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Forkhead transcription factors regulate mosquito reproduction

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Abstract

Forkhead box (Fox) genes encode a family of transcription factors defined by a 'winged helix' DNA-binding domain. In this study we aimed to identify Fox factors that are expressed within the fat body of the yellow fever mosquito *Aedes aegypti*, and determine whether any of these are involved in the regulation of mosquito yolk protein gene expression. The *Ae. aegypti* genome contains eighteen loci that encode putative Fox factors. Our stringent cladistic analysis has profound implications for the use of Fox genes as phylogenetic markers. Twelve *Ae. aegypti* Fox genes are expressed within various tissues of adult females, six of which are expressed within the fat body. All six Fox genes expressed in the fat body displayed dynamic expression profiles following a blood meal. We knocked down the 'fat body Foxes' through RNAi to determine whether these "knockdowns" hindered amino acid-induced vitellogenin gene expression. We also determined the effect of these knockdowns on the number of eggs deposited following a blood meal. Knockdown of FoxN1, FoxN2, FoxL, and FoxO, had a negative effect on amino acid-induced vitellogenin gene expression and resulted in significantly fewer eggs laid. Our analysis stresses the importance of Fox transcription factors in regulating mosquito reproduction.

Keywords

forkhead; *Aedes aegypti*; fat body; vitellogenin; yolk protein; RNAi

1. Introduction

The class of proteins termed forkhead-box (Fox) transcription factors are a large and diverse group of transcriptional regulators characterized by a conserved 110-amino acid 'forkhead box' DNA-binding domain, also referred to as the 'winged-helix' domain. This domain consists of three alpha-helices connected by two polypeptide loops, or 'wings,' which are related to the 'helix-turn-helix' domain found in homeobox-containing proteins. Forkhead box-containing proteins have been identified in eukaryotic organisms from yeast to humans, and have been shown to play important roles in various biological processes, including development, metabolism, immunoregulation, development of cancer, and aging (Carlsson & Mahlapuu, 2002; Coffe & Burgering, 2004; Jonsson & Peng, 2005; Kaufmann & Knochel, 1996; Lehmann *et al.*, 2003). The nomenclature for the chordate forkhead transcription factors has been revised, and these genes, now termed 'Fox' (after 'Forkhead box'), are divided into

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seventeen subclasses, or clades, (A to Q), based on the amino acid (AA) sequence of their conserved forkhead domain (Kaestner *et al.*, 2000). Invertebrates share several Fox genes that can be subgrouped according to the vertebrate subfamilies (Mazet *et al.*, 2003).

The first Fox protein, forkhead (FoxA), was identified as a homeotic gene in the fruit fly *Drosophila melanogaster* (Weigel *et al.*, 1989), followed by a description of its Fox DNA-binding domain (Weigel & Jackle, 1990). Several additional Fox transcription factors have been described in *Drosophila*, with greater emphasis placed on their regulation of embryonic development. *crocodile* (FoxC) is an early patterning gene expressed in the head anlagen of the *Drosophila* embryo, and is responsible for establishing the development of head structures (Hacker *et al.*, 1995). *binou* (FoxF) plays a key role in the development of the visceral mesoderm and the derived gut musculature (Zaffran *et al.*, 2001). The two FoxG genes in *Drosophila*, *sloppy paired 1* and *sloppy paired 2* (Grossniklaus *et al.*, 1992) are both involved in the regulation of early embryo segmentation (Cadigan *et al.*, 1994). *domina* (FoxN) is expressed mainly within the central and peripheral nervous systems, and *domina* mutants display multiple phenotypes including diminished vitality and fertility (Strodick *et al.*, 2000). The best-characterized invertebrate Fox gene is FoxO (*Caenorhabditis elegans*, DAF16), a target of the insulin-signaling pathway. dFoxO regulates insulin signaling in the brain and fat body, along with controlling fertility and lifespan (Hwangbo *et al.*, 2004a). FoxO manifests this effect through exhibiting transcriptional feedback control of the insulin receptor (Puig & Tjian, 2005). Together, these studies stress the importance of Fox transcription factors for regulating development and homeostasis in *Drosophila*. However, the function for a majority of Fox transcription factors of *Drosophila* and other insects are yet to be described.

