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Distinct roles of isoforms of the heme-liganded nuclear receptor E75, an insect orthologue of the vertebrate Rev-Erb, in mosquito reproduction

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Abstract

Mosquitoes are adapted to using vertebrate blood as a nutrient source to promote egg development and as a consequence serve as disease vectors. Blood-meal activated reproductive events in female mosquitoes are hormonally and nutritionally controlled with an insect steroid hormone 20-hydroxyecdysone (20E) playing a central role. The nuclear receptor E75 is an essential factor in the 20E genetic hierarchy, however functions of its three isoforms - E75A, E75B, and E75C – in mosquito reproduction are unclear. By means of specific RNA interference depletion of E75 isoforms, we identified their distinct roles in regulating the level and timing of expression of key genes involved in vitellogenesis in the fat body (an insect analogue of vertebrate liver and adipose tissue) of the mosquito *Aedes aegypti*. Heme is required in a high level of expression of 20E-controlled genes in the fat body, and this heme action depends on E75. Thus, in mosquitoes, heme is an important signaling molecule, serving as a sensor of the availability of a protein meal for egg development. Disruption of this signaling pathway could be explored in the design of mosquito control approaches.

Keywords

ecdysone; nuclear receptor; fat body; vitellogenesis; egg development; heme

1. Introduction

Mosquitoes are vectors of some of the world's most devastating diseases. Malaria, transmitted by *Anopheles* mosquitoes, causes over one million deaths annually (www.who.org). Dengue fever has become the most significant arboviral human disease, rapidly expanding in most tropical and subtropical areas of the world (Morens, 2009). The yellow fever mosquito *Aedes aegypti* has become the major vector of Dengue virus in the world. These disease vectors are adapted to using human blood as a nutrient source to

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The nucleotide sequences of *A. aegypti* E75A, E75B and E75C reported in this paper have been submitted to the GenBank™ data bank with the accession numbers AM397060, AM39706 and AM397062, respectively.

promote egg development. An understanding of mosquito reproductive biology is an important component in developing novel strategies for control of mosquito-borne disease.

In insects, vitellogenesis is an essential process in egg development, which includes massive production of yolk protein precursors (YPPs) by the fat body, a tissue analogous to the vertebrate liver and adipose tissue, and their subsequent internalization into developing oocytes for the use during embryonic development (Raikhel et al., 2005). The two major insect-specific hormones that govern vitellogenesis and egg maturation are sesquiterpenoid juvenile hormones (JH) and the steroid hormone 20-hydroxyecdysone (20E). Nutritional control mediated by the target-of-rapamycin pathway plays an essential role in regulating female reproduction (Hansen et al., 2004; Attardo et al., 2005). Recently, insulin-like peptides have also been implicated in the regulatory network controlling egg development in insects, including mosquitoes (Brown et al., 2008; Gulia-Nuss et al., 2011; Parthasarathy and Palli, 2011).

Molecular elucidation of the 20E genetic hierarchy in *Drosophila melanogaster*, has led to the identification of the ecdysone receptor as a heterodimer of two nuclear receptors, EcR and ultraspiracle (USP), which is an orthologue of vertebrate retinoid X receptor. The action of the EcR/USP is mediated by products of early genes—*br*, *E74*, and *E75*—that encode transcription factors. The 20E regulatory pathway is further refined by factors required for setting up the stage specificity of target gene activation (Thummel, 2001; King-Jones and Thummel, 2005; Beckstead et al., 2005; Nakagawa and Henrich, 2009). 20E plays a key role in the control of vitellogenesis, yolk protein precursor (YPP) production, in *A. aegypti* (Raikhel, 2004; Raikhel et al., 2005). The targets of 20E regulation in the mosquito, such as the *Vg* gene, are under direct/indirect regulation by this hormone (Kokoza et al., 2001; Martin et al., 2001). The EcR/USP heterodimer directly binds ecdysone response element in the *Vg* promoter, thereby activating its expression (Martin et al., 2001). Synergistic action of E74B, the Ets-domain protein, and Broad Z2, the C2H2-type zinc-finger DNA-binding protein, with the ecdysone complex results in a high level of *Vg* gene expression (Chen et al., 2004; Sun et al., 2004; Sun et al., 2005; Zhu et al., 2007). In addition, β FTZ-F1 enhances 20E activity by recruiting the p160/SRC coactivator FISC, which binds the EcR/USP heterodimer advancing recruitment of the transcriptional machinery to the *Vg* promoter (Zhu et al., 2006).

In *Drosophila*, the 20E-responsive *Eip75B* gene encodes three splice variants, E75A, E75B and E75C, which differ in their N-terminal regions (Segraves and Hogness, 1990). E75B isoform contains only one of the two zinc fingers making it incapable of binding DNA. It is generally accepted that E75A acts as an activator in the 20E pathway, while E75B is a heterodimer partner of the nuclear receptor hormone receptor 3 (HR3), which plays a critical role in the 20E-dependent developmental shifts (Horner et al., 1995; White et al., 1997; Lam et al., 1997). Isoform-specific E75 null mutations in *Drosophila* have revealed phenotypic differences. Germ-line clones of *E75*-null mutants, which are missing all three isoforms, lead to the arrest during mid-oogenesis (Burczak et al., 1999). *E75A* mutants exhibited reduced ecdysteroid titer, blocking developmental transition from molting to metamorphosis in *Drosophila* (Bialeski et al., 2002). *E75A* and *E75B* have opposite effects on the development choices of the *Drosophila* chamber differentiation (Terashima and Bownes, 2006).

Orthologues of *Drosophila* E75 isoforms, which are differentially expressed during development, metamorphosis and oogenesis, have been identified in other insects (Segraves and Woldin et al., 1993; Jindra et al., 1994; Palli et al., 1997; Zhou et al., 1998; Pierceall et al., 1999; Swevers et al., 2002; Keshan et al., 2006). Five E75 isoforms of the cockroach *Blattella germanica* display specific 20E responsiveness, but, their RNAi depletions have not

yielded unique responses, making the authors to conclude that these E75 isoforms play redundant roles in molting and developmental progression of this direct-developing insect (Mane-Padros et al., 2008).

