

FOXO regulates RNA interference in *Drosophila* and protects from RNA virus infection

Michael J. Spellberg and Michael T. Marr II¹

Department of Biology and Rosenstiel Basic Medical Sciences Research Center, Brandeis University, Waltham, MA 02454

Edited by Robert Tjian, Howard Hughes Medical Institute, Chevy Chase, MD, and approved October 14, 2015 (received for review August 27, 2015)

Small RNA pathways are important players in posttranscriptional regulation of gene expression. These pathways play important roles in all aspects of cellular physiology from development to fertility to innate immunity. However, almost nothing is known about the regulation of the central genes in these pathways. The forkhead box O (FOXO) family of transcription factors is a conserved family of DNA-binding proteins that responds to a diverse set of cellular signals. FOXOs are crucial regulators of cellular homeostasis that have a conserved role in modulating organismal aging and fitness. Here, we show that *Drosophila* FOXO (dFOXO) regulates the expression of core small RNA pathway genes. In addition, we find increased dFOXO activity results in an increase in RNA interference (RNAi) efficacy, establishing a direct link between cellular physiology and RNAi. Consistent with these findings, dFOXO activity is stimulated by viral infection and is required for effective innate immune response to RNA virus infection. Our study reveals an unanticipated connection among dFOXO, stress responses, and the efficacy of small RNA-mediated gene silencing and suggests that organisms can tune their gene silencing in response to environmental and metabolic conditions.

FOXO | RNAi | Argonaute | viral immunity | *Drosophila*

Organisms must be able to respond to changing conditions to survive. Radical changes in environment or metabolism can induce cellular stress signaling pathways meant to help the cell return to a normal range of function. Whereas there are dedicated transcriptional response pathways to deal with specific individual stresses such as heat shock (HSF) (1) or heavy metals (MTF-1) (2), the forkhead box O (FOXO) pathway has emerged as a general stress responsive transcription factor. Although initially characterized as downstream targets of insulin signaling, it is now clear that the FOXOs respond to multiple cellular stress signaling pathways including nutrient deprivation, oxidative stress, mitochondrial dysfunction, DNA damage, bacterial infection, and other cellular stress signals (3, 4).

The FOXO family of transcription factors is conserved from *Caenorhabditis elegans* to humans. Invertebrates have a single FOXO gene: *daf-16* in worms and dFOXO in *Drosophila* (5, 6). In mammals, the four FOXO genes (FOXO1, FOXO3, FOXO4, and FOXO6) have diversified, with some factors having a stress-specific response (7–10). Therefore, invertebrates offer a particularly attractive system for understanding FOXO function because the single isoform is responsible for all of the roles of the four mammalian FOXOs.

Small noncoding RNA pathways are capable of regulating gene expression in a sequence-specific manner (11). Effector complexes called RNA-induced silencing complexes (RISC) containing small single-stranded RNAs bound to Argonaute proteins find target mRNAs and posttranscriptionally prevent their expression. Three major small RNA pathways have been identified in animal cells, the microRNA (miRNA) pathway, the small interfering RNA pathway (siRNA), and PIWI RNA (piRNA) pathways, each contain unique components and characteristic RNA lengths that differentiate them. The pathways differ in the origin of the small RNAs and in the processing enzymes used to produce the small RNAs. Two of these pathways, miRNA and siRNA, dominate in somatic cells. In *Drosophila*, there is a clear distinction between

these pathways based on the identity of the RNaseIII-related Dicer protein used to produce the small RNA. Dicer-1 is involved in miRNA biogenesis, whereas Dicer-2 is involved in siRNA biogenesis.

It is clear that posttranscriptional regulation by small RNA pathways is important for developmental and homeostatic gene regulation (12). Both somatic small RNA pathways, miRNA and siRNA, have been implicated as important players in metabolism and various cellular stress responses (13). In addition, the siRNA pathway is an important antiviral response in invertebrates (14–17). However, the regulation of genes in these pathways is largely unexplored.

Here, we show that *Drosophila* FOXO regulates the expression of core components of the small RNA pathways in response to stress. This regulation increases the effectiveness of the small RNA silencing and provides a direct link among cellular metabolism, stress, and RNA silencing.

Results

To better understand dFOXO's contribution to stress resistance, we first sought to identify dFOXO-binding sites genome-wide by using chromatin immunoprecipitation (ChIP). We used a stably transfected *Drosophila* cell line expressing a constitutively active form of dFOXO (dFOXO^{CA}) under the control of an inducible promoter as described (6). This form of dFOXO, when expressed, is found exclusively in the nucleus and seems to be blind to negative regulation by insulin signaling (6). This strategy allows us to interrogate the dFOXO-specific effects while maintaining the cells in nutrient-rich, low-stress conditions. This approach also has the potential to capture dFOXO targets downstream of many different signaling pathways because we do not require engagement of any single stress-signaling pathway. This system

Significance

A major conclusion of this work is that the effectiveness RNA interference, previously thought to be a constant, is coupled to cellular and organismal physiology through the forkhead box O (FOXO) pathway. The FOXO transcription factors respond to metabolic changes in the cell and are a central node for multiple stress responses, viral immunity, and lifespan, all topics of broad interest. In addition, these factors have a profound influence on aging and lifespan. Much effort is being spent to identify downstream targets of FOXO that allow the extension of lifespan and slowing of aging. Directly connecting FOXO with small RNA pathways and innate viral immunity provides a previously unidentified bridge between metabolism and fitness.

Author contributions: M.J.S. and M.T.M. designed research, performed research, contributed new reagents/analytic tools, analyzed data, and wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE73827).

¹To whom correspondence should be addressed. Email: mmarr@brandeis.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1517124112/-DCSupplemental.

has been successfully used in an mRNA-based study to identify legitimate, verified dFOXO targets (6, 18).