It has been suggested that Fox factors may be involved in the regulation of mosquito reproduction, for the vitellogenin (*Vg*) gene of *Ae. aegypti* has a Fox-binding site at position -469 of its proximal promoter. *In vitro* expressed *Drosophila* FoxA can bind to the promoter of the *Vg* gene, and further, it has been suggested that a Fox transcription factor in combination with other transcriptional regulators may coordinate the tissue-specific expression of *YPP* genes in the fat body of mosquitoes (Kokoza *et al.*, 2001). Interestingly, the Fox-binding site in the *Vg* promoter is in close proximity to a CCAAT/Enhancer-binding site. A similar arrangement of binding sites has been found in the proximal promoter of the human decidal prolactin gene. Human FoxO1 interacts directly with C/EBP β , and this complex binds to the composite Fox/C/EBP β binding site to strongly activate decidal prolactin transcription (Christian *et al.*, 2002).

In this study, we identified eighteen putative Fox transcription factors from the genome of *Ae. aegypti*, along with predicting their phylogenetic relationships with other insect and human Fox factors. We determined whether the identified Fox factors are expressed within tissues of adult female mosquitoes both before and after a bloodmeal. The Fox factors determined to be expressed within the fat body were characterized further with a more extensive expression profile during the course of a gonotrophic cycle, and by determining effects of knockdown of these Fox factors on *Vg* gene expression. Using RNA interference-mediated knockdown, we identified several of these Fox factors as being involved in promoting vitellogenesis and mosquito reproduction. The results of our study lay the foundation for the study of the role of Fox factors in the regulation of reproduction in mosquitoes as well as other insects.

2. Materials and methods

2.1. Sequence Identification

Predicted cDNA and deduced AA sequences of Fox transcription factors were identified using BLAST tools at two databases: Ensembl (http://www.ensembl.org/Aedes_aegypti/blastview), and VectorBase (<http://www.vectorbase.org>). Predicted Fox cDNAs were blasted against an

EST-database at TIGR (TIGR (<http://tigrblast.tigr.org/tgi/>). The full length coding sequences of *Ae. aegypti* FoxL, and FoxO, and partial sequences of FoxN1, and FoxN2 were isolated and sequenced. Sequences were submitted to Genbank with the accession numbers: EF088380 (FoxO), EF088381 (FoxL), EF537041 (FoxN2). Nine Hymenoptera sequences (EF137264-EF137272) were provided by John Heraty. Human Fox transcription factor sequences were obtained from Kaestner *et al.* (Kaestner et al., 2000).

2.2. Phylogenetic Analyses of Sequences

Stringent cladistic analyses were conducted on the data. Four alignment programs produced competing alignments, which were evaluated, and the best two scoring alignments then subjected to cladistic analyses using parsimony, maximum likelihood, and Bayesian methodologies (see Supplemental Methods for a complete discussion).

2.3. *Ae. aegypti* Rearing and Fat Body Culture

The *Ae. aegypti* mosquito strain UGAL/Rockefeller was maintained in laboratory culture as described by Hays and Raikhel (Hays & Raikhel, 1990). The fat body culture system is described in detail elsewhere (Hays & Raikhel, 1990; Raikhel *et al.*, 1997).

2.4. RNA Isolation

For expression profiling, RNA was isolated from head (hd), thorax (tx), abdominal body wall (fb), midgut (gt), malpighian tubules (mt) and ovaries (ov) both before (PV = previtellogenic) and after a bloodmeal (24h PBM, post blood meal). Tissues of 10-50 individual mosquitoes were pooled. For the fat body expression time course, the fb was dissected from females at PV, 6, 16, 22, 24, 30, 48 and 65 hours PBM. For the fb time course, quantitative PCR (qPCR) against three groups of three fat bodies were analyzed. Total RNA was isolated by means of a commercially available modification (TRIzol, Invitrogen, Carlsbad, CA) of the one-step phenol/guanidinium thiocyanate method (14). Genomic DNA contamination was removed by treatment with Rnase-free Dnase I (Invitrogen) prior to cDNA synthesis. Reverse transcription was carried out using an Omniscript reverse transcriptase kit (Qiagen, Hilden, Germany) with a final volume of 20- μ l reaction mixture containing oligo(dT) primers and 1 μ g of total RNA at 37 °C for 1 h.

2.5. RT-PCR

The presence of the different Fox transcripts within the various organs and body parts of female *Ae. aegypti* was determined by non-quantitative PCR. The PCR program used was 15 min at 95 °C followed by 40 cycles of 95 °C for 30 s, 50 °C for 15 s, 72 °C 1 min. We used two independent primer pairs, and if possible, choose primer pairs that span an intron to address genomic DNA contamination. The sequences of the primers can be found in supplemental Table 2A.