Our previous studies have identified *A. aegypti* E75 in vitellogenic female mosquitoes and 20E regulation of its expression (Pierceall et al., 1999; Cruz et al., 2009). Similar to *Drosophila*, there are three isoforms of this nuclear receptor in the mosquito - E75A, E75B, and E75C – however; their respective roles in vitellogenesis remained unknown. In this work, we took advantage of a reverse genetic approach to specifically deplete isoforms of the nuclear receptor E75 in *A. aegypti* females. Our study has revealed distinct roles of E75 isoforms in regulating the level and timing of expression of key genes involved in mosquito vitellogenesis. We have also demonstrated that in mosquitoes heme is an important signaling molecule, serving as a sensor of the availability of a protein meal for egg development. This heme function is mediated by E75 and results in a high activation of 20E-driven gene expression in the mosquito fat body.

2. Experimental Procedures

2.1. Animals Rearing

Mosquitoes of Rockefeller/UGAL strain of *A. aegypti* were raised as described previously (Roy et al., 2007). Adult females were blood fed on anesthetized white rats. All procedures for using vertebrate animals were approved by the University of California Riverside Institutional Animal Care and Use Committee (#A20100016; 05/27/2010). All dissections were performed in *A. aegypti* physiological saline (APS) at room temperature (Roy et al., 2007).

2.2. RNA Extraction, Reverse Transcription, and Real-Time PCR

Fat bodies adhered to the abdominal wall (thereafter referred as fat body) were homogenized with a motor-driven pellet pestle mixer (Kontes) and lysed by Trizol reagent (Invitrogen). RNA was isolated according to the manufacturer's protocol. Contaminating genomic DNA was removed by treatment with RNase-free DNase I (Invitrogen). One µg of total RNA was reverse transcribed using a SuperScriptII reverse transcriptase kit (Invitrogen) in a 20-µl reaction mixture. cDNA levels in the different samples were quantified by real time PCR (qPCR) by using the iCycler iQ system (Bio-Rad). Reactions were performed in 96-well plates with a QuantiTect SYBR PCR kit (Qiagen). The sequences of the primer pairs for each of the specific RNA transcripts assayed are: E75AF1:

TGTGAAGCAGGAAGAAAGGAA; E75AR1: ATGCATGGCTCTCCTGTACC; E75BF1: CTGAATTTCGGGTGAAAATGG; E75BR1: TGCTGCTACCGCTACTGTTG; E75CF1: ACGAAAAAGACTCGCTGGAA; E75CR1: TTGAATCCTAGCCCGATCCT; ActinF: 5'-ATCATTGCTCCACCAGAACG-3'; Actin R: 5'-AAG GTA GAT AGA GAA GCC AAG.

Statistical significant difference between samples was calculated with an upaired Student's t test with unequal variance (Graphpad 5.0). All quantitative data were reported as mean+/-SEM.

2.3. Synthesis of double-stranded (ds) RNA and microinjections

The DNA fragment encoding the isoform-specific A/B domain of mosquito AaE75A, AaE75B and AaE75C were amplified using the primer sets: AaE75AF2: 5'-TAATACGACTCACTATAGGGATCTCGTACCAACAGT-3', AaE75AR2: 5'-TAATACGACTCACTATAGGGGTTCTCCCTTAGAACT-3', AaE75BF2: 5'-TAATACGACTCACTATAGGGCCAAGCAGTCAGTGTA-3', AaE75BR2: 5'-TAATACGACTCACTATAGGGACGAGTGATCGGAACT-3', AaE75CF2: 5'-

TAATACGACTCACTATAGGGCGATGTCATCATCCAT-3', AaE75CR2: 5'-TAATACGACTCACTATAGGGCATTACCACCCATACT-3', (underlining indicates the T7 RNA polymerase recognition sites) and the PCR products were used as a template for the RNA synthesis, whereas the dsRNA control was generated using the plasmid LITMUS 28iMal, containing a non-functional portion of the *Escherichia coli malE* gene. dsRNA was produced by *in vitro* transcription with the Hiscrite RNAi transcription kit (New England Biolabs, Beverly, MA). Approximately 1 µg of dsRNA in 0.2 µl of H₂O was injected in the thorax of CO₂-anesthetized female mosquitoes, 2 days after adult emergence, using a Picospritzer II (General Valve, Fairfield, NJ). The fat bodies were collected at different times for RNA analysis or *in vitro* tissue culture experiments.

2.4. In vitro fat body culture

The *in vitro* fat body culture system is described in detail elsewhere (Roy et al., 2007). 20-hydroxyecdysone (Sigma) was dissolved in ethanol. The stock solution was added to the tissue culture medium to the final concentration of 10⁻⁶ M. Hemin (Fluka) was dissolved in DMSO and added to the culture medium at the final concentration of 6 µM. The stock solutions were prepared ×1000 and added to the culture medium at 1:1000. As a control, the same volume of the carrier was added to the treatments without the hormone.

3. Results

3.1. RNA interference depletion reveals distinct roles of E75 isoforms in the adult female mosquito

To establish functions of E75 isoforms, we utilized the fat body of *A. aegypti* female mosquitoes, which is a target of 20E regulatory cascade during vitellogenesis. Quantitative RT-PCR (QRT-PCR), utilizing primers specific to unique A/B regions of each isoform, was used to establish the pattern of transcript abundance for each E75 isoform throughout the first gonadotrophic cycle in the fat body of female mosquitoes. *Vg* transcript was monitored as readout of vitellogenesis timing; in our experimental setting it peaked at 24 h post blood meal (PBM) (Fig. 7A). Transcripts of E75 isoforms exhibited similar patterns of expression, being at a baseline level during previtellogenesis (PV) and rising during vitellogenesis (PBM) (Fig. 1). They significantly elevated by 12 h PBM, reached their peaks at 24 h PBM, and then declined to their previtellogenic levels by the end of vitellogenesis at 48 h PBM (Fig. 1).

We then investigated the roles of each E75 isoform in mosquito vitellogenesis utilizing RNA interference approach (RNAi). Double-stranded RNAs, specific to unique A/B regions of each E75 isoform, were injected into thoraces of previtellogenic female mosquitoes as described in Experimental Procedures. Transcript abundance of each E75 isoform was measured in fat bodies at several times during the first vitellogenic cycle by means of QRT-PCR utilizing isoform-specific primers (Fig. 1). A specific RNAi depletion of each E75 isoform resulted in a dramatic reduction of its transcript throughout the vitellogenic cycle until 48 PBM, demonstrating efficiency of the procedure. dsRNA of the bacterial gene *Mal* was used as a control for a possible adverse effect of dsRNA injection. Its RNAi depletion (dsMal) did not have any influence on transcript levels of any E75 isoform, which were similar to those in untreated (control) mosquitoes (Fig. 1). These experiments demonstrated the specificity of RNAi depletions of individual E75 isoforms.