DNA bound to dFOXO was cross-linked and the chromatin associated was immunoprecipitated with antibodies to dFOXO (6, 18). Precipitated DNA was analyzed on Affymetrix tiling arrays and compared with input DNA to control for possible copy number variation in the cells (19). Using a cutoff of fourfold enrichment over input, we identified ~2,200 genes that were bound by dFOXO (Dataset S1). The number of targets agrees well with previously reported dFOXO ChIP studies (20–23). Comparisons with the previous ChIP studies of dFOXO binding indicate that the genes bound in this model most closely resemble those found in starved larva or the adult fat body (Dataset S2) (20–23). In total, 1,555 genes were identified in the current study that were bound by dFOXO in at least one of the

previous studies. Sequence analysis of the genomic DNA bound by dFOXO in the current work indicates that the FOXO response element (TTGTTTAC) (24), or a derivative of it, are the two most overrepresented sequence motifs in the precipitated DNA (Fig. 1A). Using the same approach, similar sequences were identified in three of the four previous studies (20–23) (Fig. S1).

Gene ontology searches for targets identified in the current study show enriched groups of genes expected for dFOXO targets such as GO:0032535: regulation of cellular component size; GO:0033554: cellular response to stress; and GO:0008340: determination of adult lifespan (Table S1). Interestingly, in addition to expected groups, genes involved in the small RNA pathways were overrepresented (GO:0016246 RNA interference; $P = 0.02$) (Table S2). Tiling array data shows dFOXO bound to the promoters of RNA-induced

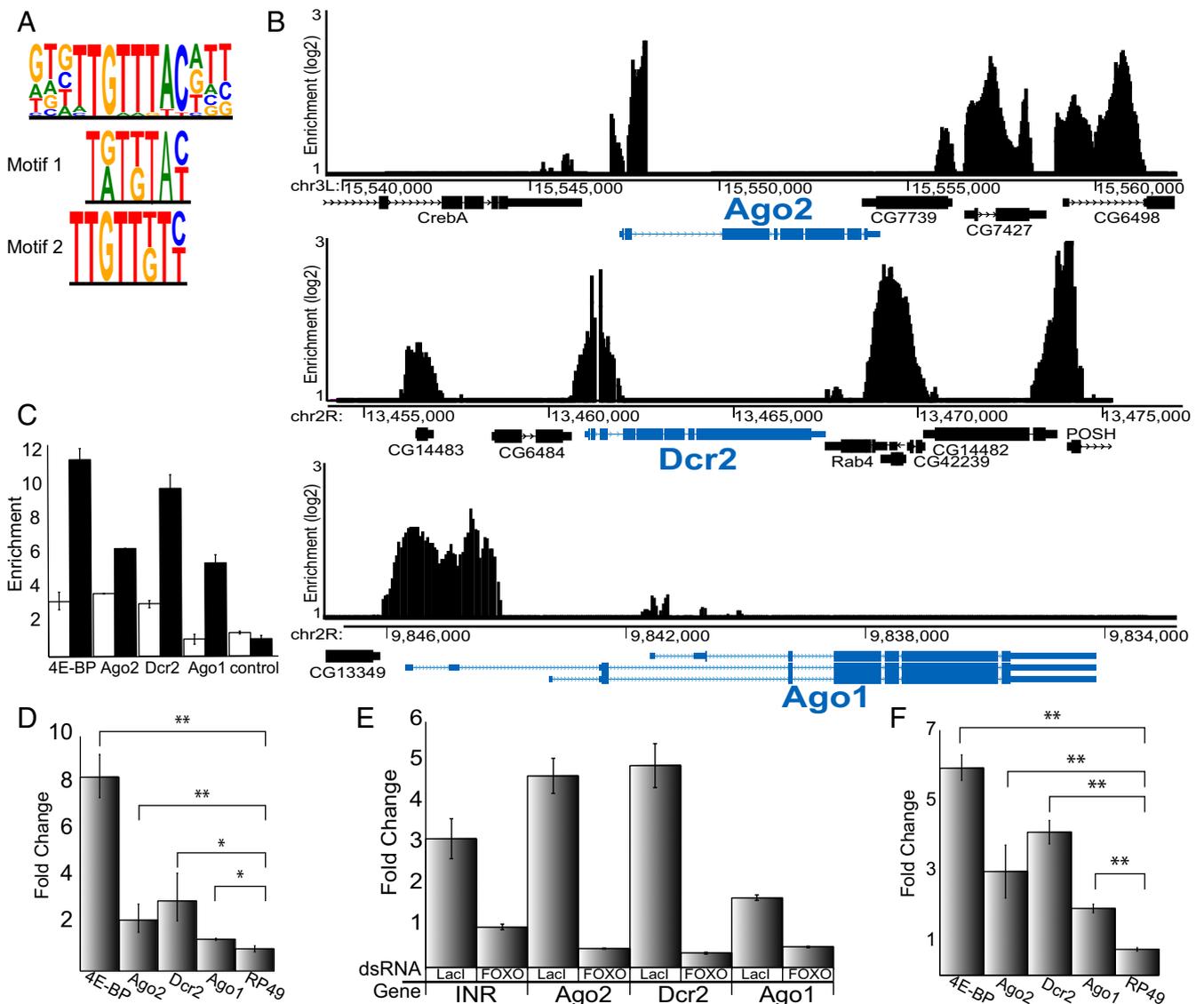


Fig. 1. dFOXO binds and stimulates expression of RNAi pathway genes. (A) Sequence motifs identified. (B) Genomic views show enrichment of precipitated DNA for three core RNAi machinery genes, Ago1, Ago2, and Dcr2. Each of these genes is oriented so the promoter of the gene is on the left. Feathered lines indicate introns; boxes indicate exons. Genomic coordinates are shown on the x axis; scale is unique for each browser view. Signal enrichment is indicated on the y axis. (C) CHIP followed by qPCR to verify dFOXO genomic binding sites. Enrichment compared with 28s rRNA gene is shown. A region 1 kb downstream of the 4E-BP gene serves as a negative control. (D) Change in mRNA of RNAi core genes upon dFOXO^{CA} expression. 4E-BP serves as positive control. RP49 is a negative control. (E) Change in mRNA of RNAi core genes upon serum starvation. LacI or dFOXO below each column indicates the dsRNA used before serum starvation. Target gene is indicated below each column pair; INR serves as positive control. (F) dFOXO transcriptional control in adult flies. Hsp70-Gal4; UAS-dFOXO male flies were heat shocked to induce dFOXO. Expression changes of core RNAi factors were assayed by RT-qPCR. All error bars are SEM, * $P < 0.05$, ** $P < 0.01$.