2.6. Quantitative PCR

Relative transcript levels were determined by qPCR, using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA). The quantity of transcripts was estimated from a standard line derived from 10-fold serial dilutions of fb cDNA of 24h PBM females (iCyclerTMiQ Optical System Software Version 3.0a). The sequences of the primers can be found in supplemental Table 2B. Analysis of variance was performed using SAS version 9.0 software (SAS Institute Inc., Cary, NC) to test for statistical differences among treatments.

2.7. RNAi

For the generation of double-stranded RNAs (dsRNA), template cDNAs were amplified with forward and reverse primers containing the T7 primer sequence at their 5' ends. The sequences of the primers can be found in supplemental Table 2C. Following amplification by RT-PCR, dsRNA was transcribed using the MEGAscript® T7 Kit and T7-RNA polymerase (Ambion, Austin, Tx). Approximately 1 µg of dsRNA contained within 0.2-0.4 µl of nuclease-free H₂O was injected into the thorax of CO₂-anesthetized female mosquitoes 2 days after adult emergence. The injected mosquitoes were then allowed to recover for 3 days before being processed for *in vitro* culture or blood feeding.

3. Results

3.1. The Fox Gene Family of transcription factors of *Ae. aegypti*

Sequences were acquired from the following databases: *Ae. aegypti*: <http://aaegypti.vectorbase.org/>; *Anopheles gambiae*: <http://agambiae.vectorbase.org/>; *D. melanogaster*: <http://flybase.net/>; *Apis mellifera* and *Homo sapiens*: <http://www.ncbi.nlm.nih.gov>.

BLAST searches conducted with Fox DNA-binding domains of *Drosophila* and vertebrate Fox factors allowed the identification of 18 loci in *Ae. aegypti* that encode proteins containing Fox domains (Table 1). All 18 predicted proteins contain only one Fox DNA-binding domain (Fig. 1). A multiple sequence alignment of the eighteen *Aedes* Fox DNA-binding core domains, performed with the ClustalW program (Thompson *et al.*, 1994), defines high conservation within regions predicted to encode alpha helices and beta strands (Fig. 2A).

Amino acid and nucleotide sequences from *D. melanogaster*, *Ae. aegypti*, *An. gambiae*, nine Hymenoptera genera and human, *H. sapiens*, were used to create 12 phylogenetic trees. The RAxML maximum likelihood tree (Figure 2B) shows that the insect Fox proteins form well-resolved subfamilies corresponding to the Fox subfamilies of humans; FoxA, FoxB, FoxC, FoxD, FoxE, FoxL2, FoxN2/3, FoxO, FoxP, and FoxQ2 are well-supported, monophyletic subfamilies across all analyses. FoxK was monophyletic across all analyses; however it lacked support in the TNT amino acid analyses. FoxF was monophyletic in all but the MrBayes ProbCons amino acid analysis and had support in only 7 analyses. FoxG was monophyletic in all but the TNT MAFFT amino acid analysis, but lacked support in all TNT analyses. FoxK was monophyletic across all analyses; however it lacked support in the TNT amino acid analyses. FoxJ, FoxL, and FoxQ and FoxN (*sensu lato* FoxL1+FoxL2, FoxQ1+FoxQ2, Fox1/4+Fox2/3) were not monophyletic assemblages. Lee and Frasch (Lee & Frasch, 2004) failed to find an orthologous *Drosophila* gene in FoxJ. Yet our analyses found their orphan taxon DM CG 12632 sister to *Ae. aegypti* 25232 m01727 (100% support in all analyses), thus tentatively ascribing it to FoxJ and their orphan DM CG 9571 was placed in Fox G in all but our TNT MAFFT amino acid analysis. (See Supplemental Results for a complete discussion).

3.2. Expression of Fox genes in adult females of *Ae. aegypti*

To determine whether the Fox transcription factors identified from the *Ae. aegypti* genome are expressed in adult females, we isolated total RNA from the following female body parts: head, thorax, fat body (abdominal body wall), midgut, malpighian tubules, and ovaries. RNA was isolated from previtellogenic mosquitoes 72 h post eclosion and 24 h post blood meal (24h PBM).

Two primer sets for each Fox factor were used to profile Fox expression within the various tissues as described within Experimental Procedures. Because of the extremely high sequence identity between FoxA1 and FoxA2 only one primer set was used that recognizes both

transcripts. Note that the reported results were observed for both primer sets for all Fox factors displayed in Fig. 3.

