Juvenile hormone (JH) has a well known role in stimulating insect vitellogenesis (i.e., yolk deposition) and oocyte maturation, but the molecular mechanisms of JH action in insect reproduction are unclear. The 78-kDa glucose-regulated protein (Grp78) is a heat shock protein 70-kDa family member and one of the most abundant chaperones in the endoplasmic reticulum (ER) where it helps fold newly synthesized peptides. Because of its prominent role in protein folding, and also ER stress, we hypothesized that Grp78 might be involved in fat body cell homeostasis and vitellogenesis and a regulatory target of JH. We report here that the migratory locust Locusta migratoria possesses two Grp78 genes that are differentially regulated by JH. We found that Grp78-1 is regulated by JH through Mcm4/7-dependent DNA replication and polypolyidization, whereas Grp78-2 expression is directly activated by the JH-receptor complex comprising methoprene-tolerant-Taiman and Taiman proteins. Interestingly, Grp78-2 expression in the fat body is about 10-fold higher than that of Grp78-1. Knockdown of either Grp78-1 or Grp78-2 significantly reduced levels of vitellogenin (Vg) protein, accompanied by retarded maturation of oocytes. Depletion of both Grp78-1 and Grp78-2 resulted in ER stress and apoptosis in the fat body and in severely defective Vg synthesis and oocyte maturation. These results indicate a crucial role of Grp78 in JH-dependent vitellogenesis and egg production. The presence and differential regulation of two Grp78 genes in L. migratoria likely help accelerate the production of this chaperone in the fat body to facilitate folding of massively synthesized Vg and other proteins.

Juvenile hormone (JH), a sesquiterpenoid secreted by corpora allata, controls many aspects of insect physiology including metamorphosis and reproduction. During the larval stage, JH prevents metamorphosis by modifying the action of 20-hydroxyecdysone at each molting (1, 2). Recent studies have revealed that JH exerts its anti-metamorphic role via its receptor, methoprene-tolerant (Met) (3, 4). Upon JH induction, Met heterodimerizes with Taiman (Tai) to transcriptionally activate Kriüppel homolog 1 (Kr-h1) (5–8), which represses the expression of genes coding for Broad-complex and E93 (3, 9–12), the triggers of pupal and adult metamorphosis. At the final larval instar, a very low titer or the absence of JH coupled with a peak of 20-hydroxyecdysonic then initiates the larva-pupa or larva-adult transition (1, 2). In adults, JH reappears to stimulate the previtellogenic development, vitellogenesis, and oocyte maturation for reproduction (2, 13–16). Vitellogenesis, the process of yolk protein precursor, vitellogenin (Vg) synthesis in the fat body, secretion into the hemolymph, and uptake by the developing oocytes, is crucial to egg production and later embryonic development (2, 13, 15, 17). JH-dependent vitellogenesis has been reported in many insect species, including the red flour beetle Tribolium castaneum, the lindeng bug Pyrrhocoris apterus, the German cockroach Blattella germanica, the Pacific beetle cockroach Diploptera punctata, and the migratory locust Locusta migratoria (2, 13, 15, 17–20). In adult female locusts, synthesis and secretion of proteins by the fat body is increased up to 20-fold during the vitellogenic phase and Vg makes up 60% of the secreted proteins (2, 21). To facilitate massive Vg synthesis, the fat body undergoes JH-dependent build-up of cellular structures such as endoplasmic reticulum (ER) and ribosomes (2). Thus, JH may act on certain genes directly and others through a cascade of effects to maintain cell homeostasis.

The 78-kDa glucose-regulated protein (Grp78), also known as immunoglobulin heavy-chain binding protein (BiP), is a member of heat shock protein 70-kDa family (22, 23). As the most abundant chaperone protein in ER lumens, Grp78 binds to newly synthesized peptides to facilitate them folding (23, 24). Surplus Grp78 interacts with inositol-requiring enzyme-1 (Ire1), protein kinase RNA-like ER kinase (Perk), and activating transcription factor-6 (Atf6) to inhibit their activity (25–28). When the unfolded or misfolded proteins accumulate, ER homeostasis is perturbed to a condition referred to as ER stress (26, 27, 29). Release of Grp78 from the complexes with Ire1, Perk, and Atf6 allows these proteins to be activated by

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1 To whom correspondence may be addressed. E-mail: hlan@ioz.ac.cn.
2 To whom correspondence may be addressed. E-mail: szhou@henu.edu.cn.
3 The abbreviations used are: JH, juvenile hormone; Met, methoprene-tolerant; Vg, vitellogenin; ER, endoplasmic reticulum; Grp78, 78-kDa glucose-regulated protein; Ire1, inositol-requiring enzyme-1; Perk, protein kinase RNA-like ER kinase; ATF6, activating transcription factor-6; UPR, unfolded protein response; Mcm, mini-chromosome maintenance; PAE, post-adult eclosion; qRT, quantitative RT; JHA, JH analog; nt, nucleotides(s); Gce, Germ cell-expressed; PDI, protein-disulfide isomerase; eIF2α, eukaryotic initiation factor 2α; Tai, Taiman.