silencing complex (RISC) core components argonaute 2 (Ago2) and argonaute 1 (Ago1) in addition to other small RNA processing genes like Dicer-2 (Dcr2) (Fig. 1B and Fig. S2). The array data were validated with direct quantitative PCR (qPCR) analysis of ChIP by using independent biological replicates. dFOXO binds the RNAi core genes at levels comparable to a well-characterized target, 4E-BP (6) (Fig. 1C). Interestingly, dFOXO is found bound to core components of all three major small RNA pathways in *Drosophila* (11): the miRNA, siRNA, and piRNA pathways (Fig. S2).

FOXO transcription factors are capable of both activating and repressing transcription (25). To determine the effect of dFOXO on the core RNAi machinery genes, RNA was extracted from cells expressing dFOXO^{CA}, and RT-qPCR was performed to determine the effect on transcription of the RNAi genes. mRNA levels of Ago2, Dcr2, and Ago1 increased in response to dFOXO^{CA} (Fig. 1D). To determine whether endogenous levels of wild-type dFOXO are sufficient to activate the expression of the core small RNA machinery genes, we used the hemocyte-derived cell line mbn2. Hemocytes are one of the major cell types involved in viral innate immunity (26). Serum starvation of these cells, expressing only endogenous levels of dFOXO, increased the expression of the core RNAi machinery genes in a dFOXO-dependent manner (Fig. 1E). To determine whether these genes represent real targets in the fly, wild-type dFOXO was expressed in adult flies by using the Hsp70-Gal4 driver. Expression of the RNAi core factors was significantly increased in flies expressing WT dFOXO (Fig. 1F). The combined ChIP and gene expression data show that activated dFOXO directly targets small RNA pathway genes and promotes their transcription.

We reasoned that if dFOXO is increasing the expression of RNAi genes, this increase could lead to increases in RNAi efficiency, an enhancement of the silencing of target genes by double-stranded RNA (dsRNA). To test this hypothesis, we use an RNAi efficiency reporter assay. In this assay, *Drosophila* S2 cells are transiently transfected with a vector expressing either dFOXO^{CA} or a control protein (GFP) control along with a firefly luciferase reporter and a nontargeted control reporter containing renilla luciferase (Fig. 2A). At the same time, the cells are also treated with a limiting dose of long dsRNA (200 bp or 90 bp) directed against firefly luciferase or a nonspecific control dsRNA. In addition, the cells were treated with short (less than 30 bp) dsRNA created by treatment of the long dsRNA with RNaseIII. These short dsRNAs that are not efficiently uptaken by the cells do not illicit an efficient RNAi response in S2 cells (27). The cells are harvested and relative luminescence is determined (Fig. 2B). Lower normalized luminescence values indicate that the targeted firefly luciferase transcript has been silenced more efficiently through the utilization of the limiting dsRNA. The results demonstrate that cells containing active dFOXO are more efficient at silencing the luciferase reporter, with the same amount of long dsRNA trigger (Fig. 2B). This result is an indication that dFOXO activation of RNAi genes leads to a functional increase in RNAi efficiency and silencing. However, short dsRNA efficiency is not improved, suggesting dFOXO cannot enhance uptake of dsRNA because this effect is limiting for endonuclease-derived dsRNA less than 30 bp (27).

To test the hypothesis that dFOXO enhances RNAi in an adult fly, we took advantage of the Transgenic RNAi Project (TRiP) resource in which transgenic flies express long hairpin dsRNA against a target gene under control of the Gal4/UAS system. This system allows tissue-specific knockdown of target mRNA when crossed to the appropriate Gal4-expressing fly. We used the classic *white* gene as an RNAi efficiency reporter. We crossed two different UAS-*white* dsRNA TRiP fly lines to flies that express Gal4 in the pigment cells of the eye (*54C-Gal4*; ref. 28). The resulting progeny have orange eyes, not white, because of inefficient knockdown of the *white* gene seen in the first generation RNAi lines (Fig. 3A). Increasing expression of the

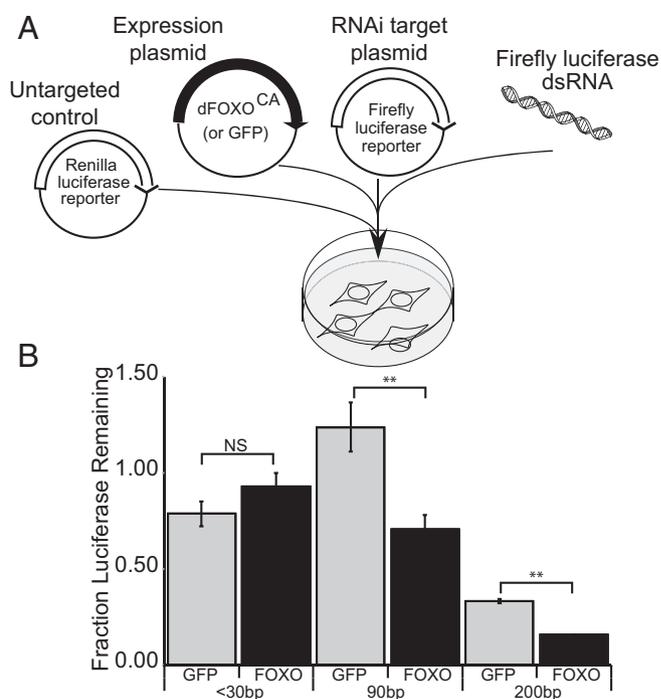


Fig. 2. RNAi efficiency is enhanced in a dFOXO-dependent manner. (A) *Drosophila* S2 cells were transiently transfected with a vector expressing dFOXO or GFP along with a firefly luciferase reporter. The cells were then incubated with limiting amount of dsRNA directed against luciferase or a nonspecific control. (B) Firefly luminescence was measured to determine knockdown efficiency. All error bars are SEM, * $P < 0.05$, ** $P < 0.01$. NS indicates not significant.