Five of the eighteen putative Fox genes were not amplified from any tissue of adult females: FoxB1, FoxC, FoxD, FoxJx, and FoxJy. Three genes, FoxB2, FoxG, and FoxP, are only expressed within the thorax of unfed mosquitoes. Three Foxes share a broad expression pattern: FoxK1, FoxK2 and FoxO. Transcripts for these three Fox factors were observed in all body regions except heads. Interestingly, transcripts for FoxK1 were expressed in heads of females 24h PBM. The remaining six Foxes displayed unique expression patterns: FoxA is expressed in the thorax, midgut, and malpighian tubules of non-bloodfed and in midgut and malpighian tubules of 24 h PBM mosquitoes; FoxF was observed in ovaries both before and after a blood meal; FoxL is expressed in thorax, fat body, and ovaries of both non-bloodfed and blood-fed mosquitoes, and in malpighian tubules following a blood-meal; FoxN1 is strongly expressed in the ovaries both before and after a blood-meal, and increases in abundance in the fat body following a bloodmeal (also see Quantitative PCR results). FoxN1 transcripts were also detected in other tissues both before and after a bloodmeal; FoxN2 was expressed exclusively within ovaries of non-bloodfed mosquitoes, and is up-regulated in the fat body, malpighian tubules, and ovaries after a bloodmeal; Lastly, FoxQ was only observed within the ovaries of non-bloodfed mosquitoes.

3.3. Fat body expression of Fox genes in blood-fed *Ae. aegypti* females

To focus on the regulation of reproductive development in the fat body, we examined the expression profiles of the six “fat body” Fox factors during a gonotrophic cycle. As determined through real-time PCR analysis, FoxK1, FoxK2, and FoxN1 displayed a similar expression profile, with an apparent decrease in transcript abundance at 6 h PBM followed by a general increase reaching their highest point at 65 h PBM (> 30 hours after vitellogenesis, and the last time point measured; Fig. 4). The expression pattern of FoxO was similar but for an apparent reduction in transcript abundance 16 h PBM, followed by a gradual increase as the gonotrophic cycle continues. FoxL transcripts declined at both 6 and 16 h PBM, rising afterwards reaching a peak at 36 h PBM, and declining thereafter. In contrast to the above described expression patterns, FoxN2 transcripts are strongly up regulated beginning at 16 h PBM peaking at 22 h PBM, remaining high thereafter until later in the gonotrophic cycle when they begin to decline (i.e., 65h PBM). The results received in our real-time PCR analysis corroborate that observed in the initial RT-PCR screen.

3.4. The effect of Fox knockdown on yolk protein gene expression

Next, we wanted to determine whether any of the Fox factors play a role in the reproductive development of *Ae. aegypti*. To this end, we employed RNA interference to knockdown each “fat body” Fox factor individually, and assess the effect the knockdown has on the ability of AAs to stimulate Vg expression *in vitro*. Knockdown of the “fat body” Fox factors was achieved by injecting dsRNA corresponding to the specific Fox factors into PV females, allowing them to recover for 3 days, after which the fat body culture experiment was performed (see below). RNA was isolated, cDNA synthesized, and RT-PCR analysis conducted to confirm an adequate reduction in transcript abundance for the respective Fox factors. RNAi-mediated knockdown of Fox transcripts was highly effective (see Fig. 5A). Interestingly, we found stimulation of fat bodies with AAs in fat body culture resulted in up regulation of FoxN2 transcription in fat bodies of control mosquitoes (compare Fig. 5A, lane 11 with FoxN2 expression in PV fat bodies, Fig.3).

Previous research in our laboratory has shown that the expression of the yolk protein gene, vitellogenin (Vg), is regulated by the nutritional target-of-rapamycin (TOR) pathway and is induced when isolated mosquito fat bodies are provided a balanced mixture of amino acids

(AAs) *in vitro* (Hansen *et al.*, 2004). In our next experiment we addressed whether any of the “fat body” Fox factors mediate the AA-stimulation of *Vg* expression. To address this question, fat bodies from females that had been injected with dsRNA corresponding to the six “fat body” Fox factors were incubated within media provided with balanced amino acids (AA-media) or media without AAs (control). The isolated fat bodies were allowed to incubate for 6 hours, after which RNA was extracted, cDNA synthesized and *Vg* transcript levels determined by qPCR. We measured three groups of three fat bodies per treatment and performed this experiment with three independent groups of mosquitoes. As shown in Fig. 5B, the AAs stimulated transcription of the *Vg* gene was significantly induced in females injected with control dsRNAs (MAL Fig. 5B). *Vg* gene induction was also observed in our negative control, FoxF (i.e., since it’s not expressed within the fat body; see Fig. 3). RNAi-mediated knockdown of FoxK1 and FoxK2 had no apparent effect on *Vg* gene expression. In contrast, knockdown of FoxL and FoxN1 resulted in a dramatic reduction in the ability of AAs to stimulate *Vg* transcription. A more impressive inhibition of AA-stimulation of *Vg* transcription was observed following RNAi knockdown of FoxN2 and FoxO. As a control the transcript levels for actin and ribosomal protein S7 were determined in the cDNA samples of the experiment above. Actin transcript levels were not affected by RNAi-mediated knockdown of Fox factors (Fig. 5C) as were the transcript levels of ribosomal protein S7 (data not shown).