8823 J. Biol. Chem. (2017) 292(21) 8823–8834
Grp78 and JH-regulated vitellogenesis

**Figure 1. Characteristics of two Grp78 genes and their expression profiles in L. migratoria.** A, gene structures of locust Grp78-1 and Grp78-2. B, the phylogenetic tree constructed with the coding sequences of Grp78 genes. Bombyx mori, silkworm; Papilio xuthus, xuthus swallowtail; Helicoverpa armigera, cotton bollworm; Diaphorina citri, Asian citrus psyllid; D. melanogaster, fruit fly; Drosophila grimshawi, Hawaiian fruit fly; Musca domestica, housefly; Ceratitis capitata, mediterranean fruit fly; Anopheles gambiae, malaria mosquito; Anopheles albimanus, another malaria mosquito; Culex quinquefasciatus, Southern house mosquito; Aedes albopictus, Asian tiger mosquito; Aedes aegypti, yellow fever mosquito; Apis dorsata, giant honeybee; Apis mellifera, European honeybee; Oxya chinensis, rice grasshopper; L. migratoria, migratory locust; T. castaneum, red flour beetle. C, relative expression levels of Grp78-1 and Grp78-2 in the selected tissues of adult female locusts at 8 days PAE. Ov, ovary; Fb, fat body; Mg, midgut; Br, brain. n = 12–16. D, absolute quantification of Grp78-1 and Grp78-2 mRNA abundance in the fat body of adult females from 0 to 10 days PAE. For each gene, different letters indicate significant difference in mRNA abundance between the developmental stages. p < 0.05 (n = 12–16).

homodimerization and phosphorylation, which triggers the unfolded protein response (UPR) to restore ER homeostasis via enhancing the folding capacity and reducing the protein load in the ER (26–29). When cells undergo severe or prolonged ER stress, apoptosis is induced (25–27, 30). In mammals, Grp78-related ER stress has been reported in obesity (31, 32), type II diabetes (32–34), cholera-induced liver injury (35, 36), heart ischemia/reperfusion damage (37), atherosclerotic lesions (38), neurodegenerative diseases (39–41), intestinal inflammation (42, 43), and cancer (44). In insects, studies on this chaperone have been limited to *Drosophila melanogaster*, in which Grp78 (also called heat shock 70-kDa protein cognate 3, Hsp70-3 or Hsc3) links to the ER stress and apoptosis in the fat body, which restrains the response of locusts to exogenous application of JH. These results provide new insights into the mechanisms of JH regulation in insect reproduction.

**Results**

**Two Grp78 genes are identified in the migratory locust.**

The sequenced locust genome (53) yielded two Grp78 genes, Grp78-1 (GenBank™ FJ472842) and Grp78-2 (GenBank™ FJ472843). The Grp78-1 gene is 7,113 bp comprised of 11 exons, whereas the Grp78-2 gene is 26,220 bp with 10 exons (Fig. 1A). Grp78-1 and Grp78-2 cDNA are 2,395 and 2,583 bp, respectively, and their proteins share 87% identity of amino acids. Phylogenetic tree construction revealed that Grp78 genes are evolutionally conserved, and locust Grp78-1 is closer to Grp78 of the rice grasshopper *Oxya chinensis* (Fig. 1B). Intriguingly, a single Grp78 gene is reported in other insect species available in the databases including NCBI, FlyBase, and VectorBase. The presence of two Grp78 genes in *L. migratoria* might...
Grp78 and JH-regulated vitellogenesis

Figure 2. Responsiveness of Grp78-1 and Grp78-2 to JH and Met. A and B, relative mRNA levels of Grp78-1 (A) and Grp78-2 (B) in the fat body of adult females treated with precocene for 10 days (P) and those further treated with methoprene or acetone (solvent control) for 12–48 h. PAE10, 10-day-old adult females as the positive control. *, p < 0.05 and **, p < 0.01 (n = 8), C, Met knockdown efficiency in the fat body of dsMet-injected adult females (iMet) at 4–8 days PAE. **, p < 0.01 and ***, p < 0.001 compared with the respective dsGFP controls (iGFP) (n = 8). D, effect of Met RNAi on the expression of Grp78-1 and Grp78-2 in the fat body on day 4–8. *, p < 0.05; **, p < 0.01; and ***, p < 0.001 compared with the respective dsGFP controls (n = 8).

Grp78-1 and Grp78-2 are expressed in response to JH and Met

To evaluate the responsiveness of Grp78 to JH, qRT-PCR was carried out using total RNA from the fat body of precocene-treated adult females for 10 days as well as those further treated with an active JH analog (JHA), methoprene for 12–48 h. Depivation of endogenous JH by ablation of corpora allata with precocene treatment caused 65 and 76% reduction of Grp78-1 and Grp78-2 mRNA levels, respectively (Fig. 2, A and B). Further application of methoprene led to a 3.5-fold increase of Grp78-1 mRNA levels at 48 h (Fig. 2A). Interestingly, Grp78-2 mRNA levels were significantly increased by 1.6-fold at 12 h post-methoprene treatment, and continually elevated by 3.2-fold at 24 h and 5.0-fold at 48 h (Fig. 2B), suggesting an earlier responsiveness of Grp78-2 to JHA. In the parallel experiment of solvent controls, acetone treatment had no significant effect on Grp78 expression (Fig. 2, A and B). We next performed Met RNAi to assess the requirement of JH receptor for the JH-dependent expression of Grp78-1 and Grp78-2. qRT-PCR demonstrated that 76–86% of Met knockdown efficiency was achieved in the fat body of dsMet-injected (iMet) adult females at 4–8 days PAE (Fig. 2C). When Met was silenced, the mRNA levels of Grp78-1 and Grp78-2 were reduced by 35–64 and 45–87%, respectively, on days 4–8 (Fig. 2D). Taken together, these data suggest that the expression of Grp78-1 and Grp78-2 are in response to JH and its receptor, Met.