RNAi machinery artificially has been found to increase efficacy of RNAi in adult *Drosophila* (29). To determine whether increased dFOXO activity can affect RNAi efficiency, we crossed the reporters to a fly line carrying a UAS-dFOXO transgene (6). This transgene increases dFOXO activity in the eye. When all three transgenes are present, UAS-*white* dsRNA; UAS-dFOXO; and the Gal4 eye driver, the efficiency of RNAi is enhanced in both TRiP lines producing flies with pale or white eyes (Fig. 3B). This result is even more impressive because there is an increase in the white gene dosage in these flies because the UAS-dFOXO transgene also carries white in addition to the endogenous white locus. UAS-dFOXO has no effect on eye pigment alone (Fig. 3C). A similar approach has been used by other laboratories to identify genes involved in the small RNA pathways (30). The cell culture and *Drosophila* eye data, taken together, show that dFOXO can enhance the efficiency of RNAi by transcriptionally up-regulating core RNAi machinery genes.

One known physiological function of RNAi is to promote viral innate immunity in invertebrates (14–17). Innate immunity is an important first line of defense against virus infection for humans. In the case of invertebrates, which lack adaptive immunity, such as *Drosophila*, it is the only defense against viruses. Among several mediators of viral immunity, the RNAi pathway is one of the most critically important pathways (14–17). Through the dicing of viral dsRNA by Dcr2 and the slicing of viral genomes or transcripts by Ago2, two of the identified dFOXO targets, RNAi can efficiently clear a virus from an infected cell (14–17). Based on the above experiments, it is possible that dFOXO transcriptionally up-regulates the RNAi pathway to increase immunity in response to the cellular stress of a viral infection.

To test whether dFOXO activity increases with infection, we used a dFOXO reporter in cultured *Drosophila* S2 cells. The cells were infected with serial dilutions of Cricket Paralysis Virus

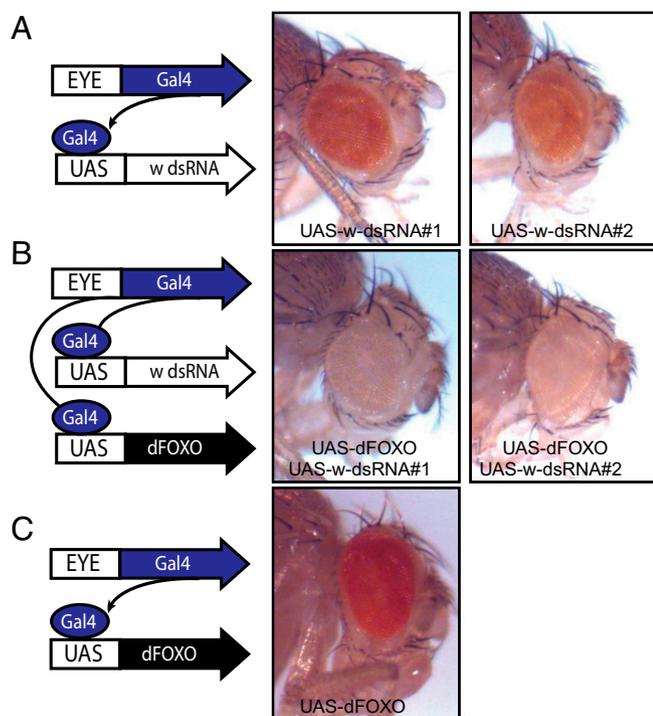


Fig. 3. dFOXO enhances RNAi in adult flies. (A) Eye-specific white dsRNA expression alone (54CGal4/UAS-white dsRNA). (B) Eye specific expression of both dFOXO and white dsRNA (54CGal4/UAS-dFOXO;UAS-white dsRNA). (C) Eye-specific dFOXO expression alone (54CGal4/UAS-dFOXO).

(CrPV). CrPV is a positive-strand RNA virus of the *Dicistroviridae* family of viruses that infects insects including *Drosophila*. Following infection, the activity of the dFOXO reporter was determined. As a control we also used serum starvation, a known physiological dFOXO stimulus, to determine the sensitivity of the reporter assay. dFOXO activity increased in a CrPV dose-dependent manner, indicating that dFOXO is activated upon viral infection (Fig. 4A). This increase is comparable to the increase seen with serum starvation. We next tested the effect of activated dFOXO on viral load in cultured cells. CrPV was used to infect naïve *Drosophila* S2 cells or S2 cells containing the inducible dFOXO^{CA} described above. Replicating virus was detected as described (31). Induction of dFOXO^{CA} reduced the replicating viral load (Fig. 4B).

To determine whether dFOXO plays a role in resistance to RNA viruses, dFOXO null (*foxo*^{Δ94}) flies and a wild-type strain of the same genetic background as the dFOXO null (w^{Dah}) were microinjected with two different RNA viruses: CrPV or Flockhouse virus (FHV), a positive-strand Nodavirus that infects insects. Survival postinjection was determined. Under both low-dose and high-dose conditions, flies lacking dFOXO are deficient in fighting off the effects of CrPV infection (Fig. 5A and B). Likewise dFOXO null flies are deficient in fighting FHV infection (Fig. 5C).

dFOXO is at the center of many stress responsive pathways. To rule out an effect of the FOXO null due to metabolic or other deficiencies, we overexpressed Dcr2 in flies depleted of dFOXO. Overexpression of Dcr2, which effectively increase RNAi activity (29), was sufficient to rescue the dFOXO effect, indicating that the effect on viral immunity in the dFOXO null flies is likely due to RNAi pathway defects, not other metabolic effects of loss of dFOXO (Fig. 5D). These data, taken together, indicate that dFOXO responds to viral infection and an intact dFOXO pathway is required for efficient viral innate immunity.

