Transcriptional Regulation of Midgut Protease Genes in *Aedes aegypti* Mosquitoes

By

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Abstract

Blood meal digestion in female mosquitoes is initiated by increased levels of protease enzymes in midgut epithelial cells that are then secreted into the midgut lumen. In *Ae. aegypti*, the level of early phase (1-6 hrs) and late phase (8-30 hrs) proteases increases dramatically after feeding as a result of translational and transcriptional control mechanisms, respectively. We have focused on coordinate expression of the late trypsin (LT), 5G1-14, serine collagenase-1 (SC-1), and Culex late trypsin homolog (CxLT) protease genes in the midgut of blood- and protein-fed mosquitoes because of their extremely high levels of expression. In order to identify transcription factors that might be required for coordinate expression of these four midgut protease genes in response to feeding, we selected candidate RNAi knock-down targets in the Target of Rapamycin (TOR) kinase and phosphoinositol-3 kinase (PI-3K) signaling pathways. Injections of dsRNA into *Ae. aegypti* females were used to decrease the expression of TOR, the TOR-associated transcription factor YY1, and the forkhead box transcription factor linked to PI-3K signaling called FOXK1. Preliminary QRT-PCR data monitoring protease transcript abundance after knockdown of FOXK1 suggests that proteases transcription may be regulated by repression rather than activation. It is possible that one of these candidate transcription factors regulates all four protease genes and allows us to impede the mosquito's ability to reproduce, thus presenting a new direction for insecticide development.

Introduction

Female *Aedes aegypti* mosquitoes require a blood meal in order to obtain nutrients necessary for ovarian follicle development. The number of eggs produced depends on the size of the blood meal, and females that consume a small meal often enter oogenic arrest and degeneration, unable to obtain enough nutrients for complete egg maturation (Lea et al., 1978). It is a common occurrence in the wild that females take several small meals from multiple hosts rather than feed to repletion on a single host in order to complete oogenesis and produce viable offspring. This feeding pattern results in the transmission of diseases between human hosts.

Ae. aegypti mosquitoes serve as vectors for virally-transmitted diseases such as Yellow Fever and Dengue, which affects millions of people annually. Countries around the world rely on fumigation methods to reduce mosquito populations in heavily infected areas. By decreasing the *Ae. aegypti* populations over several seasons with continued pesticides, the probability of viral transmission between diseased human and vector can be brought below a statistical threshold to locally eradicate the disease. However, insecticide resistant strains are increasingly documented, and broad-spectrum insecticides affect numerous arthropod populations besides mosquitoes (Georghiou, 1972). There is increasing need for novel mosquito-selective insecticides, and one strategy seeks to disrupt blood meal digestion, halting the gonotrophic cycle.

Upon consumption of a blood meal, amino acids stimulate the protein expression of early phase proteases such as Early Trypsin (ET) via the nutrient sensor Target of Rapamycin (TOR) in the mosquito midgut (Brandon et al., 2008). As early phase protease expression begins to decline around 6 hours post-blood meal (PBM), late phase

protease expression begins to increase at 8 hours PBM as a result of transcriptional regulation (Isoe et al., unpublished data). Analysis of late phase protease transcript abundance revealed that the four proteases Late trypsin (LT), 5G1-14, serine collagenase-1 (SC-1), and Culex late trypsin homolog (CxLT) simultaneously increase by greater than 100-fold immediately following a protein meal (Isoe et al., unpublished data). This suggests a model of coordinate regulation of all four proteases.

The relative contribution to total blood meal digestion by individual proteases is unknown. However, it is possible that simultaneous transcriptional inhibition of multiple proteases could hinder substantial blood meal digestion. This could be fatal to the mosquito or at least reduce the nutrient load delivered to the ovaries, decreasing egg number and viability. If a single transcription factor can be identified that regulates all four coordinately expressed late phase proteases, then it may lead to development of a digestion-based approach to mosquito population control.

Two transcription factors, Yin Yang 1 (YY1) and Forkhead box K1 (FOXK1), were selected as candidate regulators of protease gene transcription. mTOR is known to integrate both metabolic and nutrient sensing pathways (Tokunaga et al., 2004), and we suspect that TOR regulates the expression of proteases in response to blood meal. YY1 and FOXK1 were therefore chosen on the basis of their roles in the TOR and phosphoinositol-3 kinase (PI-3K) metabolic pathways. YY1, a transcription factor previously studied in mice, has been shown to transcriptionally regulate mitochondrial oxidative function for energy homeostasis by forming a physical interaction with Peroxisome-proliferator-activated receptor coactivator (PGC)-1α (Cunningham et al., 2007). In turn, mTOR has been shown to regulate YY1 by directly altering the physical

interaction and its ability to interact with PGC-1α (Dunlop et al., 2009). The clear relationship between YY1, mTOR, and energy metabolism provided reasonable ground for selecting YY1 as a candidate transcription factor for protease regulation.

The other chosen transcription factor, FOXK1 is a member of the "winged helix" transcription factor family that contains a highly conserved 110-amino acid DNA-binding domain. Recently, 18 forkhead box genes were identified in the *Ae. aegypti* genome, and FOXK1 is one of five that is expressed in midgut tissues (Hansen et al., 2007). The role of several forkhead box transcription factors in regulating vitellogenesis in mosquito fat body tissues, as well as the evidence connecting them to PI-3K metabolic signaling (such as FOXO), suggests that FOXK1 could potentially regulate midgut protease gene expression.

