New Tool for Late Stage Visualization of Oskar Protein in *Drosophila melanogaster* Oocytes

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Abstract

Determination of the anterior/posterior axis in *Drosophila melanogaster* is established in oogenesis, in part by localization of the *oskar* (*osk*) mRNA to the posterior pole of the oocyte. This mRNA is subject to translational control to ensure that it is translated only when properly localized, but the process is not fully elucidated.

In identifying genes with roles in translational control of *osk*, antibody staining is typically used to assay Osk expression. A limitation of this method is that the vitelline membrane, which surrounds the oocyte and is deposited during oogenesis, prevents access to antibodies, and so later stages of Osk expression cannot be monitored. Although early work focused on the the stage when Osk protein first appears, we now know that the majority of Osk is translated in later stages of oogenesis. My goal was to look for new factors involved in *osk* translational control by monitoring Osk::GFP expression and 'knocking down' the expression of candidate genes by using RNA interference (RNAi). An expansive collection of transgenic RNAi (TRiP) lines target a large fraction of all Drosophila genes. Knockdown is achieved by crossing a chosen TRiP line to a GAL4 driver (MAT3), which directs TRiP expression in the germline cells of the ovary.

Knockdowns can be more useful than mutants, which are often homozygous lethal or arrest oogenesis earlier than the stage we wish to visualize. Knockdowns typically partially decrease the expression of a target gene, thus avoiding the complications associated with a null mutant. To simplify the screen I recombined the MAT3 driver and *osk::GFP* transgene on to the same chromosome. This allowed me to test each TRiP line with a single cross, with the TRiP transgene from one parent and *osk::GFP* and MAT3 from the other parent. To make the

recombinant I crossed MAT3 and *osk::GFP* flies, and used a PCR assay to test the progeny of individual candidates for presence of MAT3 and Osk::GFP. To validate the knockdown approach, I crossed the *osk::GFP MAT3* recombinant TRiP lines of known regulators of *osk* mRNA to see if I obtained the same phenotypes as previously observed for mutants of these genes. It was found that these knockdowns show no change in expression of Oskar::GFP protein in TRiP lines of activators and increased expression in TRiP lines of repressors, except for that of *hephaestus*, which exhibited decreased expression. *vasa*, which is required for a late stage in Osk expression, also appears to have decreased expression. In the future, we plan to use this recombinant in crosses with other TRiP lines of known genes used in *osk* translational control such as *tudor*, *orb*, and *bruno*. The knockdown of *hephaestus* and *aubergine* should be reperformed in order to ensure that the results are accurate. In addition, this recombinant can be used in screens to find genes involved in *osk* expression during late stage oogenesis so that more detailed mechanisms of control in posterior development can be described.

Background

RNA interference

RNA interference is a natural biological process which has been repurposed as a tool in functional genomics. This gene silencing mechanism hinges on the transcription of hairpin RNAs (hpRNAs) which fold upon themselves to form double stranded DNA (dsDNA). These dsDNA strands are then processed by the RNA-induced silencing complex (RISC), which cleaves the dsDNA into siRNAs. These siRNAs are highly specific to an mRNA sequence and upon binding to the target mRNA, will direct the sequence-specific degradations of this mRNA strand (Kuttenkeuler and Boutros, 2004).

Within functional genomics, an Upstream Activation Sequence (UAS) has been added to the promoter of the hpRNA sequence to ensure that its transcription is dependent on the presence of a GAL4 driver, which specifically binds to the UAS to activate gene transcription. This system allows for tissue or cell specific activation of the RNAi, by constructing a GAL4 driver with a promoter that is activated in that specific tissue or cell. Once the RNAi gene and the GAL4 driver are located in the same genome, the activated RNAi will generate a knockdown to partially or completely diminish the target gene expression in the desired tissue. In doing this, lethal effects of diminished expression in other tissues or cells can be avoided. Stable stocks of flies that use this transgenic RNAi are known as TRiP lines and can target a large number of Drosophila genes.

Collection of TRiP Lines Utilized

In order to ensure that the *osk::GFP MAT3* recombinant didn't have any unexpected interactions with many of the proteins and mRNAs involved in translational control of *osk*, TRiP lines of major known regulators of *osk* mRNA were selected for screening. These included repressors such as *Bicaudal-C* (*Bic-C)hephaestus* (*heph*), and *Argonaute-1* (*AGO1*) and activators such as *vasa* and *staufen*. RNA binding proteins such as Staufen (Stau) and Hephaestus (Heph) are found in the same RNP particles that *osk* mRNA travels in and mediate translational control of *osk* mRNA (Martin et al. 2003). Staufen proteins mediate posterior localization of *osk*, as *stau* mutants develop *osk* particles in the anterior margin of the oocyte (Irion et al. 2006). Heph, also known as a polypyrimidine tract binding (PTB) protein, has shown the ability to bind to *osk* mRNA in a sequence specific manner and is necessary for the translational repression of this mRNA while it is in the process of localization (Besse et al. 2009). The repressor *Bic-C* is known to be a part of translational control and the absence of Bic-

C in oocytes yields premature *osk* translation prior to its posterior localization (Saffman et al. 1998). Another repressor, *argonaute*, is thought to be involved in the *osk* repression but its role is uncertain. The activator *aubergine* (*aub*) is necessary for posterior development and *osk* activation once it reaches the posterior pole (Harris and Macdonald, 2001).