Differential regulation of Grp78-1 and Grp78-2 by JH

Previously, the core E-box and E-box-like motifs with variable nucleotides at their flanking regions has been reported as the JH response elements recognized by Met (6, 14, 50, 51, 56–58). We therefore analyzed the 3-kb upstream sequences of both Grp78-1 and Grp78-2 genes. An E-box-like motif reflects an evolutionary gene duplicate in this primitive insect species.

The first gonadotrophic cycle of adult female locusts was ~11 days and vitellogenesis started from ~5 days post-adult eclosion (PAE). To explore the spatial expression patterns of two Grp78 genes in locusts, qRT-PCR was conducted using total RNA from the fat body (Fb), ovary (Ov), midgut (Md), and brain (Br) of adult females collected at 8 days PAE. As shown in Fig. 1C, Grp78-1 and Grp78-2 were expressed at similar levels in these selected tissues. As the fat body is the primary tissue for protein synthesis during vitellogenesis, we next performed absolute quantitative RT-PCR to compare the transcript levels of Grp78-1 and Grp78-2 in the fat body of adult females from 0 (the day of eclosion) to 10 days PAE. For developmental profiles, Grp78-1 mRNA levels in the fat body were significantly increased by 3.1-fold at 4 days PAE compared with that at 0 day PAE, then declined but remained significantly elevated (2.2–2.5-fold) on days 6–10 (Fig. 1D). With respect to Grp78-2, its transcript levels were significantly increased by 2.8-fold at 2 days PAE, and remained high (2.2–4.0-fold) on days 4–10 (Fig. 1D). As locust hemolymph JH titer is undetectable at eclosion but elevates significantly in the previtellogenic stage and rises to a peak during vitellogenesis (54, 55), the increase of Grp78 mRNA levels appeared to correlate with the phase of increased JH titers. Although Grp78-1 and Grp78-2 had similar expression patterns from 0 to 10 days PAE, 1 μg of total RNA of fat bodies appeared to have 0.7–2.1 × 10^5 copies of Grp78-1 mRNA and 6.0–24.4 × 10^5 copies of Grp78-2 mRNA, respectively (Fig. 1D). The data indicate that the abundance of Grp78-2 mRNA was about 10-fold higher than that of Grp78-1.
Grp78 and JH-regulated vitellogenesis

Figure 3. Differential regulation of Grp78-1 and Grp78-2 by JH. A, alignment of DNA sequences containing E-box-like motif in the promoters of Kr-h1, ribosomal protein S28 (Rps28), and Early trypsin (Et) from the mosquito A. aegypti (Aa), Kr-h1 from D. melanogaster (Dm), as well as cell-division-cycle 6 (Cdc6), Mcm4, and Grp78-2 from the migratory locust L. migratoria (Lm) (14, 50, 51, 56, 57, 88). B, relative expression levels of DmMet, DmGce, and DmTai in S2 cells treated with Drosophila Met and Tai dsRNA (iDmMet + iDmTai). *, p < 0.05; **, p < 0.01 compared with the iGFP control (n = 3). C, Western blot analysis (WB) and immunoprecipitation (IP) showing the expression of FLAG-Met (pAc5.1/Flag-Met1–3108) and V5-Tai (pAc5.1/V5-Tai1–1785 plus 4581–4961); Tai-A variant with the INDEL-1/PRD motif (52). In Grp78-1 and Grp78-2 expression in the fat bodies of 6-day-old adult females. *, p < 0.05; **, p < 0.01 compared with the respective iGFP controls (n = 8).