Based on the published evidence linking FOXK1 and YY1 to the mTOR signaling pathway and energy metabolism, we set out to investigate their role in *Ae. aegypti* midgut protease gene expression. We used an RNAi strategy coupled with quantitative real time PCR to observe their effects on protease transcription abundance following a blood meal. Our preliminary findings provide a starting point for future experiments designed to optimize this approach.

Materials and Methods

Mosquitoes and Experimental Design

The Rockefeller strain of *Ae. aegypti* mosquitoes was reared under standard conditions (see Scaraffia et al. 2003) and maintained on 10% sucrose. Mosquitoes were injected in the thorax with 2 μg of dsRNA two days post-eclosion. They were fed three days after injection with bovine blood and then dissected 24 hours after feeding. Directly after feeding, mosquitoes were observed under a microscope, and only mosquitoes with fully distended, blood-filled midguts were considered fed and used for the experiment. Only those mosquitoes with at least 70% gene knockdown were considered reliable data points, and all other mosquitoes were excluded from the data.

Making dsRNA

An approximately 500-base pair region of the Open Reading Frame (ORF) of the knockdown gene of interest was selected. Primers for the 500-bp ORF region were designed with 5'-T7 flanking promoters, and no more than 30 PCR cycles were conducted to amplify this region (to minimize gene duplication error). PCR product and 1-KB ladder were then loaded into individual lanes on a pre-cast 1% agarose gel with ethidium bromide, and PCR products were separated under a 100 Volt electric current in 1X TAE buffer. Upon completion gel electrophoresis, the gel was visualized under UV light to confirm the size of the PCR product. The product of proper size was excised from the gel with a razor and then purified using the GFX PCR DNA and Gel Band Purification Kit (GE Healthcare) according to the manufacturer's instructions. The resulting purified DNA fragment was cloned into a TA vector using the pGEM®-T Easy Vector System (Promega) and by incubating the ligation reaction at 15°C overnight.

The resulting plasmid product was transformed into DH5α competent cells by mixing the ligation product with 0.5 ml of competent cells in a 15 ml Falcon tube and then incubating for 30 minutes on ice. The cells were heat shocked at 42°C for 30 seconds and then 1 ml of LB broth was added. The competent cells were subsequently placed in a 37°C shaker for 1 hour and then centrifuged at 4000 rpm for 2 minutes, leaving a pellet of cells on the bottom of the falcon tube. The supernatant was discarded, the transformed cells were plated on an LB-ampicillin culture plate, and incubation was conducted at 37°C overnight. The next day, bacterial colonies were picked and colony PCR was performed to screen the colonies for the plasmid.

Colony PCR was performed by touching a single bacterial colony with a pipet tip and then adding it to a PCR reaction mixture; the PCR mixture includes primers for the inserted gene, but does not include any other DNA. The same colony was touched a second time and then added into a small tube containing LB and 0.1 μg/μl ampicillin. After 23 PCR cycles, colonies shown to contain the inserted gene were then grown at 37°C overnight for plasmid miniprep, which was performed with the QIAprep Spin Miniprep Kit (Qiagen) according to the manufacturer's instructions.

The purified plasmid was submitted to the Genomic Analysis and Technology Core (GATC) sequencing facility (with SP6 sequence primer). Upon return of the sequence by GATC, the plasmid sequence was verified by comparison to the genomic sequence. After identifying a plasmid with the correctly inserted DNA sequence, the plasmid was amplified by 30 PCR cycles and run on a 1% agarose gel. The amplified DNA (template) was purified with the GFX PCR DNA and Gel Band Purification Kit, purified once more with an ethanol precipitation, and then diluted to 250 ng/μl in

nuclease free water. *In vitro* dsRNA synthesis was performed with 8 µl of the concentrated template DNA per synthesis reaction included within the MEGAscript® RNAi Kit (Ambion). Manufacturer instructions were followed with the exception that we extended the *in vitro* transcription to proceed overnight. After completing the kit's final purification steps, the dsRNA was concentrated by ethanol precipitation to a final concentration of 4 µg/µl, quality was checked by running gel electrophoresis, and it was stored at -80°C until injection.

Mosquito Microinjection Procedure

A clean, glass capillary needle was filled with mineral oil and carefully attached to the metal plunger of a Nanoject II apparatus. After complete assembly of the unit, the glass capillary was filled with dsRNA (at a concentration of 4 µg/µl) by holding down the "Fill" button on the remote. This process was carefully monitored through the microscope to avoid the uptake of air into the needle. Once filled, the needle was gently inserted into the thorax of a cold-anesthetized mosquito and the "Inject" button was pushed a total of 7 times (for the delivery of approximately 1.0 µg of dsRNA). The mosquito was then carefully removed from the glass needle using a pair of forceps and transferred into a labeled cup with a mesh lid. Between injections of different samples of dsRNA, the needle was rinsed at least three times with ddH_2O to prevent contamination. After all injections were complete, the cups holding the injected mosquitoes were placed upside down (mesh-side down) over a 10% sucrose pad.

Mosquito Dissection and Total RNA Isolation

Centrifuge tubes were labeled and filled with 250 µl of TRIzol® Reagent (Invitrogen). Cold-anesthetized mosquitoes were rapidly dissected in 1X PBS under a light

microscope. The midguts were carefully removed and placed into the centrifuge tubes to yield a total of 4 midguts per tube. The midguts were briefly homogenized by hand with a pestle and then frozen at -20°C for storage.

