Mmps, whereas Blimp-1 overexpression had the opposite effect. In addition, Blimp-1 overexpression in Kc cells repressed the expression of b†tz-F1 and Mmps (Fig. 4D).

To gain additional insights into the regulation of fat body cell dissociation by E75, DHR3, and Blimp-1, we determined developmental profiles of mRNA expression in the fat body at 3-h intervals. Expression of E75, Blimp-1, and DHR3 peaked at 6 h AIW, WPP, and 3 h APF, respectively (Fig. 4E). Thus, we assume that E75 represses DHR3 transactivation of b†tz-F1 expression, and Blimp-1 directly represses b†tz-F1 expression during the larval–prepupal transition; moreover, DHR3 directly induces b†tz-F1 expression during the prepupal–pupal transition.

EcR and b†tz-F1 are required for Mmp expression

To better understand how the two 20E pulses regulate fat body cell dissociation during Drosophila metamorphosis, we first re-examined the roles of EcR and b†tz-F1 in this process (3, 15). When a dominant-negative mutant of EcR (EcR<sup>DN</sup>) was overexpressed (Lsp2-Gal4>UAS-EcR<sup>DN</sup>) or b†tz-F1 expression was reduced by RNAi (Lsp2-Gal4>UAS-b†tz-F1–dsRNA), fat body cell dissociation was inhibited at 12 h APF. By contrast, when b†tz-F1 was overexpressed (Lsp2-Gal4>UAS-b†tz-F1), fat body cell dissociation increased 10-fold at 6 h APF (Fig. 3A and Fig. S2). According to the results of Western blotting analyses, protein levels of both Mmps decreased in EcR<sup>DN</sup>-overexpressing and b†tz-F1-RNAi fat body and increased in b†tz-F1-overexpressing fat body (Fig. 3B). The results of qPCR analysis revealed that mRNA levels of both Mmps were similarly regulated by EcR and b†tz-F1 (Fig. 3, C and D). It is of note that the regulatory effect of EcR and b†tz-F1 on Mmp2 is much stronger than Mmp1 (Fig. 3, B–D). Significantly, b†tz-F1 mRNA levels in the fat body were comparatively low from 1W to 3 h APF, sharply increased until 9 h APF, and decreased at 12 h APF (Fig. 3E), indicating that the developmental profile of b†tz-F1 mRNA is similar to but slightly ahead of that for fat body cell dissociation. Finally, we determined whether b†tz-F1 overexpression in Kc cells increased Mmp expression to the same extent as that in the fat body. Again, expression of both Mmps was up-regulated, but the up-regulation of Mmp2 expression was much more significant (Fig. 3F). We next investigated whether E75, DHR3, and Blimp-1 convert signals of the first 20E pulse to b†tz-F1 at the second 20E pulse to induce Mmp expression and thus Mmp–induced fat body cell dissociation before pupation.
Identification of a F1-binding site (FBS) in Mmp2 promoter

To this end, we examined whether βtzt-F1 induces Mmp2 expression directly or indirectly using the dual luciferase assay system and Kc cells. A 3-kb Mmp2 promoter region was cloned into the pGL3-Basic vector. Upon βtzt-F1 overexpression, this promoter region supported an ~4-fold increase in luciferase activity (Fig. 5A). Five truncated regions (−3000 to −1000, −2000 to 0, −3000 to −2000, −2000 to −1000, and −1000 to 0) were cloned into the pGL3-Basic vector. Based on the results of dual luciferase assay, βtzt-F1 response elements exist in a distal region of the Mmp2 promoter (−3000 to −2000) (Fig. 5A).

Previous studies suggest the monomeric FBS consensus sequence is PyCAAGGPyCPu or PyGAAGGPyCPu (36). Three possible FBSs were predicted in the 3-kb promoter region of Mmp2: GGAAGGTCA (−2604 to −2595), AGGCCTTGA (−2326 to −2317), and TCAAGGCTG (−1254 to −1263) (Fig. 5B). After FLAG-βtzt-F1 was overexpressed in Kc cells, ChIP-qPCR was performed to examine whether βtzt-F1 directly binds to these potential FBSs to induce Mmp2 expression. As measured by qPCR, a FLAG antibody increased the precipitation of the first possible FBS but not the other two (Fig. 5C).
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A 12h APF

B 12h APF

C 6h APF

D 6h APF

E 6h APF
To verify that this sequence was a genuine FBS, we examined whether three tandem repeats of TGGGGGAAGGTCAAAT (\(\text{H11002}_{2607}\) to \(\text{H11002}_{2592}\)) bound to GST-purified \(\text{ftz-F1}\) protein in an EMSA experiment (Fig. 5D). TGGGGGAAGGTCAAAT formed a protein–DNA complex with a specific band shift that was supershifted by the addition of a \(\text{ftz-F1}\) antibody.