Discussion

Despite its importance, almost nothing is known about how the protein components of the small RNA pathways are transcriptionally regulated in the cell. Currently only studies addressing the transcriptional regulation of germ-line small RNA pathways (piRNA) have been reported (32). Nothing is reported on the transcriptional regulation of the protein components of the dominant somatic cell small RNA pathways, the miRNA and siRNA pathways. Here, we find dFOXO at the promoters of many core small RNA pathway genes. Components of the miRNA, siRNA, and piRNA pathways are all bound by dFOXO, suggesting an integrated control of the small RNA pathways.

In the current work, we focus on the core small RNA pathway genes dominant in somatic cells. We find the transcription of the *Ago2*, *Ago1*, and *Dcr2* genes is increased during dFOXO activation. The effect of this dFOXO activation is augmented RNAi efficacy even with an unchanged limiting level of the dsRNA trigger. This result suggests the RNA-mediated gene silencing response is not constant but it is tunable to cellular physiology. This notion is consistent with previous work showing enhanced RNAi-based phenotypes in a *daf-2*/INR mutant of *C. elegans* (33) and greater knockdown of target genes with dsRNA in *Drosophila* S2 cells after serum starvation (34). Both of these conditions increase FOXO (*daf-16* in *C. elegans*) activity.

It is interesting to note that Dcr1, the core miRNA dicer, does not seem to be a dFOXO target. This finding is despite the fact that the core miRNA argonaute, Ago1, is a dFOXO target. There is limited evidence for Ago1 involvement in inhibiting viral replication. However, there is evidence showing changes in the miRNA RISC and enhanced silencing by miRNAs under serum-starved conditions (35). This effect is achieved through the increased recruitment of GW182 (Gawky) to the miRNA RISC. Based on our dFOXO ChIP data, GW182 is also a dFOXO target (Fig. S2). Rather than dealing directly with a viral infection, dFOXO's up-regulation of these miRNA factors may be a stress responsive mechanism to repress translation initiation, a previously described role for dFOXO during stress (18, 36).

We also find that dFOXO is activated by viral infection to a comparable level as another well-defined physiological signal, serum starvation. Activated dFOXO can decrease viral load in cell culture and is required for effective resistance to RNA virus infection. The FOXO family of transcription factors responds to a multitude of cellular and extracellular signals (3). The current

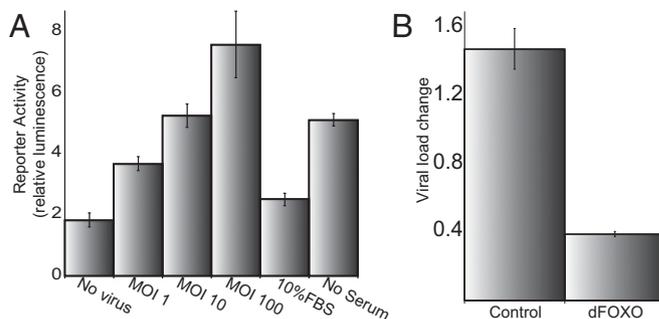


Fig. 4. dFOXO is activated upon infection and promotes viral immunity. (A) S2 cells were transfected with a dFOXO firefly luciferase reporter and a control plasmid containing the renilla luciferase gene. Cells were infected with CrPV and incubated for 24 h or serum starved. Luminescence was measured to determine dFOXO activity. (B) *Drosophila* S2 or S2 cells with a stable integration of constitutively active dFOXO under the control of the metallothionein A promoter were treated with CuSO₄ for 16 h. The cells were then infected with CrPV to an MOI of 1. After 8-h incubation, the amount of CrPV (replicating form) was measured and normalized to mock-treated expression levels to determine the change in viral load.