To isolate total RNA, the centrifuge tubes with TRIzol® were removed from the freezer and 50 µl of chloroform was added. The tubes were vortexed and then left to incubate at room temperature for approximately 2 min. The tubes were then centrifuged at 4°C and 14000 rpm for 15 min. About 85 µl of supernatant was then transferred to new tubes, and 125 µl of isopropyl alcohol was added. After mixing, they were left to incubate at room temperature for 10 min. They were then centrifuged once more at 4°C and 14000 rpm for 15 minutes, and then the supernatant was carefully discarded. A volume of 250 µl of 75% ethanol was added and mixed by inverting the tubes. The tubes were centrifuged at 4°C and 14000 rpm for 5 minutes and then the supernatant was discarded. The tubes were centrifuged once more at 4°C and 14000 rpm for 5 minutes and then totally air-dried. The pellet was then resuspended in 25 µl of nuclease-free water and the concentration was measured using a NanoDrop spectrophotometer (NanoDrop Technologies).

cDNA Synthesis and QRT-PCR

Total RNA (1.0 µg) was combined with 1.0 µl of DNaseI buffer, 0.5 µl of DNaseI, and enough DEPC water to bring the reaction volume to 10.0 µl. The reaction was incubated at room temperature for 15 minutes before 1.0 µl of EDTA was added. The reaction was incubated at 70°C for 15 minutes, and then 1.0 µl of Oligo-dT-VN was added. The reactions were incubated again at 70°C for 10 minutes and then immediately chilled on ice for 3 minutes. A cocktail consisting of 4.0 µl DEPC water, 2.0 µl buffer, 1.0 µl dNTP,

0.5 µl RNase Inhibitor, and 0.5 µl Reverse transcriptase was added to each reaction. The reactions were then incubated at 37°C for 30 minutes, 42°C for 15 minutes, and then briefly spun down. Another incubation at 42°C for 15 minutes and then 70°C for 20 minutes was performed to obtain the final cDNA product.

To use the cDNA for real time PCR, standard 1:90 or 1:3 dilutions were made. A master mix containing 5.0 µl FastStart Universal SYBR Green Master (Roche) and 3.0 µl of 1 µM primer mix per reaction well was made. This 8.0 µl mixture was pipetted into each of the reaction wells, and then 2.0 µl of preferred dilution of cDNA was pipetted in, bringing the total volume up to 10.0 µl. The QRT-PCR plate was sealed shut with a thermal adhesive sealing film and the progress of the PCR reaction was monitored over the course of 40 cycles. The data was analyzed using the GraphPad Prism program (GraphPad Software) to convert CT values of the exponential function into "real numbers" so that transcript abundance of protease genes in different samples could be more directly compared.

Single-Mosquito Knockdown Protocol

A protocol for the study of gene knockdown in individual mosquitoes is currently in refinement by Dr. Jun Isoe in order to reduce pool-related background noise. The protocol for single-mosquito experiments is essentially a scaled down version of the protocol for pooled mosquitoes described throughout this text. Due to inability to reproduce the knockdown of YY1 or FOXK1 over the course of the spring semester (despite numerous attempts), these new techniques were repeatedly attempted, but they were not effectively applied in this particular experiment.

Results

Since SC-1, 5G1-14, CxLT, and LT are coordinately expressed following a blood meal, it is possible that a single transcription factor regulates the expression of all four late phase proteases. If such a transcriptional activator is identified, it could lead to development of novel mosquito control strategies. Because this project seeks to understand the regulation of blood meal digestions, conventional methods of transcription factor study are not feasible. For instance, it is not possible to study the transcription factor in cell cultures because cultures cannot be blood fed. Alternately, creating transgenic mosquitoes is costly and time consuming, and blood meal metabolism is not yet understood well enough to rationalize the expense.

A novel approach using whole body transfection (WBT) was initially attempted to identify the transcription factor. WBT is the injection and transfection of engineered DNA plasmids into live mosquitoes tissues (Isoe et al. 2006). Using WBT, we sought to identify the transcription factor through a promoter mapping strategy followed by an affinity assay to pull-down and isolate the transcription factor. Different upstream pieces of the 5G1-14 promoter were cloned into luciferase reporter plasmids, live mosquitoes were injected with the different plasmids, and after blood feeding, luciferase assays were performed on midgut tissues to identify the most strongly activated promoter regions. Promoter regions corresponding to the highest luciferase activity reflect areas where the transcription factor binds. However, WBT eventually proved to be highly variable and we could not obtain consistent results.

We next decided to pursue an educated guess approach and turned to literature to search for already-known transcription factors related to energy metabolism, nutrient

signaling, and involvement with TOR or PI-3K signaling pathways. Using this strategy, we identified eight transcription factors: Transcription Initiation Factor 1a (Tif-1a), Yin Yang 1 (YY1), Peroxisome-proliferator-activated receptor coactivator (PGC)-1α, and the forkhead box genes FOXA1/A2, FOXK1, FOXK2, FOXN1, and FOXO (Table 1). One by one, these candidate transcription factors were knocked down and protease expression was monitored with real time PCR in pools of mosquitoes. Among the first trials of the eight transcription factors was the knockdown of FOXO.

FOXO is a known transcription factor regulating the insulin-signaling pathway (Puig et al. 2007). It has been shown that FOXO is a "major coordinator of the transcriptional response to nutrients" and so it was selected as a candidate transcription factor (Gershman et al. 2007). Analysis of the pooled data revealed that knockdown could be achieved above the required 70% threshold to achieve reliable data. However, substantial knockdown was obtained from only a few of the total pools (Figure 2). Only those pools with approximately 70% or better knockdown were analyzed for protease transcript abundance (figure 3). Analysis of LT, 5G1-14 and CxLT protease transcript abundance in mosquitoes 24 hours PBM revealed that FOXO knockdown did not have an affect on LT or CxLT, but did decrease 5G1-14 gene expression by approximately 70%.