Next, we evaluated whether a specific depletion of an individual E75 isoform affected transcript abundance of other isoforms of this nuclear receptor (Fig. 1). In E75A depletion background, E75B transcript levels were depressed at 12 and 24 h PBM as compared to the wild-type and dsMal controls (Fig. 1). In the same E75A RNAi background, E75C transcript

level was prematurely elevated to the maximum at 12 h PBM. Depletion of E75B isoform did not affect the transcript abundance of either E75A or E75C at any time of the vitellogenic cycle. Likewise, depletion of E75C isoform had no effect on either E75A or E75B transcripts.

3.2. Distinct effects of E75 isoforms on expression of 20E hierarchy genes

The same sets of samples were used for the analysis of effects of RNAi depletions of individual E75 isoforms on expression of 20E hierarchy genes. Previously, we have characterized two *Aedes* EcR isoforms, EcRA and EcRB, which have dissimilar profiles of expression in the fat body, with EcRB being maximally elevated early in vitellogenesis (4–6 h PBM) and followed by a peak of EcRA transcript (16–20 h PBM) (Wang et al., 2002). In our present study, transcript profiles of EcR isoforms were in agreement with this previous study (Figs. 2 and 3). As a control, we utilized dsE75common directed to its common region; the results were significantly different from those with E75 isoform depletions, emphasizing specific effects of depletions of individual E75 isoforms (Figs. 2A and 3A). RNAi depletion of E75A resulted in a dramatic reduction of the EcRA transcript at 12 h PBM, when it was normally at its peak of expression; EcRA transcript was then highly elevated at the time of termination of vitellogenesis, 30 and 48 h PBM, when it was normally dropped to its background level in the untreated mosquitoes or dsMal controls (Fig. 2B). RNAi depletion of E75B led to a premature decrease of EcRA transcript (Fig. 2C). E75C isoform depletion affected EcRA transcript in a complex manner; EcRA transcript was prematurely elevated at 6 h PBM, and then declined at its peak time 12 h PBM, rising dramatically at the time of termination of vitellogenesis, 30 and 48 h PBM (Fig. 2D). RNAi depletion of either E75A or E75B resulted in a decrease of EcRB transcript level at 12 h PBM (Figs. 3B and 3C). E75C depletion elevated EcRB transcript at its peak of 6h PBM, but EcRB transcript precipitously declined thereafter to the background level (Fig. 3D).

Broad isoforms (Br) play a significant role in regulation of mosquito reproduction (Chen et al., 2004; Zhu et al., 2007). In particular, BrZ2 isoform serves as an activator of Vg gene expression. RNAi depletion of E75A resulted in a complete inhibition of BrZ2 transcript throughout the vitellogenesis in the female fat body (Fig. 4B). RNAi depletion of E75B led to a very high level of BrZ2 transcript abundance at the time of termination of vitellogenesis, 30 and 48 h PBM, when its levels were normally low (Fig. 4C). E75C isoform depletion had no effect on BrZ2 expression (Fig. 4D). Control dsE75common yielded very different results from those with E75 isoform depletions (Fig. 4A),

The nuclear receptor HR3 is essential in regulating 20E-driven developmental shifts during insect development and metamorphosis (Lam et al., 1997; Thummel, 2002; Ruaud et al., 2010). In the mosquito *A. aegypti*, the 20E-responsive gene encoding the nuclear receptor HR3 is expressed during vitellogenesis (Kapitskaya et al., 2000; Li et al., 2000; and Fig. 5). We investigated whether E75 isoforms played any role in regulating HR3 expression in mosquito fat body during the vitellogenic reproductive cycle. In the E75A depletion background, HR3 transcript level was elevated throughout the vitellogenic period as compared to untreated control and Mali (Fig. 5). Depletion of E75B isoform resulted in an elevation of HR3 transcript level at the later period of vitellogenesis (24 and 30 h PBM). Interestingly, depletion of E75C isoform led to a premature elevation of the HR3 transcript only at 12 h PBM, which later declined (Fig. 5).

To confirm the *in vivo* results with RNAi depletions, we employed the *in vitro* fat body culture. Female mosquitoes were injected with individual E75 isoform-specific dsRNAs, four days after fat bodies were dissected and incubated for 6 h in a complete culture medium supplemented with amino acids in the presence or absence of 10^{-6} M 20E (Fig. 6). QRT-

PCR analysis showed that HR3 transcript was elevated in fat bodies from female mosquitoes with the dsMal background after incubation with 20E. RNAi depletion of either E75A or E75C resulted in further elevation of HR3 transcript in the presence of 20E (Fig. 6A and B). HR3 transcript was particularly high in fat bodies from E75C-depleted female mosquitoes incubated in the presence of 20E (Fig. 6B).

3.3. Distinct roles of E75 isoforms in regulating the Vg gene expression

We tested whether E75 isoforms had different effects on expression of the key *YPP* gene, *Vg*. As above we utilized isoform-specific RNAi depletion approach, and *Vg* transcript abundance was evaluated by means of QRT-PCR in fat bodies isolated from female mosquitoes at several time points during vitellogenesis. The effect of depletions of individual E75 isoforms on *Vg* transcript abundance was dramatically different (Fig. 7A). In the E75A RNAi depletion background, *Vg* transcript levels were depressed throughout the vitellogenic period as compared to wild-type control and Mal-depleted mosquitoes. Depletion of E75B isoform did not have any effect on the *Vg* transcript abundance. The *Vg* transcript level was highly elevated at 30 h PBM in fat bodies from E75C-depleted female mosquitoes as compared to wild-type control and Mal-depleted mosquitoes, in which the level of *Vg* transcript dramatically decreased at this time (Fig. 7A).