(CACGCC; nt −1661 to −1656) was identified in the promoter of Grp78-2 (Fig. 3A). However, neither E-box nor the E-box-like motif were found in the 3-kb upstream of Grp78-1. Interestingly, the flanking regions of E-box-like motif of Grp78-2 share high nucleotide identity to that of locust MCM4 (Fig. 3A), which has been experimentally demonstrated for binding by the JH-receptor complex (50). To determine whether the JH-receptor directly activates the transcription of Grp78-2, we performed luciferase assays using S2 cells co-transfected with the wild-type or mutational pGL4.10/Grp78-2 (−2068 to −24) plus pAc5.1/Flag-Met1–3108 and/or pAc5.1/V5-Tai1–1785 plus 4581–4961. It must be noted that this Tai variant is Tai-A isofrom with the INDEL-1/PRD motif (52). In Drosophila, Met has a paralogue, Germ cell-expressed (Gce; FlyBase: FBpp0292296), which shares about 40% identity of amino acids (59, 60). To diminish the interference of endogenous Met, Gce, and Tai, S2 cells were treated with dsRNA of Drosophila Met and Tai (iDmMet + iDmTai) prior to transfection of the recombinant constructs. As shown in Fig. 3B, the mRNA levels of Drosophila Met, Gce, and Tai were reduced by 62, 55, and 57%, respectively (Fig. 3B). Immunoprecipitation and Western blot analysis demonstrated that the expressed FLAG-Met and V5-Tai were dimerized in the presence of methoprene (JHA). aFLAG, anti-FLAG antibody; α-V5, anti-V5 antibody; α-GAPDH, anti-GAPDH antibody. D, luciferase reporter assays using S2 cells co-transfected with pGL4.10/Grp78-2 (−2068 to −24) plus pAc5.1 empty vector (control), co-transfected pGL4.10/Grp78-2 (−2068 to −24) + pAc5.1/Flag-Met1–3108 (Met), pGL4.10/Grp78-2 (−2068 to −24) + pAc5.1/V5-Tai1–1785 plus 4581–4961 (V5-Tai). E, effect of Mcm4 RNAi (iMcm4) or Mcm7 RNAi (iMcm7) on Grp78-1 and Grp78-2 expression in the fat bodies of 6-day-old adult females. *, p < 0.05; **, p < 0.01; n.s., no significant difference compared with the respective iGFP controls (n = 8).

In an earlier report (50), we have demonstrated that the JH-receptor acts on MCM4 and MET via Mcm4 and Mcm7. To investigate the dependence of Grp78-1 expression on Mcm4 and Mcm7, we performed RNAi of Mcm4 (iMcm4) and Mcm7 (iMcm7) using the fat body from 6-day-old adult females. qRT-PCR showed that 97 and 84% knockdown efficiency were obtained for Mcm4 and Mcm7 (Fig. 3E), respec-
Figure 4. Effects of Grp78 depletion on vitellogenesis and oocyte maturation. A–C RNAi efficiency of Grp78-1 and Grp78-2 in the fat body of 6-day-old adult females treated with dsRNA shared by both Grp78-1 and Grp78-2 (iGrp78), Grp78-1-specific dsRNA (iGrp78-1), or Grp78-2-specific dsRNA (iGrp78-2). D, Western blot analysis (left panel) and the quantitative analysis of band intensity by ImageJ (right panel) on VgA in the fat body and ovary after iGrp78, iGrp78-1, or iGrp78-2. 6d PAE, the adult females at day of eclosion was used as a negative control. Different letters indicate significant difference at p < 0.05 (n = 3). E, morphology changes of ovaries (Ov), primary oocytes (Po), and follicle cells (Fc) after iGrp78, iGrp78-1, or iGrp78-2 at 6 days PAE. Scale bar: Ov, 2.5 mm; Po, 500 μm; Fc, 10 μm. Blue, follicle cell nuclei; green, F-actin. Yellow arrows indicate the patency.

Grp78 knockdown suppresses locust vitellogenesis and oocyte maturation

To evaluate the function of Grp78 in JH-mediated vitellogenesis and oocyte maturation, we conducted RNAi via injection of dsRNA shared by both Grp78-1 and Grp78-2 (iGrp78), Grp78-1-specific dsRNA (iGrp78-1), or Grp78-2-specific dsRNA (iGrp78-2). As shown in Fig. 4A, iGrp78 gave rise to 56 and 99% reduction of Grp78-1 and Grp78-2 expression, respectively, at 6 days PAE. iGrp78-1 caused 87% reduction of Grp78-1 abundance, but had no significant effect on Grp78-2 expression (Fig. 4B). iGrp78-2 only significantly reduced the expression of Grp78-2, with 98% knockdown efficiency (Fig. 4C). The migratory locust has two coordinately expressed genes, VgA (GenBankTM KF171066) and VgB (GenBankTM KX709496) that are expressed in similar patterns (50, 51). VgA was selected as a representative. Western blot analysis and subsequent quantification of band intensity demonstrated that depletion of both Grp78-1 and Grp78-2 caused 99.1 and 99.9% reduction of VgA protein levels in the fat body and ovary, respectively (Fig. 4D). When Grp78-1 was knocked down alone, VgA protein levels were decreased by 84.6% in the fat body and 97.8% in the ovary (Fig. 4D). Grp78-2 RNAi reduced VgA proteins to 7.8 and 1.1% of the control levels in the fat body and ovary, respectively (Fig. 4D).

We next examined the phenotypes of Grp78 knockdown on oocyte maturation and ovarian development. Knockdown of both Grp78-1 and Grp78-2 resulted in blocked maturation of primary oocytes and arrested development of ovaries in all experimental locusts. Consequently, the primary oocytes and ovaries of Grp78-depleted adult females remained small on day 6 (Fig. 4E). In contrast, the primary oocytes and ovaries of iGFP controls were markedly enlarged (Fig. 4E). We measured the length of primary oocyte as an indicator of oocyte growth and maturation. Statistically, the length of primary oocytes of iGrp78 locusts was 0.9 mm, whereas that of iGFP controls was 3.7 mm. The defective phenotypes were also observed in adult females subjected to either Grp78-1 or Grp78-2 RNAi, but it was less severe than that of Grp78 RNAi. About 63% of iGrp78-1 and 75% of iGrp78-2 individuals showed significantly impaired oocyte maturation and ovarian growth. As the follicular epithelium determines the size of ovarian follicle and facilitates the transport Vg from the hemolymph to the developing oocytes via the intercellular spaces, known as patency (2, 61), we further examined the morphological change of follicular epithelium after Grp78 RNAi. Silencing of Grp78 led to significantly smaller follicle cells and nuclei as well as reduced incidence of patency in comparison with the iGFP controls (Fig. 4E). When Grp78-1 and Grp78-2 were separately knocked down, the follicle cells and nuclei became slightly smaller, and the patency was also relatively less observed (Fig. 4E).