Although FOXO does not appear to be the master transcription factor for all four proteases, it laid the foundation for future knockdown experiments. It confirmed that knockdown could be achieved and monitored when analyzing pooled mosquito data. Although knockdown worked in this trial, we occasionally began to notice flaws in the experiment. For instance, occasional pools of mosquito midguts demonstrated over-expression rather than knockdown of the target gene after injection, and large fluctuations

of protease transcript abundance was difficult to evaluate. We attributed such variable results to the pooled data; if even just one mosquito per pool of 4 were not effectively knocked down, then both the overall knockdown and the potential downstream signaling molecules would be affected, creating noise. However, we decided to follow with the same protocol for future experiments and attempt to circumvent these issues by strictly adhering to the rule where we only used data with 70% knockdown or better. Additional transcription factors were knocked down and analyzed.

In the preliminary experiment conducted for knockdown of FOXK1 and YY1, strong trends in their affects on protease gene expression became apparent.The knockdown of YY1 in blood fed mosquitoes at 24 hours PBM corresponded with a 4-fold increase in SC-1 transcript abundance (Figure 4). YY1-knockdown appears to have no effect on unfed mosquitoes (Figure 5). This suggests that YY1 is a blood meal-induced repressor of SC-1 that, upon knockdown, caused SC-1 transcript expression to increase at 24 hours PBM (Figure 6). SC-1 is a late phase protease, but its transcript abundance begins to decline earlier than most late phase proteases (at about 6 hours post blood meal). If YY1 does in fact repress SC-1 in response to blood meal, then perhaps YY1 plays a role in the premature decline in SC-1 transcript abundance. YY1 knockdown does not appear to affect the expression on 5G1, CxLT, or LT from this preliminary data.

The knockdown of FOXK1 appears to have no effect on protease expression in blood fed mosquitoes (figure 4). However, in unfed mosquitoes, the transcript abundance of SC-1 increased 2-fold, 5G1 increased 8-fold, and CxLT increased 21-fold (Figure 5). This suggests that FOXK1 is a repressor that is inactivated by blood meal. By knocking down a repressor, protease transcript abundance increased dramatically. This preliminary

data suggests that FOXK1 may function to regulate protease gene expression through de-repression (Figure 6). Because of this interesting preliminary data, FOXK1 and YY1 were selected for further investigation.

At this point in time, a protocol for knockdown and analysis in *individual* mosquitoes was created in order to reduce pool-related noise in protease expression data. Changes to dsRNA protocol, the time of injection in the mosquito life cycle, and multiple aspects of the overall experimental procedure were used. With improvements in the dsRNA protocol, a new dsRNA target region was designed to knock down a slightly different area of the open reading frame (ORF) in both genes (Figure 1 b). Numerous atttempts to perform individual mosquito knockdown were conducted, however we were unable to observe knockdown. Thinking that our dsRNA perhaps targeted an area of the gene transcript with secondary structure, preventing knockdown, we redesigned the dsRNA primers of both genes to target the 5' end of the ORF (Figure 1 c). Attempts to knock down these two transcription factors with the individual mosquito protocol were continually unsuccessful. However, our preliminary findings provide a starting point for future experiments designed to optimize this approach.

This data suggests a very interesting method of protease gene regulation by transcription factors. Rather than coordinate activation, it is possible that they are coordinately de-repressed. Our preliminary findings provide a foundation for future investigation into this regulatory mechanism.

Table 1.

Transcription Factor	**Reference Species**	**Function**	**Activator or Repressor in literature?**
Tif-1a	NA	Mediates RNA polymerase binding and transcription	Activator
PGC-1α	NA	Regulates cellular energy metabolism in humans	Activator
YY1	Mouse	Regulates mitochondrial oxidative function for energy homeostasis	Activator and Repressor
FOXA1/A2	mosquito	Binds to the promoter and regulates the vitellogenin gene	Activator and Repressor
FOXK1	mosquito	Diverse roles in different organisms, notably development	Activator and Repressor
FOXK2	mosquito	Diverse roles in different organisms, notably development	Activator and Repressor
FOXN1	mosquito	Diverse role in different organisms; implicated in vitality and fertility	Activator and Repressor
FOXO	mosquito	Transcriptional feedback regulator of the insulin receptor	Activator and Repressor

Figure 1. The FOXK1 ORF, shown as the big white box, is 2078 base pairs long. The regions of the ORF shown in blue with primer arrows drawn above the ORF represent areas targeted by dsRNA, and the regions in orange with primer arrows shown below the ORF represent areas monitored for transcript abundance via real time PCR. A) Original dsRNA target region. Notice that the QRT-PCR primers are internal to the dsRNA target region. B) The second dsRNA target region, located in the middle of the ORF. The dsRNA primers amplify 874-1433 and QRT-PCR primers amplify 1321-1535 of the ORF. C) Third set of primers targeting the 5' region. The dsRNA primers amplify 1-545 and the QRT-PCR primers amplify 454-578 of the ORF.