Next, we employed the *in vitro* fat body culture to confirm the *in vivo* results. The experiments were performed as described above for HR3 (Fig. 6). The *Vg* transcript was highly elevated in fat bodies from female mosquitoes with dsMal background after incubation with 20E (Fig. 7B). In contrast, RNAi depletion of E75A impaired 20E ability to activate the *Vg* gene in fat bodies incubated *in vitro* in the presence of 20E (Fig. 7B). Similarly to the *in vivo* experiments, depletion of E75B had no effect on *Vg* transcript abundance. Incubation of fat bodies from female mosquitoes with E75C depletion background in the presence of 20E resulted in an elevation of *Vg* transcript to a considerably higher level than in dsMal control (Fig. 7B). Thus, *in vitro* experiments confirmed *in vivo* results, indicating that the observed effects were due to E75 isoform depletions in the fat body tissue.

In the next experiment, we examined whether the effect of E75 isoforms on expression of the *Vg* gene was mediated by additional factors. As above, fat bodies from female mosquitoes with E75 isoform-specific depletions were incubated for 6 h in a complete culture medium supplemented with amino acids in the absence or presence of 10^{-6} M 20E (Fig. 8). *Vg* transcript was elevated in fat bodies from female mosquitoes with dsMal background after incubation with 20E; however, it was completely repressed when the medium was supplemented with 1 μ M of the protein inhibitor cycloheximide (Chx), which is in agreement of the 20E hierarchy action in activation of the *Vg* gene (Deitsch et al., 1995). RNAi depletion of E75A significantly reduced the 20E-mediated activation the *Vg* gene, its transcript abundance was further suppressed with the addition of Chx (Fig. 8, dsE75A). In fat bodies from female mosquitoes with E75C depletion background, the *Vg* transcript, which was highly activated in the presence of 20E, was reduced to a background in the presence of 20E and Chx, similarly to that of Mal (Fig. 8, dsE75C). The latter indicated that 20E activation of *Vg* in the absence of E75C was due to an additional factor(s) suppressed by E75C.

3.5. Effect of heme on the expression level of the Vg gene

E75 is a thiolate-ligated heme-containing protein, and heme is required for stability of this nuclear receptor (Reinking *et al.*, 2005). Mosquitoes are blood-feeding organisms that metabolize heme as a by-product of blood digestion and likely use it for regulatory purposes. To investigate a possible effect of heme on transcriptional events in the mosquito fat body,

we utilized an *in vitro* fat body culture (Fig. 9). The Vg transcript level was elevated in the fat bodies of untreated previtellogenic female mosquitoes incubated for 6 h in the presence of amino acids and further increased with the addition of 10^{-6} M 20E, which is in agreement with our previous report (Hansen et al., 2004). When heme (at the concentration of 6 μ M of hemin) was added into an amino acid containing medium, there was an increased in Vg transcript abundance compared to this medium alone. However, incubation of previtellogenic fat bodies with a combination of amino acid, 20E and hemin increased the Vg transcript level in about 7-fold as compared to the effect of an amino acid containing medium supplemented with 20E only (Fig. 9A).

Next, we investigated whether E75 was involved in the heme-mediated increase of Vg transcript abundance. In dsMal controls, addition of hemin was as potent as in the wild-type untreated mosquitoes (Figs. 9B and 9C). Although RNAi depletion of E75A reduced the 20E-mediated activation the Vg gene, its transcript abundance increased with the addition of hemin, remaining much lower than in the dsMal control (Fig. 9B). This response to hemin is likely due to an incomplete depletion of E75A mRNA and some residual presence of its protein. In fat bodies from female mosquitoes with E75C depletion background, the Vg transcript, which was highly activated by 20E as compared to dsMal control, was not elevated in the presence of hemin at the statistically significant level (Fig. 9C).

4. Discussion

Our study utilizing specific RNAi depletion of E75 isoforms has revealed their distinct roles in mosquito vitellogenesis. We conducted a detailed time course analysis of E75 isoform transcript levels after their RNAi depletions showing a remarkable effectiveness of this technique in lowering transcript abundance until 42 h PBM. This experimental setting has provided an important background for interpretation of consequences of RNAi depletions of E75 isoforms. RNAi depletion has shown that E75A coordinates expression of E75B and E75C. In the vitellogenic mosquito fat body, E75A is essential for proper timing of E75B activation; however, it serves as a repressor of E75C, preventing its premature expression. Whether E75A action on transcriptional units encoding other E75 isoforms is direct remains to be elucidated. We have not observed any effect of depletion of either E75B or E75C on transcript abundance of other E75 isoforms. Thus, E75A plays an essential role in regulation of other E75 isoforms, and their miss-regulation as a consequence E75A RNAi depletion should be taken in account in interpretation of RNAi effects.

Although two isoforms of *A. aegypti* EcR, EcRA and EcRB, have different expression profiles, their respective roles in vitellogenesis are not clear (Wang et al., 2002). At the protein level, both EcR isoforms are present equally in the fat body during vitellogenesis, making it more difficult to interpret their respective functions (Zhu et al., 2003). Distinct effects of RNAi depletion of E75 isoforms on expression of EcR isoforms have shed some light on their regulation. It appears that all three E75 isoforms are important for proper timing of EcRA expression. Effects of RNAi depletion of E75A and E75C isoforms on EcRA transcript abundance are particularly striking, generally shifting the pattern of EcRA expression to the end of vitellogenesis. The action of E75 isoforms on EcR isoform expression is likely complex and involved direct and indirect actions. E75A could directly activate EcRA at the beginning of vitellogenic cycle, thus its depletion leads to repression of EcRA. However, at the end of vitellogenesis, this action is likely indirect. Considering that effects of RNAi depletion of E75A and E75C at this time were similar, it is possible that E75A action on EcRA is indirect and involved miss-expression of E75C. In turn, such a dramatic change in EcRA expression pattern undoubtedly has serious consequences in regulation of 20E-mediated events in the fat body. Another possible explanation is that because E75A depletion represses E75B expression and this, in turn, results in premature

elevation of HR3 transcript; HR3 could activate untimely expression of EcRA at the termination of vitellogenesis. More detailed RNAi analysis is required to delineate such regulatory links. Interestingly, effects of E75 isoform depletions on EcRB transcript abundance were not so dramatic. Nevertheless, the results have shown that E75A and E75C are required for maintaining EcRB transcript level at its physiological level.