3.5. RNAi-mediated Fox knockdown reduces mosquito fecundity

20 female mosquitoes received injections of Fox dsRNA 1 day after emergence. After a five day recovery period a blood meal was then provided. The following day, blood-fed females were transferred to individual containers. To stimulate egg deposition, wet filter paper was placed into these containers four days after the blood meal. As can be seen in Fig. 6, knockdown of FoxK1 and FoxK2 had no visible effect on the number of eggs deposited per female. In contrast, knockdown of FoxL, FoxN1, FoxN2, and FoxO resulted in a significant reduction in eggs deposited.

4. Discussion

The study presented here includes the identification of eighteen genes encoding putative Fox transcription factors in *Ae. aegypti*, which represents the foundation from which to address the role that Fox transcription factors play in the regulation of mosquito reproduction. We further focused on Fox factors expressed within the fat body, an insect tissue homologous to vertebrate liver and fat tissues.

We searched the newly completed genome of *Ae. aegypti* for the DNA-binding motive present in Fox transcription factors. This search resulted in the identification of eighteen loci that encode putative Fox factors. Similar searches resulted in the identification of 18 loci from the genome of the malaria mosquito *An. gambiae* (Mazet *et al.*, 2003), and 16/17 loci from the genome of the fruit fly *D. melanogaster* (Lee & Frasch, 2004; Mazet *et al.*, 2003). Of possible significance, the number of Fox factors predicted to occur in these three dipteran insects is less than half of that proposed for humans (40 Fox loci for humans are listed at the following web site: <http://www.biology.pomona.edu/Fox.html>) (Kaestner *et al.*, 2000). Past studies have used neighbor-joining to construct evolutionary hypotheses. Neighbor-joining (NJ) is a methodology for the construction of phylogenetic hypotheses that should be used only to produce a starting tree upon which more thorough searches can be performed (Swofford *et al.*, 1996). Nevertheless, our more rigorous analyses share some features in common with those of past (Lee & Frasch, 2004; Mazet *et al.*, 2003).

The Fox subfamilies are a collection of orthologous and paralogous genes (Mazet *et al.*, 2003), with the latter gene duplication events evident in both humans and insects. In *Ae. aegypti* we found a recent duplication event of the FoxA gene, as compared to *An. gambiae*,

which has a single copy. Like Mazet *et al.*, we found no orthologous insect genes in the FoxE, FoxH, FoxI, FoxJ, FoxM, and FoxQ1 subfamilies. We found that FoxA, FoxB, FoxC, FoxD, FoxE, FoxL2, FoxN2/3, FoxO, and FoxP are well-supported, monophyletic subfamilies. FoxG and FoxK were monophyletic in all analyses, yet did not receive support in all analyses. FoxF was monophyletic in all but two analyses. FoxJ, FoxL1, and FoxQ and FoxN (*sensu lato* FoxQ1+FoxQ2, Fox1/4+Fox2/3) were not monophyletic assemblages. Future stringent cladistic analyses with increased taxon sampling, especially in instances where Fox subfamilies are represented by single taxa, should better resolve our understanding of Fox relationships and their evolution. The presence of paralogous Fox genes and their expression in different tissues has a profound effect on their successful use as phylogenetic markers for both vertebrates and invertebrates. (See Supplemental Discussion for a complete discussion).

An initial assessment of whether the eighteen Fox genes identified from the *Ae. aegypti* genome are expressed within adult female mosquitoes was addressed by conducting RT-PCR against female tissues both before and after a bloodmeal (24 h PBM; the time post bloodmeal that vitellogenic gene expression is at its peak). The number of PCR cycles was optimized to ensure the detection of marginally expressed Fox genes. The results of RT-PCR analysis define a diverse expression pattern for most of the *Ae. aegypti* Fox factors throughout the body of female mosquitoes. Importantly, of the eighteen *Ae. aegypti* Fox factors identified, only twelve were detected in adult female mosquitoes. Whether the five Fox factors not observed within our study are expressed at other life stages of the mosquito (e.g., embryonic, larval), or are male-specific transcription factors will require further study. Taken together, a surprisingly high number of the predicted Fox factors are expressed in adult female mosquitoes, many of them expressed differentially before and after a bloodmeal. This suggests that most members of the Fox family of transcription factors are expressed dynamically during the gonotrophic cycle of adult female mosquitoes.