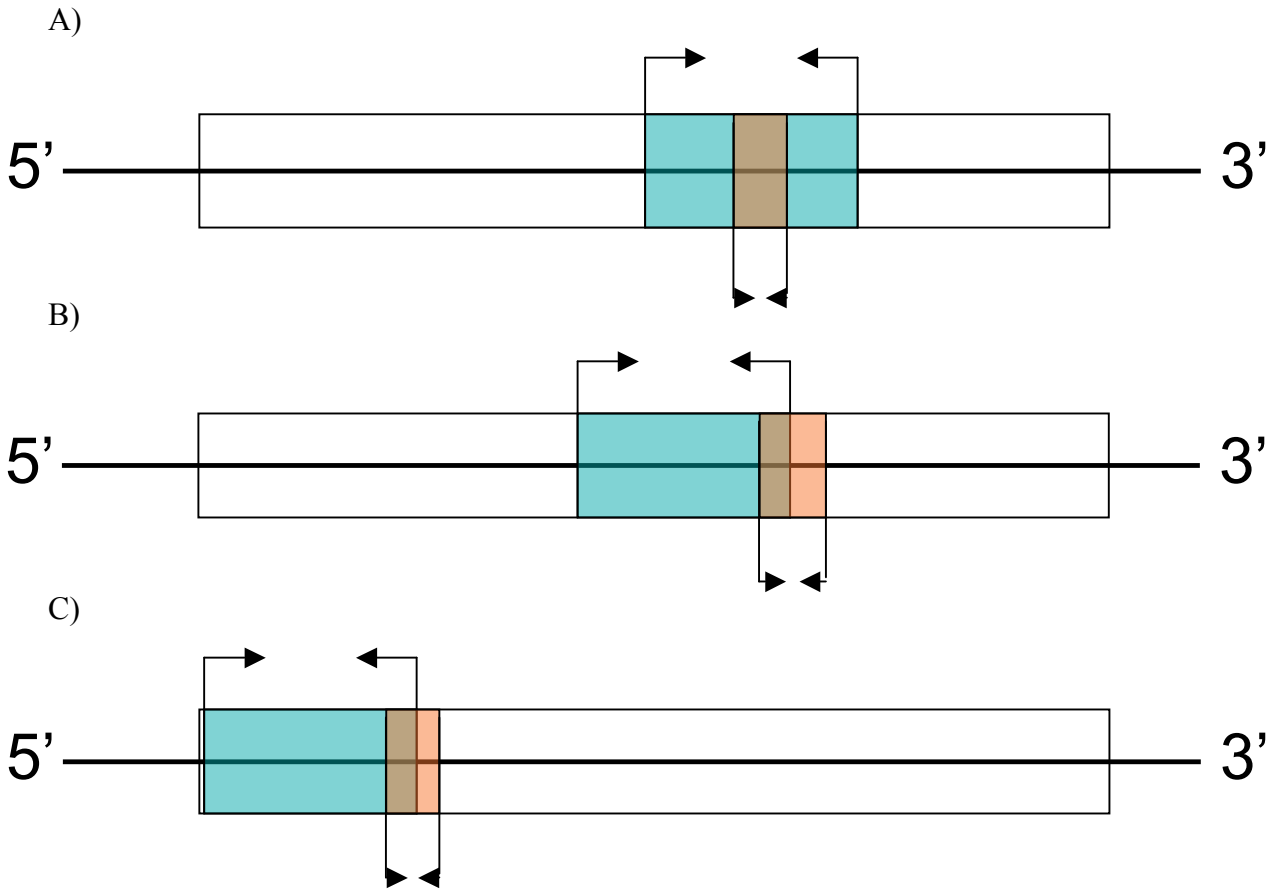


Figure 2. Knockdown observed in each pool of 4 mosquitoes injected with dsFOXO. Pools with knockdown of 70% were used for monitoring protease transcript abundance. Pools 1, 4, and 5 from this experiment were used to monitor protease transcript abundance.