Broad (*br*) is an essential gene implicated in the 20E regulatory hierarchy (Karim *et al.*, 1993). However, *br* has also been linked to JH action during larval–pupal metamorphosis and it acts as “the pupal specifier” (Zhou and Riddiford, 2002; Suzuki *et al.*, 2008). Our studies have shown that *br* is essential for regulating 20E-dependent vitellogenic periods of female mosquito reproductive cycles and Br isoforms play distinct roles in these events (Chen *et al.*, 2004; Zhu *et al.*, 2007). BrZ2 isoform serves as an activator of *Vg* gene expression. Results of RNAi depletions of E75 isoforms have shown that their effects on the BrZ2 transcript abundance are distinct. EcRA appears to be an activator of BrZ2 expression required for maintaining BrZ2 transcript abundance, while E75B is important for a timely repression of this Br isoform. The latter could be modulated via de-repression of HR3. Interestingly, based on E75C isoform depletion results, this E75 isoform does not appear to play a role in regulation of BrZ2 expression.

HR3 is recognized as a central regulatory knot in 20E-driven shifts during insect development and metamorphosis, responsible for directing timely shutting down of early genes regulated by a preceding 20E peak and a sequential activation of *βFTZ-F1*, *E74A* and *E75A* during the subsequent pulse of 20E (Lam *et al.*, 1997; White *et al.*, 1997; Thummel, 2001; Ruaud *et al.*, 2010). In the mosquito *A. aegypti*, vitellogenic expression of the *HR3* gene occurs before expression of the competence factor *βFTZ-F1*, suggesting involvement of these factors in orchestrating stage-specific transitions during vitellogenic cycles (Kapitskaya *et al.*, 2000; Li *et al.*, 2000). RNAi depletion analysis has shown that all three E75 isoforms are important for maintaining coordinated *HR3* expression in the fat body during vitellogenesis; their depletions led to elevated miss-expression of the *HR3* gene. In particular, depletion of E75B resulted in a significant increase of HR3 transcript at later times of vitellogenesis (24 and 30 h PBM), while HR3 transcript was prematurely elevated after E75C depletion. In turn, the elevated miss-expression of the *HR3* gene likely results in miss-regulation of multiple genes involved in 20E hierarchy. In *Manduca sexta*, E75A along with HR3 inhibits expression of the *HR3* gene (Hiruma and Riddiford, 2004). Further molecular studies are required to mechanistically understand the effect of E75 isoforms on the *HR3* gene during mosquito vitellogenesis.

Because of a systemic nature of RNAi technique in mosquitoes, *in vitro* experiments are essential in establishment of whether an observed response is due to a direct effect of gene depletion in a tissue of interest or it is response to an obstruction of a regulatory network, which involves other organs or tissues. An *in vitro* assay of HR3 transcript abundance in fat bodies from E75A and E75C depleted female mosquitoes confirmed inhibitory roles of these E75 isoforms in regulation of the *HR3* gene, showing that their effects are due to depletion of E75 isoforms in this tissue. Taken together, these experiments suggest that E75 isoforms play a combinatorial role in regulating time-specific, 20E-dependent expression of the *HR3* gene in the fat body during vitellogenesis.

Depletions of individual E75 isoforms had dramatically different effects on the expression of the 20E-regulated target gene, *Vg*. It appears that E75A serves as a potent activator of *Vg* gene expression, while E75C is essential for a timely repression of expression of this gene. *In vitro* experiments have confirmed that these effects of E75 isoforms occurred as a result of depletion of E75 isoforms in the fat body. *In vitro* experiments were also instrumental in determining whether the effect of E75C isoform on expression of the *Vg* gene was mediated

by an additional factor(s). In particular, in fat bodies from female mosquitoes with E75C depletion background, the *Vg* transcript, which was highly activated by 20E, was reduced to a background in the presence of Chx. This experiment suggests that other factors of 20E regulatory hierarchy are involved in mediating E75C action. A precise network of regulating factors determining not only activation of 20E-dependent *Vg* gene expression, but also its timely repression, remains to be determined.

Heme is a ligand for E75, which serves as a sensor for this molecule (Reinking et al., 2005). Heme has been identified as a physiological ligand for vertebrate orthologues of insect E75 nuclear receptor, Rev-erba and Rev-erbβ (Raghuram et al., 2007). It reversibly binds to ligand-binding domains of these nuclear receptors enhancing their stability. Heme regulates recruitment of the co-repressor NCoR to Rev-erba coordinating repression of its target genes (Raghuram et al., 2007; Yin et al., 2007; Wu et al., 2009). Mosquitoes are adapted to a very rapid egg development based on their biology as blood-feeding organisms. In a process of blood digestion, they metabolize massive amount of heme as a by-product, posing a question of whether mosquitoes utilize heme for their physiological functions. Our *in vitro* experiments have shown that a combinatorial action of 20E and heme results in a very high level of *Vg* gene expression in the *Aedes* fat body. Several amino acids are essential for the initiation of 20E-mediated gene expression in the mosquito, providing a link between blood feeding and egg development (Hansen et al., 2004; Attardo et al., 2006). The data, presented in this work, suggest that heme functions as an important signaling molecule serving as a sensor of the availability of a blood meal for egg development. Testing the effect of heme in the fat body after RNAi depletions of E75A and E75C has confirmed that the heme-mediated effects on *Vg* expression is due to involvement of this nuclear receptor. Thus, heme is required for mediating 20E action via E75 in the mosquito and intimately linked to the availability of blood. Disruption of this signaling pathway could be explored in the design of mosquito control approaches.

Highlights

Egg development in female mosquitoes is hormonally and nutritionally controlled with an insect steroid hormone 20-hydroxyecdysone (20E) playing a central role. The nuclear receptor E75 is an essential factor in the 20E genetic hierarchy, however functions of its three isoforms - E75A, E75B, and E75C – in mosquito reproduction are unclear.

By means of specific RNA interference depletion of E75 isoforms, we identified their distinct roles in regulating the level and timing of expression of key genes involved in vitellogenesis in the fat body (an insect analogue of vertebrate liver and adipose tissue) of the mosquito *Aedes aegypti*.

Heme is required in a high level of expression of 20E-controlled genes in the fat body, and this heme action depends on E75. Thus, in mosquitoes, heme is an important signaling molecule, serving as a sensor of the availability of a protein meal for egg development.