Six different Fox factor transcripts were expressed within abdominal fat body tissue; of likely importance, FoxN2 was expressed only after a bloodmeal. The Fox factors expressed within midgut and malpighian tubules displayed a similar pattern of expression in the adult female mosquito, six of which were observed in these tissues both before and after a blood meal. Two other Fox factors are expressed within the malpighian tubules following a bloodmeal, but are absent from the midgut (FoxL and FoxN2). Eight different Fox factors are expressed in ovaries of non-bloodfed females, including the insect-specific FoxQ. FoxQ represents the only Fox factor expressed exclusively within ovaries, as the other seven Fox factors expressed within ovaries are also expressed in other tissues.

The insect fat body, also called 'fat' (Kapahi *et al.*, 2004) is a multifunctional tissues that participates in intermediate metabolism, including protein, AA, lipid and carbohydrate synthesis and storage, detoxification, and immunity. In mosquitoes, the fat body is the sole source of yolk protein synthesis during vitellogenesis (Clements, 1992). To our knowledge, only one Fox factor has been described in the fat body of insects thus far, FoxO of *D. melanogaster* (dFoxO) (Giannakou *et al.*, 2004; Hwangbo *et al.*, 2004b).

From our initial RT-PCR analysis, we identified six different Fox factors that are expressed in the fat body of non-bloodfed and bloodfed adult female mosquitoes (see above). In addition to FoxO, two members of the FoxK and the FoxN subfamilies, and FoxL are expressed within this tissue. The expression profiles of all six of these Fox factors are highly dynamic during the gonotrophic cycle (Fig. 4). Four Fox factor transcripts (FoxK1, FoxK2, FoxN1, FoxO) begin a steep rise in expression at the vitellogenic termination phase, the period when yolk protein production ceases and the fat body returns to the previtellogenic state of arrest. The observed increase in these four Fox factors coincides with the rise in juvenile hormone titer at this later stage of the gonotrophic cycle. Thus, one could hypothesize the expression of these

four Fox factors may be regulated by juvenile hormone; although, one could also propose that a decline in ecdysone levels observed during these later stages of the gonotrophic cycle is responsible for the observed increase in these four Fox factors.

The expression profile of FoxN2 is fundamentally different from all other “fat body” Fox factors. It is similar to that observed for Vg, where both are weakly expressed in the fat body of non-bloodfed mosquitoes, becoming strongly up regulated PBM. However, the increase in FoxN2 expression after a bloodmeal trails that observed for Vg expression. Moreover, at the termination of vitellogenesis, Vg transcript abundance decreases significantly, whereas FoxN2 transcripts are still abundant.

RNAi-mediated knockdown of four of the six “fat body” Fox factors had a profound effect on AA-induced Vg gene expression (Fig. 5). Of particular interest is that RNAi-mediated knockdown of FoxK1 and FoxK2 had no significant effect on Vg expression, even though both of these Fox factors are highly expressed in the fat body in addition to other tissues. A broad expression pattern could indicate a general role in gene regulation; however, our results clearly show that Vg gene expression is not diminished after knockdown of either FoxK. One possible explanation is that the FoxKs are transcriptional repressors (i.e., their reduction has no observable effect on AA-induction of Vg). Such a regulatory role of the FoxKs will require further study.

We found that FoxL, FoxN1, FoxN2, and FoxO are required for activation of Vg gene expression in mosquitoes, given that RNAi-mediated knockdown of these transcripts resulted in a significant reduction in AA-induced Vg expression in fat bodies *in vitro* (Fig. 5B). Further, the RNAi-mediated knockdown of these four Fox factors resulted in a strong reduction of egg deposition (Fig. 6). Whether these transcription factors are direct activators of Vg expression, upstream to another Vg transcriptional activator, or even an upstream repressor of other transcriptional repressors will certainly require further examination; although, Fox factors have been shown to act mostly, but not exclusively, as transcriptional activators (Carlsson & Mahlapuu, 2002). Our results suggest that AA-TOR signaling may be mediated through these four Fox factors. It is noteworthy that since the six fat body-expressed Fox genes are also expressed in several other tissues, the knockdown effect on Vg gene expression (Fig. 5) and egg numbers (Fig. 6) may reflect RNAi-induced effects of non-fat body cells.