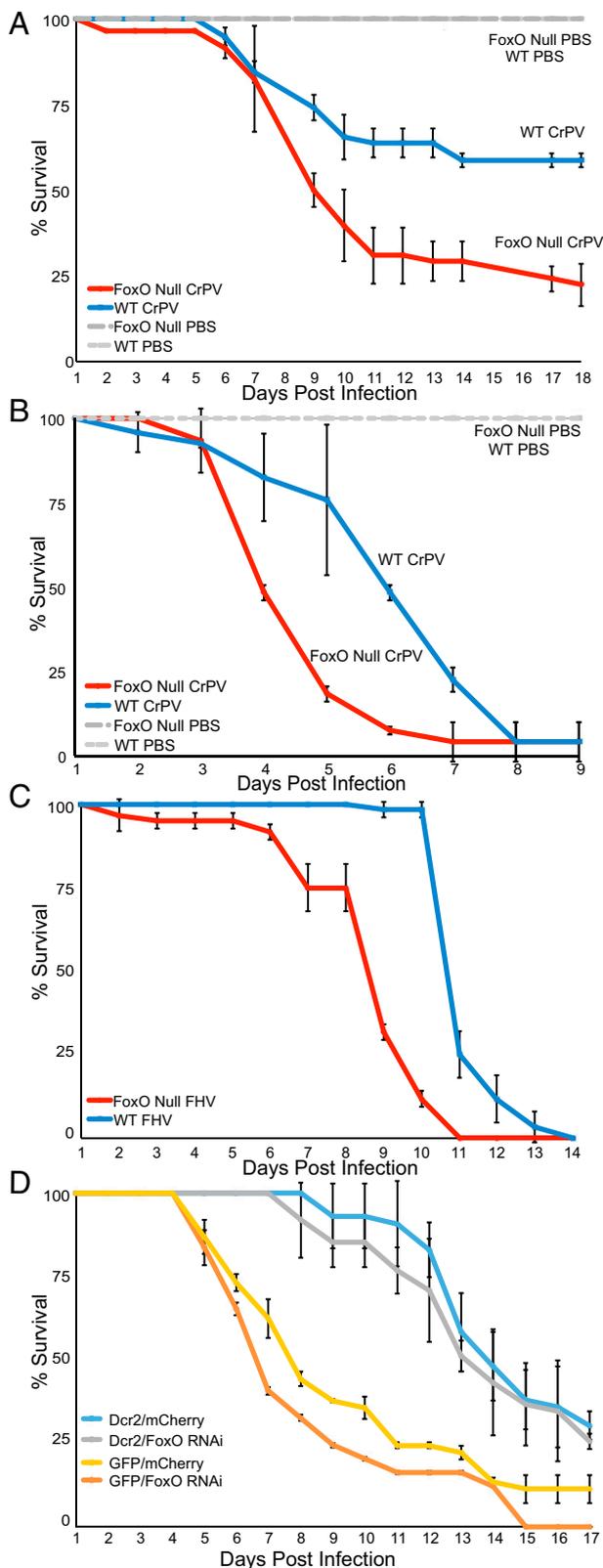


Fig. 5. dFOXO is required for defense against RNA virus. Adult *Drosophila* dFOXO nulls (FOXO Δ 94) and white Dahomey (wDah) wild-type controls were injected in the abdomen with 50 nL of a CrPV solution. PBS injections were used as a control for the effects of injection. Flies were injected with either 100 TCID₅₀ (A) or 300 TCID₅₀ (B), and survival following infection was scored daily. (C) The same injection scheme except 5 × 10⁵ PFU of FHV was used. (D) CrPV was injected into adults depleted of dFOXO by RNAi or depleted of dFOXO and expressing Dcr2. GFP and mCherry serve as nonspecific controls.

study shows dFOXO provides a link among cellular physiology, the RNAi pathway, and innate immunity enhancing the effectiveness of silencing and allowing the RNAi pathway to respond dynamically to changes in cellular homeostasis.

The importance of the RNAi pathway for viral immunity in invertebrates is well defined. However, the role of RNAi in viral immunity for mammals is still an open question. The mammalian cellular innate immune system differs from lower organisms, relying strongly on the IFN response during a viral infection. However, in cell types that lack a fully developed IFN response, RNAi plays an important role in viral defense (37, 38). Additionally, several viruses that infect mammalian cells contain genes that suppress the RNAi response (39). This result suggests an ongoing battle between RNAi-based innate immunity and viruses. There is a growing appreciation for the role of FOXOs in mammalian immune regulation (40, 41). If conservation of the function of FOXO-small RNA regulation exists in mammals, there are potential therapeutic benefits.

Materials and Methods

Fly Lines. w^{Dah} and FOXO Δ 94 were described (42). Other lines were obtained from Bloomington stock center. Transgenic RNAi Project (TRiP) lines: (white, 28980 and 25785), (FOXO, 32427), (mCherry, 35785). The 54C-Gal4 driver has been described (28) (27328). UAS-foxo has been described (6) (9575). UAS-Dcr2 (25708) and a UAS-GFP (42713) were obtained from Bloomington stock center.

dFOXO ChIP. The ChIP was done as described (18) except enrichment was normalized to the 28s gene. For genome-wide analysis, precipitated and input DNA were treated as described (43). Affymetrix *Drosophila* tiling arrays were processed as described (43). A cutoff of fourfold enrichment over background was used to define dFOXO-bound regions. Genes within 400 bp of the binding sites were defined as possible targets for gene ontology searches. Gene ontology searches were performed by using the web based DAVID (44) server (david.ncifcrf.gov). DNA motifs were identified by using the web-based MEME-ChIP server (meme-suite.org/tools/meme-chip). Array data has been deposited, GEO accession no. GSE73827.

Cell Culture, RNA Extraction, and RT-qPCR. *Drosophila* S2 cells with a stable transfection of an inducible constitutively active FOXO (321s) (6) were maintained in Schneider's Insect media with 10% (vol/vol) FBS. Cells were plated at 1 × 10⁶ cells per mL, and induced by addition of 500 μM CuSO₄ for 16 h. During induction, the media was supplemented with 5 μg/mL bovine insulin. Total RNA was extracted by using TRI Reagent according to manufacturer's protocol (MRC). First-strand cDNA synthesis was done using oligo-dT on 5 μg of DnaseI-treated RNA. The final concentrations of the cDNA reaction were 50 mM Tris-HCl (pH 8.3), 50 mM KCl, 3 mM MgCl₂, 10 mM DTT, 400 μM dNTPs, 1–3 μg RNA, 500 ng of primers, 200 units of Moloney Murine Leukemia Virus Reverse Transcriptase. cDNAs were diluted 1:10 in Tris EDTA, pH 8. qPCR used 4 μL of cDNA, GoTaq qPCR Master Mix (Promega), and 100 nM gene-specific primers (Table S3) in a 20-μL reaction. Target gene expression was calculated as a fraction of RP49 and normalized to mock-treated expression levels. All experiments were performed at least twice in triplicate.

RNAi Gene Expression Analysis. *Drosophila* Mbn2 cells were maintained in Schneider's Media with 10% FBS. For serum-starvation experiments, 1 × 10⁶ cells were incubated with 25 μg/mL dsRNA against dFOXO or LacI as a negative control. After 3 d, the cells were washed three times with media without serum and left to incubate for 12 h. As a control, separate Mbn2 cells were maintained in serum-containing media supplemented with 5 μg/mL bovine insulin. Total RNA, cDNA synthesis, and qPCR analysis were completed as described above. Target gene fold expression was calculated as a fraction of RP49 and normalized to unstarved expression levels. All experiments were performed at least twice in triplicate.

Fly Heat Shock, RNA Extraction, and RT-qPCR. Ten Hsp70-Gal4;UAS-dFOXO male flies were heat shocked in a circulating water bath at 37 °C for 1 h then incubated at 25 °C for 6 h. A separate group of 10 Hsp70-Gal4;UAS-dFOXO male flies were left unheatshocked as a control. Total RNA was harvested and RT-qPCR was performed as described above.