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References

- Attardo GM, Hansen IA, Raikhel AS. Nutritional regulation of vitellogenesis in mosquitoes: Implications for anautogeny. *Insect Biochem. Mol. Biol.* 2005; 35:661–675. [PubMed: 15894184]
- Attardo GM, Hansen IA, Shiao SH, Raikhel AS. Identification of two cationic amino acid transporters required for nutritional signaling during mosquito reproduction. *J. Exp. Biol.* 2006; 209:3071–3078. [PubMed: 16888056]
- Beckstead RB, Lam G, Thummel CS. The genomic response to 20- hydroxyecdysone at the onset of *Drosophila* metamorphosis. *Genome Biol.* 2005; 6(12):R99. [PubMed: 16356271]
- Bialicki M, Shilton A, Fichtenberg C, Segraves WA, Thummel CS. Loss of the ecdysteroid-inducible E75A orphan nuclear receptor uncouples molting from metamorphosis in *Drosophila*. *Dev. Cell.* 2002; 3:209–220. [PubMed: 12194852]
- Brown MR, Clark KD, Gulia M, Zhao Z, Garczynski SF, Crim JW, Suderman RJ, Strand MR. An insulin-like peptide regulates egg maturation and metabolism in the mosquito *Aedes aegypti*. *Proc Natl Acad Sci U S A.* 2008; 105:5716–5721. [PubMed: 18391205]
- Burszczak M, Freeman MR, Carlson JR, Bender M, Cooley L, Segraves WA. Ecdysone response genes govern egg chamber development during mid-oogenesis in *Drosophila*. *Development.* 1999; 126:4581–4589. [PubMed: 10498692]
- Chen L, Zhu J, Sun G, Raikhel AS. The early gene Broad is involved in the ecdysteroid hierarchy governing vitellogenesis of the mosquito *Aedes aegypti*. *J. Mol. Endocrinol.* 2004; 33:743–761. [PubMed: 15591032]
- Cruz J, Sieglaff DH, Arensburger P, Atkinson PW, Raikhel AS. Nuclear receptors in the mosquito *Aedes aegypti*: annotation, hormonal regulation and expression profiling. *FEBS J.* 2009; 276:1233–1254. [PubMed: 19183228]
- Deutsch KW, Chen JS, Raikhel AS. Indirect control of yolk protein genes by 20-hydroxyecdysone in the fat body of the mosquito, *Aedes aegypti*. *Insect Biochem. Molec. Biol.* 1995; 25:449–454. [PubMed: 7742832]
- Gulia-Nuss M, Robertson AE, Brown MR, Strand MR. Insulin-like peptides and the Target of Rapamycin coordinately regulate blood digestion and egg maturation in the mosquito *Aedes aegypti*. *Plos One.* 2011; 6:e20401. [PubMed: 21647424]
- Hansen IA, Attardo GM, Park J-H, Peng Q, Raikhel AS. TOR-mediated amino acid signaling in mosquito anautogeny. *Proc. Natl. Acad. USA.* 2004; 101:10626–10631.
- Hiruma K, Riddiford LM. Differential control of MHR3 promoter activity by isoforms of the ecdysone receptor and inhibitory effects of E75A and MHR3. *Dev. Biol.* 2004; 272:510–521. [PubMed: 15282165]
- Horner MA, Chen T, Thummel CS. Ecdysteroid regulation and DNA binding properties of *Drosophila* nuclear hormone receptor superfamily members. *Dev. Biol.* 1995; 168:490–502. [PubMed: 7729584]
- Jindra M, Sehnal F, Riddiford LM. Isolation, characterization and developmental expression of the ecdysteroid-induced E75 gene of the wax moth *Galleria mellonella*. *Eu. J Biochem.* 2004; 221:665–675.
- Kapitskaya MZ, Li C, Miura K, Segraves WA, Raikhel AS. Expression of the early-late gene encoding a nuclear receptor HR3 suggests its involvement in regulating the vitellogenic response to ecdysone in the adult mosquito. *Mol. Cell. Endocrinol.* 2000; 160:25–37. [PubMed: 10715536]
- Karim FD, Guild GM, Thummel CS. The *Drosophila* Broad-Complex plays a key role in controlling ecdysone-regulated gene expression at the onset of metamorphosis. *Development.* 1993; 118:977–988. [PubMed: 8076529]
- Keshan B, Hiruma K, Riddiford LM. Developmental expression and hormonal regulation of different isoforms of the transcription factor E75 in the tobacco hornworm *Manduca sexta*. *Dev. Biol.* 2006; 295:623–632. [PubMed: 16697364]
- King-Jones K, Thummel CS. Nuclear receptors--a perspective from *Drosophila*. *Nat. Rev. Genet.* 2005; 6:311–323. [PubMed: 15803199]