Figure 4. \(\text{E75, DHR3, and Blimp-1 regulate the expression of Mmps and ftz-F1.}\) A, fat body cell dissociation in animals in which \(\text{E75A}\) was overexpressed, \(\text{DHR3}\) expression was reduced by RNAi, combined \(\text{E75A}\) overexpression and \(\text{DHR3}\) RNAi, and \(\text{Blimp}\) was overexpressed at 12 h APF (left panel). Fat body cell dissociation in animals in which \(\text{DHR3}\) was overexpressed, \(\text{E75}\) expression was reduced by RNAi, combined \(\text{DHR3}\) overexpression and \(\text{E75}\) RNAi, and \(\text{Blimp}\) expression was reduced by RNAi at 6 h APF (right panel). B, \(\text{ftz-F1}\) and \(\text{Mmp}\) expression in the fat body of \(\text{E75A}\) overexpression (left panel), \(\text{DHR3}\) RNAi (middle panel), and combined \(\text{E75}\) RNAi and \(\text{DHR3}\) overexpression animals (right panel). D, \(\text{ftz-F1}\) and \(\text{Mmp}\) expression levels in the \(\text{Blimp}\) RNAi fat body (left panel); \(\text{ftz-F1}\) and \(\text{Mmp}\) expression in the \(\text{Blimp}\) overexpressing fat body (middle panel) and \(\text{Kc}\) cells (right panel). E, the columns show developmental changes in mRNA levels of \(\text{E75}\) (left panel), \(\text{Blimp}\) (middle panel), and \(\text{DHR3}\) (right panel) in the fat body, and the curve shows the degree of fat body dissociation in \(\text{w}^{\text{1118}}\) animals at 3-h intervals.

Figure 5. Identification of a FBS in \(\text{Mmp2}\) promoter. A, dual luciferase assay. Kc cells were co-transfected with a \(\text{ftz-F1}\) expression construct (EGFP was used as a control), along with pCI3-basic plasmids containing \(\text{Mmp2}\) promoter regions of different lengths. After 48 h of transfection, dual luciferase assays were performed. The luciferase activity fold change is defined as the relative luciferase activity induced by \(\text{ftz-F1}\) overexpression compared with EGFP overexpression. B, locations of three \(\text{ftz-F1}\) putative binding sites within the \(\text{Mmp2}\) 3-kb promoter. C, ChIP-qPCR. At 48 h after transfection, FLAG-\(\text{ftz-F1}\)-overexpressing Kc cells were fixed and subjected to ChIP using a FLAG mouse monoclonal antibody. Mock immunoprecipitations with preimmune serum were performed as negative controls. The precipitated DNA (different fragments in the 3-kb promoter region of \(\text{Mmp2}\)) and input were analyzed by qPCR to detect binding ability. D, EMSA. We used a three times repeated TGGGGGAAGGTCAAAT sequence (KBS), corresponding to a site located in the \(\text{H11002}_{2604}\) to \(\text{H11002}_{2595}\) region upstream of the \(\text{Mmp2}\) transcription start site. A Cy5-labeled \(\text{ftz-F1}\) binding site was added to a mixture with GST-\(\text{ftz-F1}\) fusion proteins, in the presence (lane 4) or absence (lane 5) of an unmodified competitor. A supershift band was observed when a FTZ-F1 antibody was added into the reaction mixture.
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Competition assays showed that the specific band disappeared upon the addition of 100-fold molar excess of an unlabeled probe but not a nonspecific competitor. Thus, a genuine FBS was identified from the Mmp2 promoter.

Altogether, βftz-F1 expression is activated during the prepupal–pupal transition because of elaborate control exerted by the 20E–triggered transcriptional cascade, including E75, Blimp-1, and DHR3. When JH titer declines, the prepupal peak of 20E suppresses Mmp–induced fat body cell dissociation through the 20E primary-response genes, E75 and Blimp-1, which inhibit βftz-F1 expression indirectly or directly. Until 20E titer declines, βftz-F1 expression is induced by the 20E early–late response gene DHR3; then βftz-F1 directly activates Mmp expression and causes Mmp–induced fat body cell dissociation occurring from 6 h APF to 12 h APF.