Because locust vitellogenesis and oocyte maturation strictly depend on JH, we next treated Grp78 RNAi locusts with methoprene to examine whether the defective oocyte maturation, follicular epithelium development, and ovarian growth could be rescued. After methoprene treatment, the expression of neither Grp78-1 nor Grp78-2 was significantly increased (Fig. 5, A and B). As shown in Fig. 5C, additional administration of metho-
Grp78 and JH-regulated vitellogenesis

Figure 5. Methoprene treatment fails to rescue the defective phenotypes caused by Grp78 RNAi. GFP dsRNA (iGFP) or Grp78 dsRNA (iGrp78) was injected within 12 h post-adult eclosion and boosted on day 5. Methoprene (M) or acetone (A) was applied on day 6, and the effects were examined on day 8. A and B, the relative expression of Grp78-1 and Grp78-2 in the fat body. ***, p < 0.001; n.s., no significant difference (n = 8). C, comparison of ovaries (Ov), primary oocytes (Po), and follicle cells (Fc) of three groups. Scale bar: Ov, 2.5 mm; Po, 500 μm; Fc, 10 μm. Blue, follicle cell nuclei; green, F-actin. Yellow arrows indicate the patency.

Grp78 and JH-regulated vitellogenesis and oocyte maturation

Using the migratory locust, we demonstrated in this study that loss of Grp78 function resulted in substantial reduction of Vg, accompanied with arrested oocyte maturation and ovarian growth. Further treatment of the JH analog on Grp78-depleted locusts failed to restore the defective phenotypes to the normal levels. These data provide the evidence that Grp78 is essential for JH-stimulated vitellogenesis and egg production. The maturation of pre-vitellogenic adult insects is characterized by the enhanced protein synthesis in the fat body required for oocyte maturation and successful reproduction (2, 13). The secretory proteins like Vg are synthesized, modified, and folded in the ER. It has been documented that Grp78 in the ER lumens functions as a chaperone to facilitate protein folding and to inhibit protein aggregation (27, 29, 30). Grp78 also binds to ER stress sensor proteins including Ire1, Perk, and Atf6 to prevent the activation of UPR (27, 29, 30). In addition, Grp78 binds to Ca²⁺ in the ER and maintains ER Ca²⁺ homeostasis (71, 72). Previous studies have shown that reduction of Grp78 expression in mammalian cells activates UPR and ER stress leading to apoptosis, whereas overexpression of Grp78 protects against ER stress-induced apoptosis (73–77).

In the present study, knockdown of Grp78 via RNAi in adult female locusts led to ER stress and apoptosis in the fat body. It is likely that loss of Grp78 leads to the accumulation of unfolded or misfolded proteins including Vg, which are transported into cytoplasm to be degraded by proteasome, known as the process of ER-associated degradation (26, 27, 78, 79). It is also likely that depletion of Grp78 gives rise to activation of Ire1 and Perk. Consequently, Perk phosphorylates eukaryotic initiation factor 2α (eIF2α) to attenuate protein translation globally (80), whereas Ire1 degrades mRNAs in the ER through its RNase activity known as regulated Ire1-dependent decay of mRNA (39). As a consequence, Grp78-depleted fat bodies had significantly declined Vg, as well as other regulatory proteins that directly or indirectly regulate Vg synthesis. The locust ovary is panoistic. Vg and other forms of yolk precursor synthesized in the fat body are released into hemolymph and transported to maturing oocytes through the patency in the follicular epithelium (2, 13). The reduced yolk protein precursor production from Grp78-depleted fat bodies might consequently result in arrested oocyte maturation and ovarian growth. We also demonstrated that silencing of Grp78 resulted in smaller follicle cells and nuclei. The follicular epithelium became shrunk and the incidence of patency was reduced. The blocked development of follicular epithelium might alternatively limit the uptake of Vg by the ovary.