- Kokoza VA, Martin D, Mienaltowski M, Ahmed A, Morton C, Raikhel AS. Transcriptional regulation of the mosquito *Aedes aegypti* vitellogenin gene by a blood-meal triggered cascade. *Gene*. 2001; 274:47–65. [PubMed: 11674997]
- Kokoza VA, Raikhel AS. Targeted gene expression in the transgenic *Aedes aegypti* using the binary Gal4-UAS system. *Insect Biochem. Mol. Biol.* 2011; 41:637–644. [PubMed: 21536128]
- Lam GT, Jiang C, Thummel CS. Coordinating of larval and prepupal gene expression by DHR3 orphan receptor during *Drosophila* metamorphosis. *Development*. 1997; 124:1757–1769. [PubMed: 9165123]
- Li C, Kapitskaya MZ, Zhu JS, Miura K, Segraves WA, Raikhel AS. Conserved mechanism for stage specificity of the mosquito vitellogenic response to ecdysone. *Dev. Biol.* 2000; 224:96–110. [PubMed: 10898964]
- Mane-Padros D, Cruz J, Vilaplana L, Pascual N, Belles X, Martin D. The nuclear hormone receptor links molting and developmental progression in the direct-developing insect *Blattella germanica*. *Dev. Biol.* 2008; 315:147–160. [PubMed: 18207139]
- Martin D, Wang SF, Raikhel AS. The vitellogenin gene of the mosquito *Aedes aegypti* is a direct target of the ecdysteroid receptor. *Mol. Cell. Endocrinol.* 2001; 173:75–86. [PubMed: 11223179]
- Morens DM. Dengue fever and dengue hemorrhagic fever. *Infect. Pediatr. Dis. J.* 2009; 28:635–636.
- Nakagawa Y, Henrich VC. Arthropod nuclear receptors and their role in molting. *FEBS J.* 2009; 276:6128–6157. [PubMed: 19796154]
- Palli SR, Ladd TR, Ricci AR, Sohi SS, Retnakaran A. Cloning and developmental expression of Choristoneura hormone receptor 75: a homologue of the *Drosophila* E75A gene. *Dev. Genet.* 1997; 20:36–46. [PubMed: 9094210]
- Parthasarathy R, Palli SR. Molecular analysis of nutritional and hormonal regulation of female reproduction in the red flour beetle, *Tribolium castaneum*. *Insect Biochem. Mol. Biol.* 2011; 41:294–305. [PubMed: 21288489]
- Pierceall WE, Li C, Biran A, Miura K, Raikhel AS, Segraves WA. E75 expression in the mosquito ovary and fat body suggests reiterative use of ecdysone-regulated hierarchies in development and reproduction. *Mol. Cell. Endocrinol.* 1999; 150:73–89. [PubMed: 10411302]
- Raghuram S, Stayrook KR, Huang P, Rogers PM, Noise AK, McClure DB, Burris LL, Khorasanizadeh S, Burris TP, Rastinejad F. Identification of heme as the ligand for the orphan nuclear receptors REB-ER α and REV-ERB β . *Nature Struct. Mol. Biol.* 2007; 14:1207–1213. [PubMed: 18037887]
- Raikhel, AS. Vitellogenesis of disease vectors, from physiology to genes. In: Marquardt, WC., editor. *Biology of Disease Vectors*. Academic Press: Elsevier; 2004. p. 329-346.
- Raikhel, AS.; Brown, M.; Belles, X. Hormonal control of reproductive processes. In: Gilbert, L.; Gill, S.; Iatrou, K., editors. *Comprehensive Insect Physiology, Biochemistry, Pharmacology and Molecular Biology*. Vol. Vol.3. Endocrinology, Elsevier Press; 2005. p. 433-491.
- Reinking J, Lam MM, Pardee K, Samson HM, Liu S, Yang P, Williams S, White W, Lajoie G, Edwards A, Krause HM. The *Drosophila* nuclear receptor E75 contains heme and is gas responsive. *Cell*. 2005; 122:195–207. [PubMed: 16051145]
- Roy SG, Hansen IA, Raikhel AS. Effect of insulin and 20-hydroxyecdysone in the fat body of the yellow fever mosquito, *Aedes aegypti*. *Insect Biochem. Mol. Biol.* 2007; 37:1317–1326. [PubMed: 17967350]
- Ruaud AF, Lam G, Thummel CS. The *Drosophila* nuclear receptors DHR3 and β FTZ-F1 control overlapping developmental responses in late embryos. *Development*. 2010; 137:123–131. [PubMed: 20023167]
- Segraves WA, Hogness DS. The E75 ecdysone-inducible gene responsible for the 75B early puff in *Drosophila* encodes two new members of the steroid receptor superfamily. *Genes Dev.* 1990; 4:204–219. [PubMed: 2110921]
- Segraves WA, Woldin C. The E75 gene of *Manduca sexta* and comparison with its *Drosophila* homolog. *Insect Biochem. Mol. Biol.* 1993; 23:91–97. [PubMed: 8485520]
- Sun G, Zhu J, Chen L, Raikhel AS. The early gene E74B isoform is a transcriptional activator of the ecdysteroid regulatory hierarchy in mosquito vitellogenesis. *Mol. Cell. Endocrinol.* 2004; 218:95–105. [PubMed: 15130514]

- Sun G, Zhu J, Chen L, Raikhel AS. Synergistic action of E74B and Ecdysteroid Receptor in activating a 20-hydroxyecdysone effector gene. *Proc. Natl. Acad. USA*. 2005; 102:15506–15511.
- Suzuki Y, Truman JW, Riddiford LM. The role of Broad in the development of *Tribolium castaneum*: implications for the evolution of the holometabolous insect pupa. *Development*. 2008; 135:569–577. [PubMed: 18171684]
- Swevers S, Eystathioy T, Iatrou K. The orphan nuclear receptors BmE75A and BmE75C of the *Bombyx mori*: hormonal control and ovarian expression. *Insect Biochem. Mol. Biol.* 2002; 32:1643–1652. [PubMed: 12429116]
- Terashima J, Bownes M. E75A and E75B have opposite effects on the apoptosis/development choice of the *Drosophila* egg chamber. *Cell Death Differentiation*. 2006; 13:454–464.
- Thummel CS. Molecular mechanism of developmental timing in *C. elegans* and *Drosophila*. *Dev. Cell*. 2001; 1:453–465. [PubMed: 11703937]
- Wang SF, Li C, Zhu JS, Raikhel AS. Differential expression and regulation of mosquito ecdysteroid receptor isoforms by 20-hydroxyecdysone. *Mol. Cell. Endocrinol.* 2002; 196:29–42. [PubMed: 12385823]
- White KP, Hurban P, Wanatabe T, Hogness DS. Coordination of *Drosophila* metamorphosis by two ecdysone-induced nuclear receptors. *Science*. 1997; 276:114–117. [PubMed: 9082981]
- Wu, n; Yin, L.; Hanniman, EA.; Joshi, S.; Lazar, MA. Negative feedback maintenance of heme homeostasis by its receptor, Rev-erb α . *Genes Dev.* 2009; 23:2201–2209. [PubMed: 19710360]
- Yin L, Wu N, Curtin JC, Qatanani M, Szewergold NR, Reid RA, Waitt GM, Parks DJ, Pearce KH, Wicely GB, Lazar MA. Rev-erb α , a heme sensor that coordinates metabolic and circadian pathways. *Science*. 2007; 318:1786–1789. [PubMed: 18006707]
- Zhou B, Hiruma K, Jindra M, Shinoda T, Segraves WA, Malone F, Riddiford LM. Regulation of the transcription factor E75 by 20-hydroxyecdysone and juvenile hormone in the epidermis of the tobacco hornworm, *Manduca sexta*, during larval molting and metamorphosis. *Dev Biol.* 1998; 193:127–138. [PubMed: 9473318]
- Zhou X, Riddiford LM. Broad specifies pupal development and mediates the ‘status quo’ of juvenile hormone on the pupal-adult transformation in *Drosophila* and *Manduca*. *Development*. 2002; 129:2259–2269. [PubMed: 11959833]
- Zhu J, Chen L, Sun G, Raikhel AS. The competence factor β Ftz-F1 potentiates ecdysone receptor activity via recruitment of a p160/SRC coactivator. *Mol. Cell. Biol.* 2006; 26:9402–9412. [PubMed: 17015464]
- Zhu J, Chen L, Raikhel AS. Distinct roles of Broad isoforms in regulation of 20-hydroxyecdysone effector gene, vitellogenin, in the mosquito *Aedes aegypti*. *Mol. Cell. Endocrinol.* 2007; 267:97–105. [PubMed: 17303321]

Fig. 1. Effect of RNAi depletions of E75 isoforms on their transcript levels in fat bodies of *A. aegypti* female mosquitoes

dsRNAs of AaE75A, AaE75B, AaE75C or Mal were injected into one-day-old female mosquitoes and transcript abundance was measured by means of QRT-PCR using E75 isoform-specific primers. Data were normalized using S7 ribosomal RNA. Data representing means $\pm$ standard errors of the means from three independent experiments are shown. * Indicates statistical significance < 0.05 . PV –previtellogenic stage; PBM – post blood meal stage.