While the FoxO subfamily of transcription factors has been studied extensively in both vertebrates and invertebrates, the other Fox factors shown to be involved in AA-induction of Vg transcription in our system (FoxL, FoxN1, and FoxN2) have received little attention. So far, no data is available on the role of FoxL in *D. melanogaster*; however, FoxL2 has been shown to play an important role in vertebrate reproduction where human females carrying mutations in this gene suffer premature ovarian failure syndrome (Uhlenhaut & Treier, 2006). The *Drosophila* homologue of *Ae. aegypti* FoxN1 and FoxN2 are *jumeaux* (Cheah *et al.*, 2000) and *domina* (Strodicke *et al.*, 2000), respectively. *jumeaux* mutants display defects in the central nervous system, while homozygous *domina* mutants show several developmental effects as well as a severely diminished vitality and fertility.

Vertebrates have several FoxO proteins, many being identified as part of the insulin/insulin-like growth factor/Akt signaling pathway. The FoxO factors of vertebrates act as regulators of metabolism, cellular differentiation, and immunity (Accili & Arden, 2004; Coffey & Burgering, 2004). As opposed to the many FoxO factors observed in vertebrates, a single FoxO gene is predicted from the genomes of *D. melanogaster*, *Ae. aegypti*, and *An. gambiae*. Importantly, the dFoxO protein has been linked with insulin signaling in adult brain and fat body (Boutros *et al.*, 2004; Giannakou *et al.*, 2004; Hwangbo *et al.*, 2004a). Over-expression or reduction of dFoxO affected body size and lifespan of the flies.

In conclusion, we identified eighteen Fox transcription factors from the genome of the yellow fever mosquito, *Ae. aegypti*, along with determining their expression pattern in adult females. In the present study we observed that a great majority of the identified Fox factors are expressed within adult female mosquitoes. This observation is intriguing for the vast majority of Fox genes described so far have only been shown to have a role in embryonic development in vertebrates and invertebrates. Significantly, four of the six Fox factors expressed within the mosquito adult fat body are essential for the ability of AAs to stimulate Vg expression

Database Deposition: Sequences reported in this paper have been deposited in the GenBank™ database with the accession numbers EF088380 (FoxO), EF088381 (FoxL), EF537041 (FoxN2), EF587762 (FoxK1), and EF137264-137272 (hymenoptera Fox sequences).

List of supplemental online material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AA

	amino acid
BM	blood meal
Fox	forkhead box
PBM	post blood meal
PV	previtellogenic
RNAi	RNA interference
TOR	target of rapamycin
vg	vitellogenin
YPP	yolk protein precursor

Fig. 1. *Aedes aegypti* Fox genes. A) Designated name. B) Number of AAs (#AA). C) Schematic location of protein domains: Fox, winged helix DNA-binding domain; FHA, forkhead-associated domain; Zn, zinc finger domain. D) Scheme of genomic organization. Exons are marked with boxes, total gene length is indicated above.

Fig. 2. Phylogenetic analysis of the *Aedes aegypti* Fox genes. A) Alignment of the DNA binding domain from *Ae. aegypti* Fox factors. Alpha helices, H; beta sheets, S; wings, W. Identical residues in at least 9 of 18 sequences are shaded black, similar residues are shaded grey. B) RAxML best-known likelihood tree (-Ln -9340.947535) for the MAFFT generated alignment

of amino acid data and 500 replicates. Bootstrap values for 500 replicates are shown, with those in large font signifying subfamily support.

Fig. 3.

Expression of *Aedes aegypti* Fox genes in different tissues of previtellogenic female mosquitoes (PV) and 24 h after a blood meal (PBM). Total RNA of head, hd; thorax, tx; fat body, fb; midgut, gt; malpighian tubules, mt; and ovaries, ov was isolated, treated with DNase I to remove DNA contamination and subsequently used as template for RT-PCR. Below each gel image is a synopsis of RT-PCR results for three independent repeats: +, transcripts detectable in all samples, -, transcript absent in all samples; (-), transcripts absent in two of three samples. Amplification of actin cDNA was used to confirm cDNA integrity (last line).

Fig. 4.

Fox gene expression in the fat body of *Aedes aegypti* without (PV) and 6, 16, 22, 24, 36, 48, and 65 h PBM. Relative Fox mRNA levels were determined by qPCR. Three groups of twenty fat bodies were used per time point, samples from three biological replicates were analyzed, and their mean separated by Tukey-Kramer HSD ($p \leq 0.05$). Means with the same letter are not significantly different.

Fig. 5.