Grp78 depletion disturbs the homeostasis of fat body cells

The chaperons including the 94-kDa glucose-regulated protein (Grp94, GenBank™ KJ472841), protein-disulfide isomerase (PDI, GenBank™ KX683312), 170-kDa glucose-regulated protein (Grp170, GenBank™ KX683313), and Grp78 have been used as the markers of ER stress because of their transcriptionally regulated by the Ire1 (GenBank™ KX683314) pathway in the UPR (26, 44, 65–71). We conducted double knockdown by injecting the dsRNA mixture of Grp78 and Ire1 (iGrp78 + Ire1) and examined the effects at 6 days PAE. In the iGrp78 + Ire1 group, Grp94, PDI, and Grp170 mRNA levels were significantly reduced by 71, 59, and 62%, respectively, compared with the iGrp78 group (Fig. 6C). In parallel experiments, depletion of Ire1 alone (89% knockdown efficiency) did not alter the expression of Grp78-1, Grp78-2, Grp94, PDI, Grp170, or VgA (Fig. 6D). Taken together, these data indicate the presence of ER stress in the Grp78-depleted fat body and the involvement of Ire1 in the induction of UPR by Grp78 depletion. To examine the possible apoptosis in the fat body cells caused by Grp78 depletion, we performed TUNEL assays using adult female locusts treated with dsGrp78 at 6 days PAE. As shown in Fig. 6E, apoptotic nuclei were apparently detectable in Grp78-depleted fat body cells, indicating an important role of Grp78 in the maintenance of fat body cell homeostasis.

Discussion

Grp78 and JH-stimulated vitellogenesis and oocyte maturation

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**Grp78 and JH-regulated vitellogenesis**

Differential regulation of Grp78 genes by JH

Different from a single Grp78 gene in other insect species whose genome sequences are available in the public databases, two Grp78 genes were identified in the migratory locust. The genome size of migratory locust is about 6.5 Gb, which is characterized by a large number of repeated sequences (53). Phylogenetic tree construction suggests that Grp78-2 is likely a new gene evolutionally derived from the locust genome. Our qRT-PCR showed the expression of both Grp78-1 and Grp78-2 was significantly increased in the fat body during the vitellogenic phase, which was in accord with the high levels of JH titer in this stage (54, 55). Notably, the mRNA levels of Grp78-1 and Grp78-2 were significantly reduced in adult females chemically allatectomized with precocene treatment, whereas further administration of JH analog induced their expression. Moreover, Met RNAi resulted in a significant decrease of Grp78-1 and Grp78-2 expression. These results together suggest that the expression of Grp78-1 and Grp78-2 is dependent on JH and Met in the fat body of adult female locusts.

Interestingly, the mRNA levels of Grp78-2 were about 10-fold higher than that of Grp78-1. Furthermore, Grp78-2 expression was significantly increased at 12 h post-JHA treatment, whereas significant induction of Grp78-1 expression was observed at 48 h. The delayed response of Grp78-1 to JHA treatment suggests the requirement of additional protein synthesis for Grp78-1 transcription. Analysis of 3-kb upstream sequences of Grp78-1 and Grp78-2 revealed that only the Grp78-2 promoter possesses a JH response element (E-box-like motif) recognized by the JH-receptor. Given the marked differences in these two genes, it might have been predicted that the JH-receptor directly regulates Grp78-2 but not Grp78-1. This hypothesis is supported by our luciferase reporter assays that demonstrated the activation of Grp78-2 transcription by Met/Tai in the presence of JHA. During locust vitellogenesis, JH induces extensive DNA replication to produce up to 16-ploid cells in the fat body for massive Vg synthesis (81, 82). Our previous report (50) has demonstrated that the JH-receptor complex acts on Mcm4 and Mcm7 for DNA replication and polyploidization. In this study, RNAi and qRT-PCR showed that Grp78-1 expression was significantly reduced in Mcm4- or Mcm7-depleted adult females, suggesting that Grp78-1 is regulated by the JH-Met-Mcm4/7 pathway. It has been reported...
Grp78 and JH-regulated vitellogenesis

Figure 7. A proposed model for the regulation and function of two Grp78 genes in JH-stimulated locust vitellogenesis. Grp78-1 is regulated by the JH-Met-Mcm4/7 pathway, whereas Grp78-2 is directly activated by the JH-receptor complex. The large amount of Grp78 in the ER facilitates the folding of massively synthesized Vg and other proteins and maintains cell homeostasis. Loss of Grp78 function induces ER stress, which activates the Ire1-dependent decay (RIDD) of mRNA and Perk-eIF2α pathway. RIDD results in the degradation of Vg mRNA. The activated eIF2α inhibits protein translation, leading to reduced synthesis of Vg and other regulatory proteins that directly or indirectly regulate Vg synthesis. Consequently, Vg in the fat body and ovary are substantially reduced, and the oocyte maturation and egg production are blocked.

that Grp78 is transcriptionally regulated by Atf6 and Ire1-activated X-box binding protein 1 (Xbp1) during the UPR through an ER stress-dependent manner (28). Grp78 is also regulated by insulin-like growth factor-1, Leptin, and miR-376a (83–86). Our data thus extends the view in Grp78 regulation by demonstrating the differential regulation of two Grp78 genes in locusts.