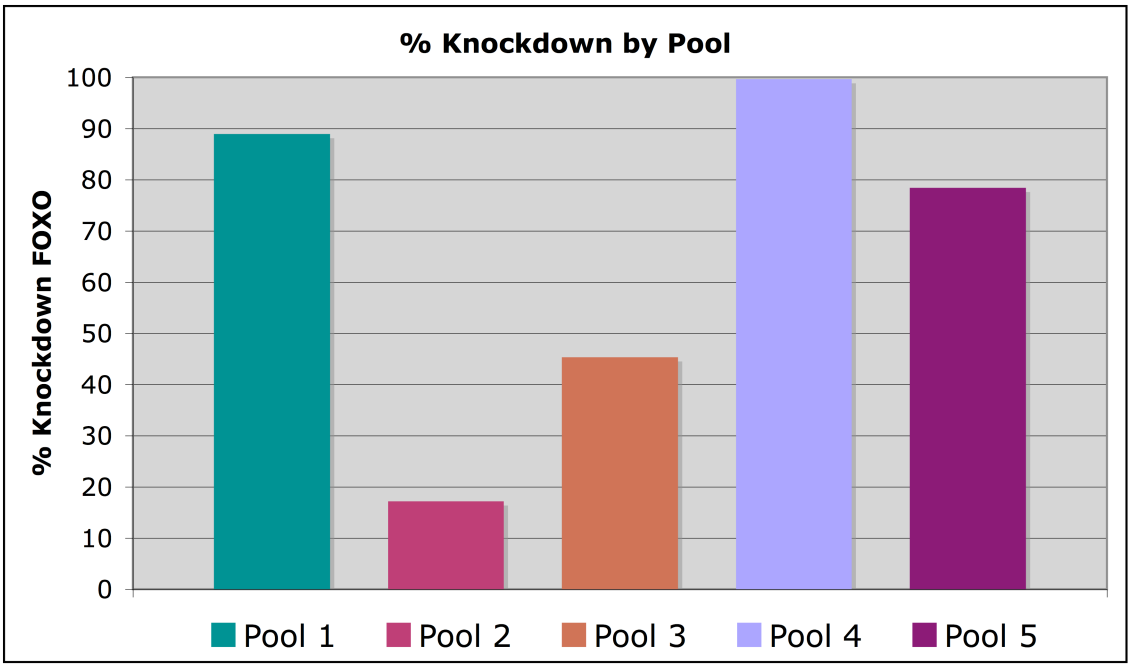


Figure 3. Pools with greater than 70% knockdown were used to monitor protease transcript abundance. Knockdown of FOXO appears not to have an affect on LT or CxLT transcript abundance. However, knockdown of FOXO appears to reduce 5G1-14 transcript abundance by approximately 70%.

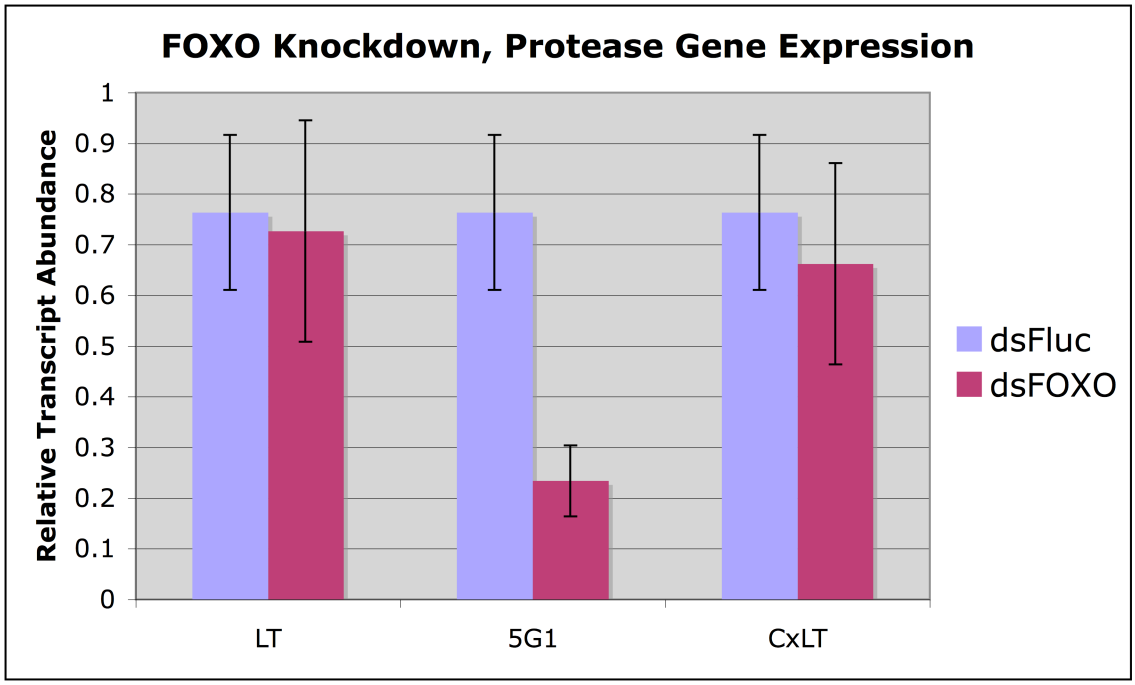


Figure 4. Mosquitoes injected with dsRNA were blood fed to repletion and their midgut tissues were dissected 24 hours later. The midguts were pooled into groups of 4 for analysis by real time PCR. Transcript abundance was then normalized to set the control equal to 1. Knockdown of FOXK1 did not affect protease transcript abundance in blood fed mosquitoes. Knockdown of YY1 caused an approximately 4-fold increase in SC-1 transcript abundance, but did not affect 5G1-15, CxLT and LT.