Kr-h1 activates and βftz-F1 inhibits timp expression

As mentioned in our previous reports (2), overexpression of timp in the fat body (Lsp2-Gal4>UAS-timp) completely blocks its cell dissociation, resulting in pupal lethality. By contrast, precocious fat body cell dissociation was observed in the timp mutant (timp^28) (Fig. S5B). The above results agree with the notion that timp inhibits the enzymatic activity of Mmps in the fat body (2). Thus, we investigated whether and how JH- and 20E-mediated regulation of timp expression and thus the enzymatic activity of Mmps.

The mRNA levels of timp were down-regulated at 6 h APF in the fat body of animals lacking JH signaling and up-regulated at 12 h APF in the fat body of Kr-h1–overexpressing animals (Fig. 6A). By contrast, the mRNA levels of timp were up-regulated in EcR_{DN}–overexpressing and ftz-F1-RNAi fat body at 12 h APF and down-regulated in βftz-F1-overexpressing fat body at 6 h APF (Fig. 6B). The experimental data indicate that Kr-h1 transduces JH signaling to activate and βftz-F1 mediates 20E signaling to inhibit timp expression, which could inhibit the enzymatic activity of Mmps and thus fat body cell dissociation.

Finally, we examined the enzymatic activity of Mmps in the above genotypes. Mmps activity significantly increased at 6 h APF in the fat body of the timp mutant, animals lacking JH signaling, and βftz-F1-overexpressing animals and decreased at 12 h APF in the fat body of timp-overexpressing, EcR_{DN}–overexpressing, βftz-F1-RNAi, and Kr-h1–overexpressing animals (Fig. 6C). Thus, the enzymatic activity of Mmps for each genotype results from changes in the expression of both Mmps and timp. In summary, JH and 20E coordinately and precisely control Mmps activity at both mRNA and enzymatic levels so that fat body cell dissociation occurs within 6 h before pupation (Fig. 7).

Discussion

MMPs and TIMPs play crucial roles in regulating tissue remodeling in both vertebrates and Drosophila (4–6). Our previous work has demonstrated the collaborative functions of Mmp1 and Mmp2 in inducing fat body cell dissociation in Drosophila (2). timp mutant adults show autolized tissue in the abdominal cavity and inflated wings, a phenotype consistent with the role of timp in BM integrity and remodeling (33). The current study clarified the role of timp in inhibiting the enzymatic activity of Mmps and thus, Mmp–induced fat body cell dissociation (Fig. 6 and Fig. S4). In mammals, Mmps activity in vivo is controlled at different levels, including the regulation by gene expression, the zymogens activation, and the inhibition of active enzymes by TIMPs (33, 34). These studies unify the important inhibitory roles of timp/TIMP in regulating tissue remodeling in both Drosophila and mammals. In addition to regulating Mmp expression (Figs. 1–5), JH and 20E signals differentially regulate timp expression, with the stimulatory role of Kr-h1 and the inhibitory role of βftz-F1 (Fig. 6). Because timp inhibits the enzymatic activity of Mmps in the Drosophila fat body (Fig. 6), we conclude that JH and 20E coordinately control Mmps activity at both the mRNA and enzymatic levels (Fig. 7).

We previously reported the requirement of both JH and its receptors to inhibit fat body cell dissociation in Drosophila (19, 29). Here, we demonstrated the ability of Kr-h1 to transduce JH signaling to decrease Mmp expression and to induce timp expression during larval–prepupal transition (Figs. 1 and 6). Moreover, a KBS was identified in the Mmp1 promoter, indicating that Kr-h1 directly represses Mmp1 expression (Fig. 2). Interestingly, Kr-h1 expression gradually increases from IW to 3 h APF when induced by JH and 20E in an overlapping manner (Fig. 1F), thus inhibiting the enzymatic activity of Mmps and Mmp–induced fat body cell dissociation during the larval–prepupal transition. Moreover, Kr-h1 acts as an anti-metamorphic factor by inhibiting 20E signaling (16, 17). We propose, in addition to directly affecting the expression of Mmps and timp, that Kr-h1 might also indirectly regulate their expression by inhibiting 20E signaling (Fig. 7).