RNAi Enhancement in S2 cells. S2 cells were seeded at 0.5×10^6 cells per mL into 24-well plates. Cells were transiently transfected by using Effectene (Qiagen) with the following plasmids: Constitutively active FOXO under the mini actin promoter or GFP under the mini actin promoter as a control, Firefly luciferase under the metallothionein A promoter, and Renilla luciferase under the mini actin promoter as an internal control for transfection efficiency and cell number. After transfection, cells were incubated for 24 h to allow for FOXO expression. After incubation, the media was replaced with fresh media supplemented with $5 \mu\text{g/mL}$ dsRNA against Firefly luciferase or LacI as a control. Double-stranded RNA smaller than 30 base pairs was produced by treatment of the long dsRNA with NEB ShortCut RnaseIII. Cells were incubated for 4 h with the dsRNA. The media was replaced with fresh media supplemented with $500 \mu\text{M}$ CuSO_4 . After 4 h, the cells were lysed with $1 \times$ passive lysis buffer (Promega). Samples were analyzed by using a dual luciferase assay (Promega). Biological replicates were done in triplicate.

RNAi Enhancement in *Drosophila* Eye. A fly expressing Gal4 in the pigment cells of the eye 54CGal4 (Bloomington 27328) was crossed to two fly lines with a transgenic allele to express dsRNA against the *white* gene under UAS control (Bloomington 28980 or Bloomington 25785). Progeny of this first cross were then crossed to a fly with FOXO under UAS control (Bloomington 9575). Pictures of individual fly eyes were taken and PCR was done on each fly separately to determine the presence of the three transgenic alleles (Gal4, dsRNA, and FOXO) (line 27328;25785, $n = 16$; line 27328;28980, $n = 21$) (27328/9575; 25785, $n = 14$; line 27328/9575;28980, $n = 16$). Phenotypic penetrance was 100%. Progeny from the first cross (Gal4 to dsRNA) and Gal4 crossed to FOXO were used as a control. Presence of the transgenes was determined by PCR.

Viral Treatment. Twenty-four to thirty flies (5–7 d old) were starved on $1 \times$ PBS/Agar for 24 h or allowed to feed ad libitum as a control. The following day,

the flies were injected with 50 nL of a viral suspension of CrPV in $1 \times$ PBS at 6×10^6 TCID₅₀ per mL or $1 \times$ PBS alone as a control. The FHV infections were done in the same manner except 5×10^5 PFU per injection were used. After infection flies were placed normal fly food (both originally starved and fed flies). Flies were transferred to new food every 3 d, and dead flies were scored daily. The Dcr2 overexpression/FOXO knockdown CrPV infections were completed in the same manner as the FOXO null and wild-type infections.

CrPV Viral Load Measurements. The 321 cells (6) were seeded in six-well plates at 1×10^6 cells per well. CuSO_4 was added to $500 \mu\text{M}$ and media was supplemented with $5 \mu\text{g/mL}$ bovine insulin. After 16 h, media was removed and replaced with media containing CrPV at an MOI of 1. After 8 h, the media was removed and total RNA was extracted from mock treated and induced cells. cDNA synthesis proceeded as described with the following exceptions: $1.5 \mu\text{g}$ of total RNA was reverse transcribed with a tagged primer to the CrPV negative strand, along with a primer for RP49 as a control. CrPV negative strand fold change was calculated as a fraction of RP49 and normalized to mock-treated expression levels. All experiments were performed at least twice in triplicate.

ACKNOWLEDGMENTS. We thank the TRiP at Harvard Medical School (NIH/National Institute of General Medical Sciences Grant R01-GM084947) for providing transgenic RNAi fly stocks; the Bloomington Stock center for providing fly stocks; Peter Sarnow's laboratory for the generous gift of CrPV; Anette Schneemann's laboratory who generously provided titred FHV; Michael Rosbash, Nelson Lau, Sharon Marr, and members of the M.T.M. laboratory for comments on the manuscript. M.J.S. was partially funded through a Genetics Training Grant from the NIH, and part of this work was funded through New Scholar In Aging Grant AG-NS-0535-09 from the Ellison Medical foundation (to M.T.M.).