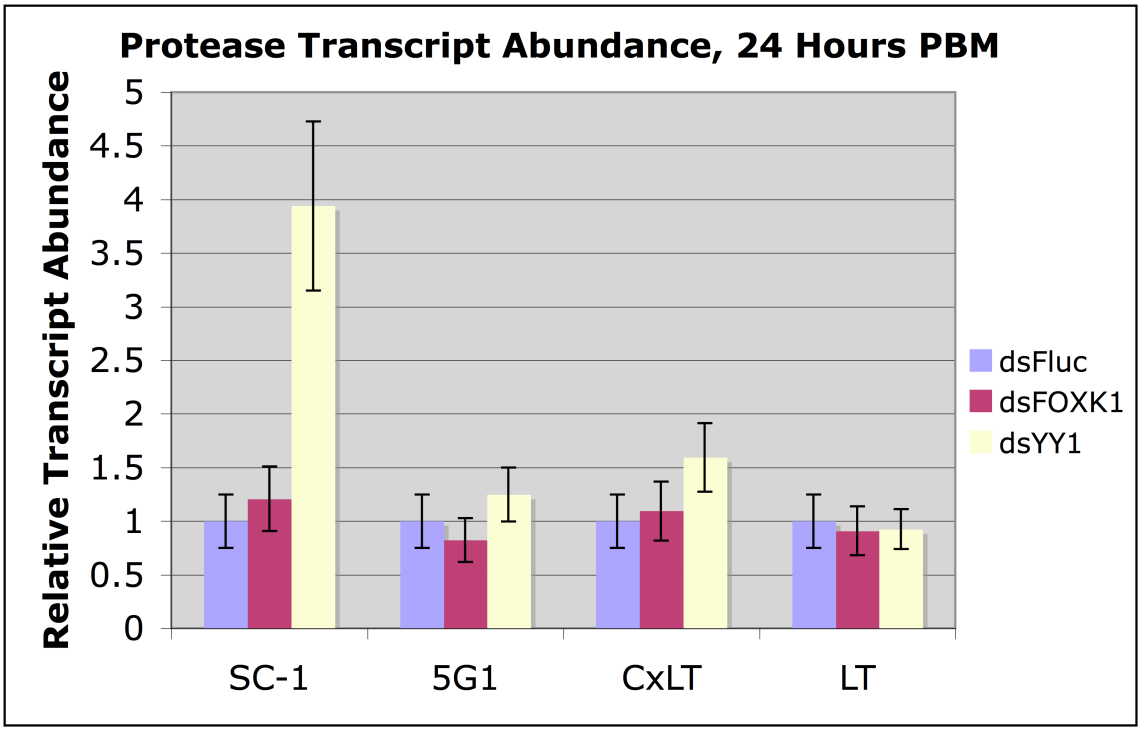


Figure 5. Mosquitoes were injected with dsRNA and were their midgut tissues were dissected 2-3 days after injection. Midguts were pooled into groups of 4 and relative protease transcript abundance was monitored and normalized to set the control equal to 1. Knockdown of FOXK1 shows approximately 2-fold increase in SC-1 transcript abundance, 8-fold increase in 5G1-14 transcript abundance, and 21-fold increase in CxLT transcript abundance compared to the control.

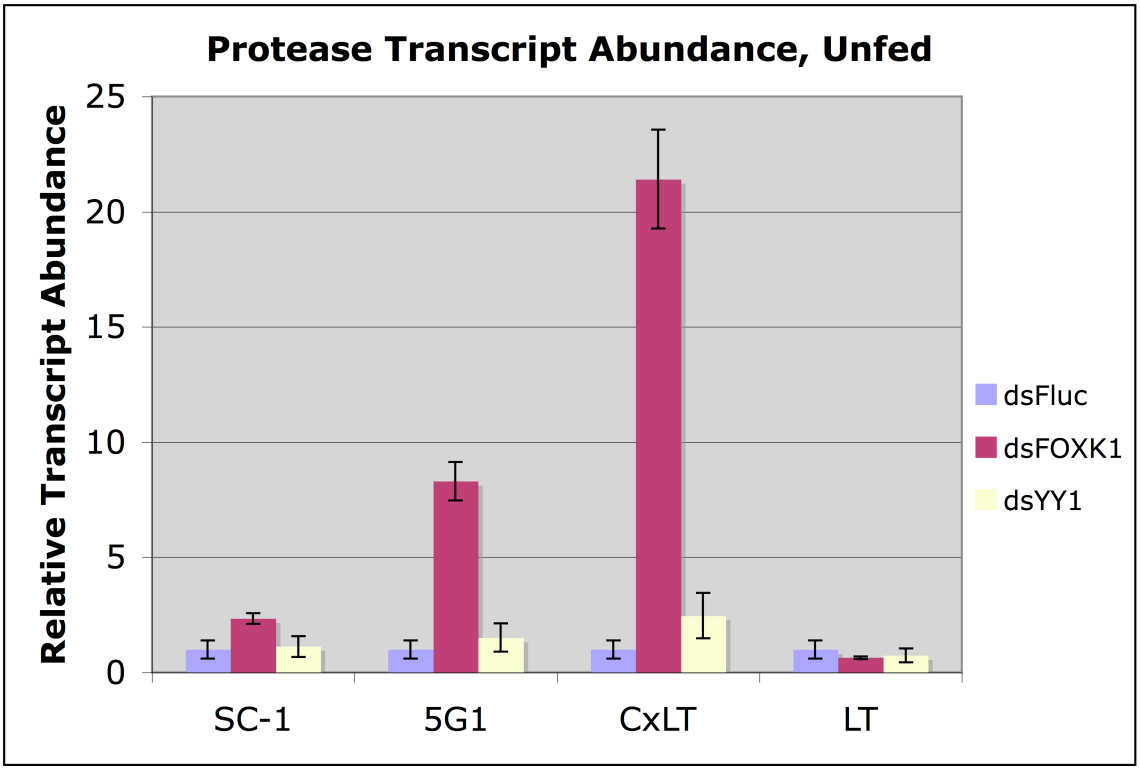
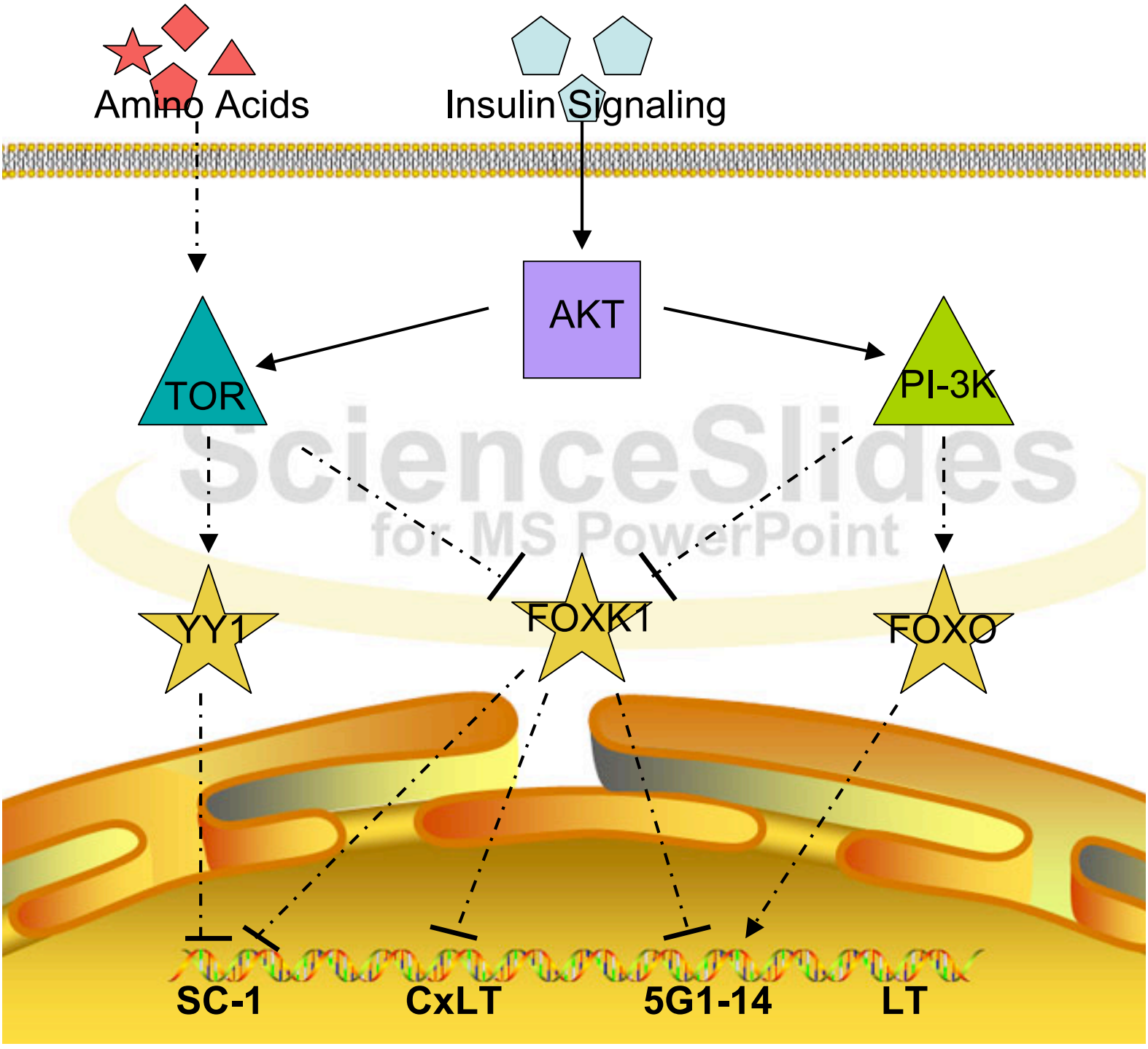