Although knockdown of Grp78-1 or Grp78-2 suppressed locust vitellogenesis and oocyte maturation, loss of both Grp78-1 and Grp78-2 function resulted in much severer defects. These observations address the importance of both Grp78-1 and Grp78-2 in JH-dependent female locust reproduction. An adult female locust possesses a pair of ovaries made up of approximately a total of 80 ovarioles. Our study suggests that the migratory locust has adapted a mechanism with two differentially regulated Grp78 genes to facilitate the proper folding of massively synthesized Vg and other proteins, and to protect fat body cells from possible ER stress during vitellogenesis. The direct activation of Grp78-2 by the JH-receptor appears to be a more efficient production of this chaperone protein. On the basis of our findings, we propose a model for the regulation and function of locust Grp78-1 and Grp78-2 in JH-stimulated vitellogenesis and oocyte maturation (Fig. 7). During insect vitellogenesis, JH acts via its receptor to directly activate the transcription of Grp78-2, while up-regulating Grp78-1 expression through the Met-Mcm4/7 pathway. Grp78 in the ER binds to newly synthesized Vg and other proteins to facilitate their folding and maintain cell homeostasis. Lack of Grp78 induces ER stress and UPR, consequently activating the ER-associated degradation of unfolded proteins including Vg, the Ire1-dependent decay of mRNA (RIDD) including Vg mRNA, and the Perk-eIF2α pathway. The phosphorylated eIF2α attenuates translation globally, leading to reduced synthesis of Vg as well as other regulatory proteins that directly or indirectly regulate Vg production. Together, these coordinately result in substantial reduction of Vg in the fat body, accompanied by blocked oocyte maturation and ovarian growth.

**Experimental procedures**

**Experimental animals**

The colony of migratory locusts in the gregarious phase was reared under a photoperiod of 14 light:10 dark and at 30 ± 2 °C as previously described (50). The diet included a continuous supply of wheat bran with fresh wheat seedlings provided once daily. The JH-deprived adult females were achieved by inactivation of corpora allata with topical application of 500 µg (100 µg/µl dissolved in acetone) precocene III (Sigma) per locust to the dorsal neck membrane within 12 h post-eclosion. To restore JH activity, s-(+)-methoprene (Santa Cruz Biotechnology) was topically applied at 150 µg (30 µg/µl dissolved in acetone) per locust 10 days post-precocene treatment. Topical application of acetone (5 µl per locust) alone was used as the solvent control (50).

**Phylogenetic analysis**

The coding sequences of Grp78 genes available in the GenBank™ database of NCBI were collected. Multiple sequence alignments were performed with ClustalW and the phylogenetic tree was constructed using MAGA6 with the Neighbor Joining method.

**RNA isolation and qRT-PCR**

Total RNA from locust tissues and *Drosophila* S2 cells was extracted with TRIzol reagent (Invitrogen). First-strand cDNA was reverse-transcribed using FastQuant RT Kit with gDNase (Tiangen). qRT-PCR was performed on the Mx3005P detection system (Agilent) using SuperReal PreMix Plus (SYBR Green) Kit (Tiangen), initiated at 95 °C for 2 min, then 40 cycles at 95 °C for 20 s followed by 58 °C for 20 s and 68 °C for 1 min. The relative expression levels were calculated using the 2−ΔΔCt method, with locust Ribosomal protein 49 (Rp49) and *Drosophila* β-actin as the internal controls. Melting curve analysis was conducted to verify the specificity of amplification. The qRT-PCR products were sequenced for the confirmation of primer specificity. For absolute quantification (87), qRT-PCR products of Grp78-1 and Grp78-2 were separately cloned into pGM-T vector (Tiangen), confirmed by sequencing, and serially diluted to serve as the reference standards. Grp78-1 and Grp78-2 transcripts in the fat body were quantified with the standard curves derived from the calibration experiments by plotting the natural log of threshold cycle (Ct) against that of serially diluted recombinant plasmids. Primers used for qRT-PCR are listed in Table 1.

**RNA interference (RNAi)**

cDNA templates were amplified by PCR, cloned into pGM-T easy vector (Tiangen), and confirmed by sequencing. Double-stranded RNA (dsRNA) was then synthesized by *in vitro* transcription with T7 RibomAX Express System (Promega) following the manufacturer’s instruction. For RNAi in locusts, adult females within 12 h after eclosion were intra-abdominally...
injected with 15 μg of dsRNA (5 μg/μl dissolved in nuclease-
free ddH2O) and boosted on day 5. For RNAi in S2 cells, 
_Drosophila Met_ (FlyBase: FBpp0073368) and 
Primers used for qRT-PCR or RNAi

| Gene    | Forward primer | Reverse primer |
|---------|----------------|----------------|
| qRT-PCR |                |                |
| Met     | CAAGCTGACACCGTTCCA | GCTTGGCTTCACTACCC |
| Tm4     | GCCGAAATCGCGAATTCACCG | GCACTGTACTAACGCTGTT |
| Tm7     | GCAGGATGATGAGTGTTG | GCACTTTCTCACTACCC |
| Grp78   | GTTGGGCTGGTTACTGACA | TGTTGGCTTCACTACCC |
| Grp78-1 | ACTCTGGGTGTGTTGCTT | GCACTTTCTCACTACCC |
| Grp78-2 | ACTCTGGGTGTGTTGCTT | GCACTTTCTCACTACCC |
| Irel    | ACTCTGGGTGTGTTGCTT | GCACTTTCTCACTACCC |
| Rp49    | ACTCTGGGTGTGTTGCTT | GCACTTTCTCACTACCC |
| DmMet   | ACTCTGGGTGTGTTGCTT | GCACTTTCTCACTACCC |
| DmGce   | ACTCTGGGTGTGTTGCTT | GCACTTTCTCACTACCC |
| DmTat   | ACTCTGGGTGTGTTGCTT | GCACTTTCTCACTACCC |
| R-actin | ACTCTGGGTGTGTTGCTT | GCACTTTCTCACTACCC |