- Morimoto RI (2011) The heat shock response: Systems biology of proteotoxic stress in aging and disease. *Cold Spring Harb Symp Quant Biol* 76:91–99.
- Günther V, Lindert U, Schaffner W (2012) The taste of heavy metals: Gene regulation by MTF-1. *Biochim Biophys Acta* 1823(9):1416–1425.
- Eijkelenboom A, Burgering BM (2013) FOXOs: Signalling integrators for homeostasis maintenance. *Nat Rev Mol Cell Biol* 14(2):83–97.
- Salih DA, Brunet A (2008) FoxO transcription factors in the maintenance of cellular homeostasis during aging. *Curr Opin Cell Biol* 20(2):126–136.
- Ogg S, et al. (1997) The Fork head transcription factor DAF-16 transduces insulin-like metabolic and longevity signals in *C. elegans*. *Nature* 389(6654):994–999.
- Puig O, Marr MT, Ruhf ML, Tjian R (2003) Control of cell number by *Drosophila* FOXO: Downstream and feedback regulation of the insulin receptor pathway. *Genes Dev* 17(16):2006–2020.
- Salih DA, et al. (2012) FoxO6 regulates memory consolidation and synaptic function. *Genes Dev* 26(24):2780–2801.
- Greer EL, et al. (2007) The energy sensor AMP-activated protein kinase directly regulates the mammalian FOXO3 transcription factor. *J Biol Chem* 282(41):30107–30119.
- Moylan JS, Smith JD, Chambers MA, McLoughlin TJ, Reid MB (2008) TNF induction of atrogin-1/MAFbx mRNA depends on Foxo4 expression but not AKT-Foxo1/3 signaling. *Am J Physiol Cell Physiol* 295(4):C986–C993.
- Puigserver P, et al. (2003) Insulin-regulated hepatic gluconeogenesis through FOXO1-PGC-1alpha interaction. *Nature* 423(6939):550–555.
- Ghildiyal M, Zamore PD (2009) Small silencing RNAs: An expanding universe. *Nat Rev Genet* 10(2):94–108.
- Stefani G, Slack FJ (2008) Small non-coding RNAs in animal development. *Nat Rev Mol Cell Biol* 9(3):219–230.
- Leung AK, Sharp PA (2010) MicroRNA functions in stress responses. *Mol Cell* 40(2):205–215.
- Galiana-Arnoux D, Dostert C, Schneemann A, Hoffmann JA, Imler JL (2006) Essential function in vivo for Dicer-2 in host defense against RNA viruses in *Drosophila*. *Nat Immunol* 7(6):590–597.
- van Rij RP, et al. (2006) The RNA silencing endonuclease Argonaute 2 mediates specific antiviral immunity in *Drosophila melanogaster*. *Genes Dev* 20(21):2985–2995.
- Wang XH, et al. (2006) RNA interference directs innate immunity against viruses in adult *Drosophila*. *Science* 312(5772):452–454.
- Zambon RA, Vakharia VN, Wu LP (2006) RNAi is an antiviral immune response against a dsRNA virus in *Drosophila melanogaster*. *Cell Microbiol* 8(5):880–889.
- Olson CM, Donovan MR, Spellberg MJ, Marr MT, 2nd (2013) The insulin receptor cellular IRES confers resistance to eIF4A inhibition. *eLife* 2:e00542.
- Lee H, et al. (2014) DNA copy number evolution in *Drosophila* cell lines. *Genome Biol* 15(8):R70.
- Teleman AA, Hietakangas V, Sayadian AC, Cohen SM (2008) Nutritional control of protein biosynthetic capacity by insulin via Myc in *Drosophila*. *Cell Metab* 7(1):21–32.
- Bai H, Kang P, Hernandez AM, Tatar M (2013) Activin signaling targeted by insulin/dFOXO regulates aging and muscle proteostasis in *Drosophila*. *PLoS Genet* 9(11):e1003941.
- Alic N, et al. (2014) Interplay of dFOXO and two ETS-family transcription factors determines lifespan in *Drosophila melanogaster*. *PLoS Genet* 10(9):e1004619.
- Alic N, et al. (2011) Genome-wide dFOXO targets and topology of the transcriptomic response to stress and insulin signalling. *Mol Syst Biol* 7:502.
- Furuyama T, Nakazawa T, Nakano I, Mori N (2000) Identification of the differential distribution patterns of mRNAs and consensus binding sequences for mouse DAF-16 homologues. *Biochem J* 349(Pt 2):629–634.
- Schmidt M, et al. (2002) Cell cycle inhibition by FoxO forkhead transcription factors involves downregulation of cyclin D. *Mol Cell Biol* 22(22):7842–7852.
- Costa A, Jan E, Sarnow P, Schneider D (2009) The Imd pathway is involved in antiviral immune responses in *Drosophila*. *PLoS One* 4(10):e7436.
- Saleh MC, et al. (2006) The endocytic pathway mediates cell entry of dsRNA to induce RNAi silencing. *Nat Cell Biol* 8(8):793–802.
- Nagaraj R, Banerjee U (2007) Combinatorial signaling in the specification of primary pigment cells in the *Drosophila* eye. *Development* 134(5):825–831.
- Dietzl G, et al. (2007) A genome-wide transgenic RNAi library for conditional gene inactivation in *Drosophila*. *Nature* 448(7150):151–156.
- Lee YS, Carthew RW (2003) Making a better RNAi vector for *Drosophila*: Use of intron spacers. *Methods* 30(4):322–329.
- Stevanovic AL, Johnson KN (2015) Infectivity of *Drosophila* C virus following oral delivery in *Drosophila* larvae. *J Gen Virol* 96(Pt 6):1490–1496.
- Li XZ, et al. (2013) An ancient transcription factor initiates the burst of piRNA production during early meiosis in mouse testes. *Mol Cell* 50(1):67–81.
- Wang D, Ruvkun G (2004) Regulation of *Caenorhabditis elegans* RNA interference by the *daf-2* insulin stress and longevity signaling pathway. *Cold Spring Harb Symp Quant Biol* 69:429–431.
- Clemens JC, et al. (2000) Use of double-stranded RNA interference in *Drosophila* cell lines to dissect signal transduction pathways. *Proc Natl Acad Sci USA* 97(12):6499–6503.
- Wu PH, Isaji M, Carthew RW (2013) Functionally diverse microRNA effector complexes are regulated by extracellular signaling. *Mol Cell* 52(1):113–123.
- Marr MT, 2nd, D'Alessio JA, Puig O, Tjian R (2007) IRES-mediated functional coupling of transcription and translation amplifies insulin receptor feedback. *Genes Dev* 21(2):175–183.
- Li Y, Lu J, Han Y, Fan X, Ding SW (2013) RNA interference functions as an antiviral immunity mechanism in mammals. *Science* 342(6155):231–234.
- Maillard PV, et al. (2013) Antiviral RNA interference in mammalian cells. *Science* 342(6155):235–238.
- Svoboda P (2014) Renaissance of mammalian endogenous RNAi. *FEBS Lett* 588(15):2550–2556.
- Hedrick SM, Hess Michelini R, Doedens AL, Goldrath AW, Stone EL (2012) FOXO transcription factors throughout T cell biology. *Nat Rev Immunol* 12(9):649–661.
- Coffer PJ, Burgering BM (2004) Forkhead-box transcription factors and their role in the immune system. *Nat Rev Immunol* 4(11):889–899.
- Slack C, Giannakou ME, Foley A, Goss M, Partridge L (2011) dFOXO-independent effects of reduced insulin-like signaling in *Drosophila*. *Aging Cell* 10(5):735–748.
- Sims HI, Chirn GW, Marr MT, 2nd (2012) Single nucleotide in the MTF-1 binding site can determine metal-specific transcription activation. *Proc Natl Acad Sci USA* 109(41):16516–16521.
- Huang W, Sherman BT, Lempicki RA (2009) Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc* 4(1):44–57.