Figure 6. The hypothetical signaling pathway for protease gene regulation from preliminary data is shown below. Solid arrows indicate that the exact signaling pathway is known. Dashed arrows indicate that signaling pathway is incomplete. Arrows indicate activation and bars indicate repression.



Discussion

Initial observation of coordinate gene expression of the four late phase protease genes SC-1, 5G1-14, CxLT, and LT following a blood meal led us to predict that transcription factors could be coordinately activating them. Should a coordinate activator be identified, this could lead to further development of novel insecticides. However, preliminary evidence with the knockdown of FOXK1 and YY1 implies that late phase protease expression may be heavily regulated by inactivation of a transcriptional repressor, de-repression.

Transcriptional de-repression has been clearly identified to play a role in blood meal induced mosquito metabolism in fatbody tissues. Studies on the Broad gene (BR) have shown that the Z1 and Z4 isoforms act as repressors of the Vitellogenin gene (Vg) by directly binding to the gene promoter. Knockdown of these two isoforms caused enhanced Vg expression and extended expression over the course of blood meal digestion period (Zhu et al. 2007). Another example, the GATA transcription factor (AaGATAr) recognizes and binds to GATA motifs upstream of yolk protein precursor genes (YPPs) in order to actively repress them during pre-vitellogenic stages of mosquito development. Within half an hour of blood meal digestion, AaGATAr is repressed and relieved from binding in order for vitellogenesis to occur (Raikhel et al. 2002). Numerous studies have shown that repression systems are frequently employed by the mosquito to regulate time-dependent metabolic pathways.

Studies of the forkhead box genes in non-midgut mosquito tissues have concluded that FOX transcription factors play important roles in regulating mosquito metabolism, however those roles have not been clearly identified. Knockdown of FOXK1 and FOXK2

in blood fed mosquito fatbody tissues showed no affect on vitellogenesis, despite high gene expression in those tissues (Hansen et al. 2007). The authors concluded that the FOXK genes to play a role in transcriptional repression, requiring further study. Our observed up-regulation of protease gene expression in the midgut following FOXK1 knockdown is therefore consistent with its hypothesized role as a repressor in mosquito tissues. This suggests that a repression system such as the one demonstrated for the GATA transcription factor may be employed by the mosquito in order to regulate the time-dependent digestion of a blood meal.

In conclusion, preliminary data on the forkhead box transcription factor FOXK1 as well as YY1 transcription factor suggests that blood meal digestive enzymes may be regulated by a mechanism of de-repression. Such as repression system has been observed in other time-dependent metabolic processes. Further study on these as well as other candidate transcription factors is important to clarify the role of such a regulatory mechanism in mosquito digestion.

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