Two consecutive 20E pulses control timely metamorphosis in Drosophila (8). Together with previous findings (3, 15), our results show that the conserved 20E transcriptional cascade precisely controls the timing of Mmp–induced fat body cell dissociation (Fig. 7). In general, the first 20E signal pulse plays an inhibitory role during the larval–prepupal transition; however, it is a prerequisite for the expression of βftz-F1, which induces the second 20E signal pulse during the prepupal–pupal transition and the expression of Mmps. Because of the requirement for the first 20E signal pulse, blockade of the 20E receptor prevents fat body cell dissociation (Fig. 3). When JH titer declines, the prepupal peak of 20E activates expression of two 20E primary-response genes, E75 and Blimp-1, to inhibit fat body cell dissociation: E75 represses DHR3 transactivation of βftz-F1 expression, and Blimp-1 directly represses βftz-F1 expression. During the prepupal–pupal transition, DHR3 directly induces βftz-F1 expression from 6 h APF to 12 APF (Fig. 4). Before pupation, βftz-F1 induces Mmp expression and represses timp expression (Figs. 3 and 6). Moreover, an FBS was identified in the Mmp2 promoter, demonstrating that βftz-F1 directly induces Mmp2 expression (Fig. 5). Finally, within 6 h before pupation, Mmp1 and Mmp2 cooperatively induce fat body cell dissociation, with each assuming a distinct role (2).

Insect metamorphosis is coordinately controlled by JH and 20E, whereas the hormonal control of tissue remodeling is strictly context-specific. Different larval tissues and adult organs might have distinct, yet precise, developmental fates and timing (7, 8, 16, 17). Our knowledge regarding this question is poor. Based on previous preliminary information, we clarified
the detailed molecular mechanisms by which JH and 20E precisely control the developmental timing of Mmp–induced fat body cell dissociation at both mRNA and enzymatic levels in Drosophila, and we provided a working model of hormonal control of tissue remodeling in animals (Fig. 7). In summary, at first, Kr-h1 transduces JH signaling to inhibit Mmp–induced fat body cell dissociation. When JH titer declines, the prepupal peak of 20E suppresses Mmp–induced fat body cell dissociation through the 20E primary-response genes, E75 and Blimp-1, which inhibit βftz-F1 expression indirectly or directly. Until 20E titer declines, βftz-F1 expression is induced by the 20E early–late response gene DHR3; then βftz-F1 directly activates Mmp expression and inhibits timp expression and causes Mmp–induced fat body cell dissociation occurring from 6 h APF to 12 h APF. The JH and 20E titers are depicted according to Dubrovsky 2005 (30).

Figure 7. Model showing developmental timing of Mmp–induced fat body cell dissociation is coordinately and precisely controlled by JH and 20E in Drosophila. During larval–prepupal transition, the anti-metamorphic factor Kr-h1 transduces JH signaling to directly inhibit Mmp expression and to activate timp expression and thus suppresses Mmp–induced fat body cell dissociation. When JH titer declines, the prepupal peak of 20E suppresses Mmp–induced fat body cell dissociation through the 20E primary-response genes, E75 and Blimp-1, which inhibit βftz-F1 expression indirectly or directly. Until 20E titer declines, βftz-F1 expression is induced by the 20E early–late response gene DHR3; then βftz-F1 directly activates Mmp expression and inhibits timp expression and causes Mmp–induced fat body cell dissociation occurring from 6 h APF to 12 h APF. The JH and 20E titers are depicted according to Dubrovsky 2005 (30).

Figure 6. JH signaling promotes timp expression, and βftz-F1 inhibits timp expression. A, timp expression in the fat body of the JH signaling-deficient animals at 6 h APF (left panel) and in the fat body of Kr-h1–overexpressing animals at 12 h APF (right panel). B, timp expression in the fat body of EcRΔN, overexpressing and βftz-F1 RNAi animals at 12 h APF (left panel) and in the fat body of βftz-F1-overexpressing animals at 6 h APF (upper panel). C, Mmps enzymatic activity in the fat body of JH signaling-deficient animals and βftz-F1-overexpressing animals at 6 h APF (upper panel) as well as in the fat body of EcRΔN-overexpressing, βftz-F1 RNAi, and Kr-h1–overexpressing animals (lower panel).

Quantitative measurements of fat body cell dissociation

The degree of fat body cell dissociation was measured as previously described in detail (2). Fat body tissues at 6 h APF from different genotypes were used to evaluate whether premature fat body dissociation happened. Fat body tissues at 12 h APF from different genotypes were used to evaluate whether delayed fat body cell dissociation happened. In this assay, 10 animals...
were used for each independent genotype, and three independent replications were carried out.