RNAi-mediated knockdown of specific Fox factors affects *Vg* gene expression in mosquito *in vitro* fat body culture. A) RNAi-mediated knockdown of Fox transcripts is highly effective. Mosquitoes were injected with control dsRNA (MAL) or dsRNA for specific Fox factors. Three days post injection fat bodies were isolated and subjected to fat body culture (see below). RNA from fat bodies incubated in AA-containing medium was isolated and used as template for RT-PCR, using gene specific primers for each Fox factor. Samples marked with '-' are control RNAs isolated from MAL-injected mosquitoes. Actin primers were added to the PCR reaction to confirm the integrity of the cDNA. B) RNAi-mediated knockdown of specific Fox factors decreases *Vg* gene expression. As above, mosquitoes were treated with dsRNAs and

allowed to recover for three days. Fat bodies were then isolated and incubated in fat body culture medium with and without balanced mixture of AAs. *Vg* mRNA levels were determined by qPCR as described above. Data represent means $\pm$ S.E. of triplicate samples. C) Actin gene expression is not altered by RNAi-mediated knockdown of specific Fox factors. Actin mRNA levels in the samples used in B) were determined by qPCR. Data represent means $\pm$ S.E. of triplicate samples.

Fig. 6.

RNAi-mediated knockdown of specific Fox factors decreases egg deposition. FoxL-, FoxN1-, FoxN2-, and FoxO-knockdown mosquitoes lay significantly fewer eggs than control mosquitoes (MAL). 20 females were analyzed per group. Similar results were obtained in three independent experiments. We used the nonparametric Mann-Whitney (Wilcoxon) test to determine significant differences in mean egg numbers for knockdown mosquito groups compared with the MAL-injected control group. Groups with statistical significant difference in mean egg numbers are marked with a ‘*’.

Table 1The Fox genes of *Aedes aegypti*

Name (<i>Ae. aegypti</i>)	Peptide ID (Vectorbase) (Ensembl)	<i>Anopheles gambiae</i> homologue	<i>Drosophila melanogaster</i> homologue	<i>H. sapiens</i> homologue
AaegFOXA1	AAEL003173 24913.m05300	ENSANGP00000022150	CG10002-PA	FOXA1 / FoxA3
AaegFOXA2	AAEL003163 24913.m05296	ENSANGP00000022150	CG10002-PA	FOXA1 / FoxA3
AaegFOXB1	AAEL002951 24906.m07447	ENSANGP00000020897	CG11922-PA	FOXB1 / AF055080
AaegFOXB2	AAEL002954 24906.m07390	ENSANGP00000024098	CG11921-PA	FOXB1 / AF055080
AaegFOXC	AAEL013354 25660.m00976	ENSANGP00000013521	<i>Crocodile</i> / CG5069-PA	FOXC1 / NP_001444
AaegFOXD	AAEL014284 25890.m00685	ENSANGP00000010631	AAL89941	FOXD4 / AAI03887
AaegFOXF	AAEL009031 25196.m01728	ENSANGP00000013579	<i>binou</i>	FOXF1 / S51624
AaegFOXG	AAEL001912 24878.m05327	ENSANGP00000025119	<i>sloppy paired 2</i>	FOXG1b / NP_005240
AaegFOXJx	AAEL009489 25232.m01727	ENSANGP00000018609	CG12632-PB	FOXJ3 / EAX07167
AaegFOXJy	AAEL000041 24834.m11750	ENSANGP00000018609	CG12632-PB	FOXJ1 / NP_001445
AaegFOXK1	AAEL008958 25193.m02647	ENSANGP00000019039	CG11799-PA	FOXK2 / NP_852095
AaegFOXK2	AAEL010565 25320.m01678	ENSANGP00000019039	CG11799-PA	FOXK2 / NP_852095
AaegFOXL	AAEL005741 25005.m03834	ENSANGP00000013200	CG5069-PA	FOXK2 / AF301906
AaegFOXN1	AAEL008832 25184.m02360	ENSANGP00000029729	CG12690	FOXN2 / P32314
AaegFOXN2	AAEL009903 25270.m02096	ENSANGP00000022569	<i>domina</i>	FOXN1 / NP_003584
AaegFOXO	AAEL012847 25582.m00975	ENSANGP00000008606	FOXO/ AFX/ AAL28078	FOXO3A / CAI16405
AaegFOXP	AAEL000250 24837.m08743	ENSANGP00000026333	CG11799-PA	FOXP4 / CAH72540
AaegFOXQ	AAEL000236 24837.m08879	ENSANGP00000026956	AAY5196	-