Fig. 2. Effect of RNAi depletions of E75 isoforms on expression of Ecdysone receptor A (EcRA) RNAi depletion of E75 isoforms was performed as in Fig. 2 and the same samples were used for measuring EcRA transcript abundance by means of QRT-PCR. Data were normalized using S7 ribosomal RNA and represented as means $\pm$ standard errors of the means from three independent experiments. PV –previtellogenic stage; PBM – post blood meal stage. RNAi depletion of E75 using dsRNA specific to a common region (dsE75com) was also performed as a control. Data are expressed in fold induction relative to S7. Data (means $\pm$ standard errors of the means) from three independent experiments are shown. * Indicates statistical significance < 0.05.

Fig. 3. Effect of RNAi depletions of E75 isoforms on expression of Ecdysone receptor B (EcRB) RNAi depletion of E75 isoforms was performed as in Fig. 2 and the same samples were used for measuring EcRB transcript abundance by means of QRT-PCR. Data were normalized using S7 ribosomal RNA and represented as means $\pm$ standard errors of the means from three independent experiments. PV –previtellogenic stage; PBM – post blood meal stage. RNAi depletion of E75 using dsRNA specific to a common region (dsE75com) was also performed as a control. Data are expressed in fold induction relative to S7. * Indicates statistical significance < 0.05 .

Fig. 4. Effect of RNAi depletions of E75 isoforms on expression of Broad isoform Z2 (BrZ2)
 RNAi depletion of E75 isoforms was performed as in Fig. 2 and the same samples were used for measuring BrZ2 transcript abundance by means of QRT-PCR. Data were normalized using S7 ribosomal RNA and represented as means $\pm$ standard errors of the means from three independent experiments. PV –previtellogenic stage; PBM – post blood meal stage. RNAi depletion of E75 using dsRNA specific to a common region (dsE75com) was also performed as a control. Data are expressed in fold induction relative to S7. * Indicates statistical significance < 0.05.

Fig. 5. Effect of RNAi depletions of E75 isoforms on expression of the nuclear receptor HR3
 RNAi depletion of E75 isoforms was performed as in Fig. 2 and the same samples were used for measuring HR3 transcript abundance by means of QRT-PCR. Data were normalized using S7 ribosomal RNA shown in fold induction relative to S7. Data (means $\pm$ standard errors of the means) from three independent experiments are shown. PV –previtellogenic stage; PBM – post blood meal stage. RNAi depletion of E75 using dsRNA specific to a common region (dsE75com) was also performed as a control. * Indicates statistical significance < 0.05 .

A **B**

Fig. 6. Effect of RNAi depletions of E75A and E75C isoforms on HR3 transcript abundance in fat bodies of *A. aegypti* female mosquitoes in vitro

Fat bodies from female mosquitoes were treated as in Fig. 2, dissected four days after dsRNAs injections and incubated in a complete culture medium in the absence or presence of 20E (10^{-6} M). HR3 transcript abundance was measured by means of QRT-PCR. Data (means $\pm$ standard errors of the means) from three independent experiments are shown. Data are expressed in fold induction normalized to S7. * Indicates statistical significance < 0.05 .

Fig. 7. Effect of RNAi depletions of E75 isoforms on vitellogenin (Vg) transcript abundance in fat bodies of *A. aegypti* female mosquitoes

dsRNAs of AaE75A, AaE75B, AaE75C or Mal were injected into 1 day-old female mosquitoes and transcript abundance was measured by means of QRT-PCR using Vg-specific primers. (A) Vg transcript abundance was assayed in fat bodies in indicated hours at the previtellogenic stage (PV) or after blood feeding (PBM). (B) Fat bodies from female mosquitoes treated as in (A) were dissected four days after dsRNAs injections and incubated in a complete culture medium in the absence or presence of 20E (10^{-6} M) for 6 h. For both (A) and (B), data (means $\pm$ standard errors of the means) from three independent experiments are shown. Data are expressed in fold induction normalized to S7. * Indicates statistical significance < 0.05 .

dsE75A

dsE75C

Fig. 8. Effect of cycloheximide (Chx) on the Vg transcript abundance in fat bodies of *A. aegypti* female mosquitoes after RNAi depletions of E75A and E75C isoforms

Fat bodies from female mosquitoes, treated as in Fig. 8A, were dissected 4 days after dsRNAs injections and incubated in a complete culture medium in the absence or presence of 20E (10^{-6} M) for 6 h. Fat bodies from dsE75A, dsE75C and dsMal controls were also incubated in the presence of 20E (10^{-6} M) with the addition of 1 mM Chx. For both (dsE75A) and (dsE75C) data represent means $\pm$ standard errors of the means from three independent experiments. Data are expressed in fold induction normalized to S7. * Indicates statistical significance < 0.05 .

Fig. 9. Heme is required for a high level of Vg gene expression
A) Fat bodies from 3-day-old previtellogenic female mosquitoes and incubated in a culture medium without or with amino acids (AA) in the absence or presence of 20E (10^{-6} M) for 6 h. Fat bodies were also incubated in the presence of 6 μ M of hemin. B) Fat bodies from dsE75A depleted and dsMal control female mosquitoes were incubated as in (A). C) Fat bodies from dsE75C depleted and dsMal control female mosquitoes were incubated as in (A). For all experiments, QRT-PCR results represent means $\pm$ standard errors of the means from three independent experiments. Data are expressed in fold induction normalized to S7. * Indicates statistical significance < 0.05 .