**Table 1**

Primer sequences used for qRT-PCR or RNAi

- **qRT-PCR**
  - _Met_
  - _Tm4_
  - _Tm7_
  - _Grp78_
  - _Grp78-1_
  - _Grp78-2_
  - _Irel_
  - _Rp49_
  - _DmMet_
  - _DmGce_
  - _DmTat_
  - _R-actin_

- **RNAi**
  - _Grp78-1_
  - _Grp78-2_
  - _Grp78-
  - _Met_
  - _Tm4_
  - _Tm7_
  - _Irel_
  - _Rp49_
  - _DmMet_
  - _DmGce_
  - _DmTat_

**Western blot analysis and immunoprecipitation**

Total proteins from the fat body and ovarian of 6-day-old adult female locusts were collected using the ice-cold lysis buffer con-
taining 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1 mM DTT, 1% Nonidet P-40, 1 mM PMSF, 1 mM NaF, and a protease inhibitor mixture (Roche Applied Science). Lysates were cleared by centrifugation at 14,000 × g for 10 min, frac-
tioned on 8% SDS-PAGE, and transferred to PVDF mem-
branes (Millipore). Extracted proteins were quantified by BCA protein assay kit (Pierce). Western blot analysis was performed

##### Luciferase reporter assay

The promoter region of _Grp78-2_ (nt −2068 to −24) was cloned into pGL4.10 vector (Promega) and confirmed by sequencing. To obtain the mutant of the E-box-like motif, the sequence CACGCG was substituted by ACATAT (forward primer, 5'-TCTACGAGCTATCTGAATGCAGTAGTGCGTGT; reverse primer, 5'-TCTACGAGCTATCTGAATGCAGTAGTGCGTGT) using the Q5 Site-directed Mutagenesis Kit (New England Biolabs) and confirmed by sequencing. S2 cells were pre-
treated with _Drosophila Met_ and _Tai_ dsRNA for 48 h, and then transfected with pGL4.10-Grp78-2 (−2068 to −24) plus pAc5.1/Flag-Met (1–3108) and/or pAc5.1/V5-Tai using Lipofectamine 2000 (Invitrogen) for 48 h, followed by treatment with 10 μM methoprene for 6 h. Cells were then lysed with the ice-cold lysis buffer and cleared by centrifugation. Western blotting was conducted using anti-FLAG or anti-V5 (MBL) antibody. For immunopre-
cipitation, the pre-cleared lysates were incubated with anti-V5 antibody for 60 min at 4 °C. The immunocomplexes were then captured with protein A-agarose (Sigma) and eluted in Laem-
ml sample buffer, followed by Western blotting with anti-
FLAG antibody.

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treated with _Drosophila Met_ and _Tai_ dsRNA for 48 h, and then transfected with pGL4.10-Grp78-2 (−2068 to −24) plus pAc5.1/Flag-Met (1–3108) and/or pAc5.1/V5-Tai (1–1785 plus 4581–4961) using Lipofectamine 2000 (Invitrogen). After 48 h,
Grp78 and JH-regulated vitellogenesis

S2 cells were further treated with 10 μM methoprene for 6 h. The Dual-luciferase Reporter Assay System and a GloMax 96 Microplate Luminometer (Promega) were employed to measure the luciferase activity.

Tissue imaging, cell staining, and confocal microscopy

The ovary and ovisac were imaged with Nikon D7000 camera and Olympus CKX41 microscope. The length of primary oocytes was measured with Image Pro PLUS software. For cell staining, sheath-free ovarioles were fixed in 4% paraformaldehyde, and then permeabilized in 0.3% Triton X-100. F-actin and nuclei were stained with 0.165 μM phalloidin/Alexa Fluor 488 (Invitrogen) and 5 μM Hoechst 33342 (Sigma), respectively. The images were captured with ZEISS LSM 710 confocal microscope and processed with ZEN2012 software (Carl Zeiss).

Apoptosis assay

Apoptosis assays were carried out using In Situ Cell Death Detection kit (Roche Applied Science) according to the manufacturer’s protocol. Briefly, the fat body sections were fixed in 4% paraformaldehyde for 15 min at room temperature, followed by treatment with proteinase K (10 μg/ml) for 40 min at 37 °C. Nuclei were stained with 5 μM Hoechst 33342 (Sigma). Apoptosis was detected with Terminal dUTP Nick End Labeling (TUNEL) reaction mixture. Images were captured with ZEISS LSM 710 confocal microscope and analyzed with ZEN2012 software (Carl Zeiss).

Data analysis

Statistical analyses were performed using Student’s t test or one-way analysis of variance with SPSS20.0 software. Significant difference was considered at p < 0.05. Values were reported as mean ± S.E.

Author contributions—S. Z. conceived the study. M. L., D. L., and Z. W. performed the experiments and acquired the data. M. L. and S. Z. analyzed the data and wrote the manuscript. W. G. and L. K. provided technical support.

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