**Cell culture and transient transfection**

*Drosophila* Kc cells were cultured in Schneider’s *Drosophila* medium (Sigma–Aldrich) supplemented with 5% fetal bovine serum (HyClone). The pActin-GAL4 plasmid was constructed in our laboratory. *Kr-h1 and Blimp-1* cDNA was cloned into the pUAST vector to obtain pUAST-Kr-h1-V5 and pUAST-Blimp-1, respectively. The pUAST-3×flag-βtzt-F1 (37) construct was generously provided by Dr. Rosa Barrio. Transient transfection in Kc cells was performed as previously described in detail (2, 24).

**qPCR and Western blotting**

qPCR and Western blotting were performed as described previously (2). Rp49 was chosen as the reference gene for qPCR analysis. Our previous studies showed that Western blotting detected four major bands (46, 52, 64, and 74 kDa) and two major bands (90 and 120 kDa) for Mmp1 and Mmp2, respectively, in the *Drosophila* fat body (2).

**Dual luciferase assay**

To identify KBS, a 3-kb region of the *Mmp1* promoter upstream of the transcription start site was cloned into the Smal and BglII sites of the pGL3-Basic vector. Likewise, to identify βtzt–FBS, a 3-kb region of the *Mmp2* promoter upstream of the transcription start site was cloned into the Smal and BglII sites of the pGL3-Basic vector. Deletions and mutations in *Mmp1* promoter regions were also constructed in the pGL3-Basic vector. The −1473 to −1308 fragment, the KBS CTCATAACCTATGCCACAT and 4×CTCATAACCTATGCCACAT sequence were cloned into the Smal and BglII sites of the pGL3-promoter vector. After transient transfection into Kc cells with the pGL3 reporter vector and the pRL reference vector, dual luciferase assays were performed as previously described (13, 24, 39).

**Electrophoretic mobility shift assay**

*Kr-h1* cDNA was inserted into a pET28a vector with a 6×His tag at the N terminus. Then the plasmid was transformed into the *Escherichia coli* Rosetta strain. The transformed bacterial cells were grown in LB medium supplemented with kanamycin (0.1 mg/ml) at 37 °C and induced by 0.25 mM isopropyl β-D-thiogalactopyranoside for 12 h at 16 °C. The cells were harvested and resuspended in buffer A (20 mM Tris–HCl (pH 8.0) and 100 mM NaCl) supplemented with 1 mM PMSF. Cells were lysed using a high-pressure cell disruptor at 18,000 p.s.i., and the lysate was centrifuged at 16,000 × g for 45 min. The supernatant was loaded onto a Ni2+–nitrilotriacetic acid affinity column (Qiagen) and washed with buffer A plus 20 mM imidazole. Proteins were eluted with buffer A plus 250 mM imidazole and purified further by gel filtration using a Superdex 200 column (GE Healthcare) in buffer A. Peak fractions were collected and concentrated for subsequent studies (40). βtzt-F1 cDNA was cloned into the pGEX-4T-1 vector, and GST-βtzt-F1 proteins were expressed and purified in Rosetta cells using standard methods as previously described (32). EMSA was performed according to our previous publication (41).

**Hormonal control of Mmp–induced fat body cell dissociation**

At 48 h after transfection, 3×FLAG-βtzt-F1-overexpressing Kc cells were fixed and subjected to ChIP using the Pierce™ agarose ChIP kit (26156; Thermo) and the FLAG mouse monoclonal antibody (F3156; Sigma–Aldrich). Mock immunoprecipitations with preimmune serum were performed as negative controls. The precipitated DNA and input were analyzed by qPCR to detect binding ability (13, 39).

**Statistics**

Experimental data were analyzed with analysis of variance and Student’s t test. Analysis of variance is shown as the bars labeled with different lowercase letters as significantly different (p < 0.05; t test: *, p < 0.01; ***, p < 0.001). Throughout the paper, values are represented as the means ± standard deviation of 3–10 independent experiments.

**Author contributions**—S. Li, J. W., and Q. J. conceived the study and wrote the paper. Q. J., S. Liu, D. W., Y. C., W. G. B., and S. Li performed and analyzed the experiments. All authors reviewed the results and approved the final version of the manuscript.

**References**

Hormonal control of Mmp–induced fat body cell dissociation