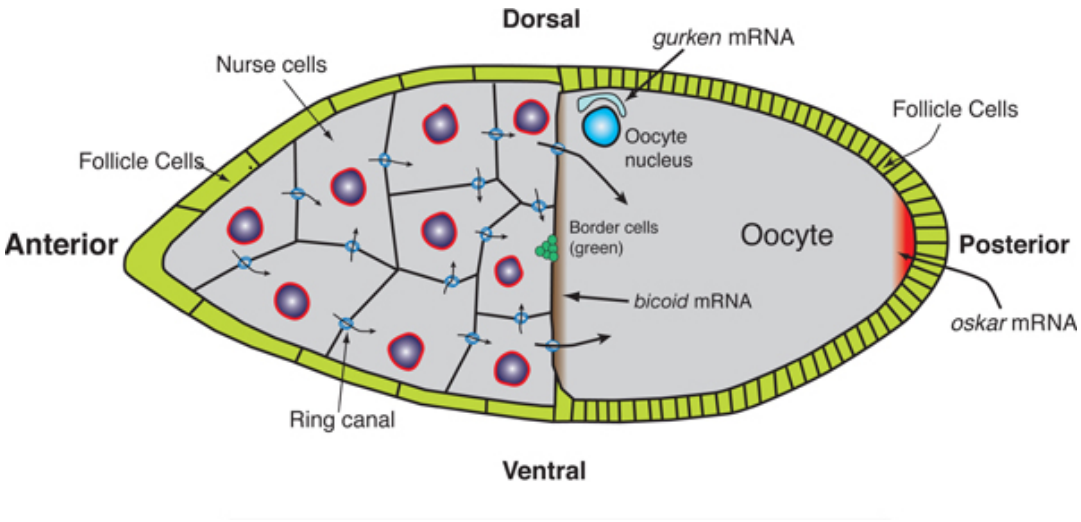

Figure 1. Wild type expression of endogenous *osk* mRNA in a *D. melanogaster* oocyte traveling from the nurse cells and into the oocyte before being localized at the posterior pole. (Gonsalvez and Long, 2012)

Each of these is necessary for appropriate expression of the *osk* mRNA in its repression, localization, and activation at the posterior pole of the oocyte. One can see the orientation of this mRNA within a developing oocyte (Stage 10A) in Figure 1

Results

The recombinant line of flies carrying both the GFP-tagged oskar and a GAL-4 driver required the crossing of flies with the transgene for GFP-tagged *oskar* (9173) and those carrying

the GAL4 driver MAT3. Using the cross w; $\frac{9173}{TM3Sb}$ ×w; $\frac{MAT3}{TM2}$ flies of the genotype w; $\frac{9173}{MAT3}$were generated. Females of this genotype were collected to cross with wild type (w118) males. The resulting flies were screened to find recombinant males with the predicted eye color specific for w; $\frac{9173\ MAT3}{+}$, which were then crossed to females of doubly balanced lines to generate a stable line of flies with both the transgene for GFP-tagged Oskar and the GAL4 driver. The recombinant males were then removed from the population and tested by mixing the fly sample DNA with target oligonucleotides and performing PCR to amplify the target DNA if the gene is in the sample. The results are shown in Figure 2 and indicate that four of the five potential recombinants contained both MAT3 and 9173 genes. Of these, the third recombinant had a more robust population and was therefore chosen as the stock population for the remaining tests.

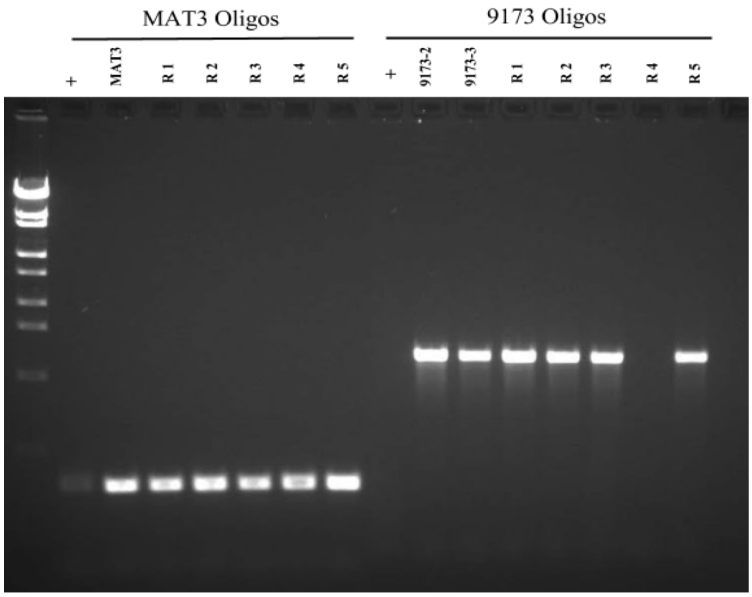

Figure 2. Agarose gel displaying the presence or absence of the desired genes using oligonucleotides specific for the GFP-tagged oskar and the GAL4 driver MAT3. Potential recombinants are described as R1, R2, R3, R4, and R5.

The recombinant was crossed to the TRiP lines of *hephaestus, vasa, staufen, aubergine, Argonaute-1* and *Bicaudal-C* to drive RNAi activity and generate knockdowns of these target genes and observe any changes in Oskar expression. The results can be seen in Figure 3.

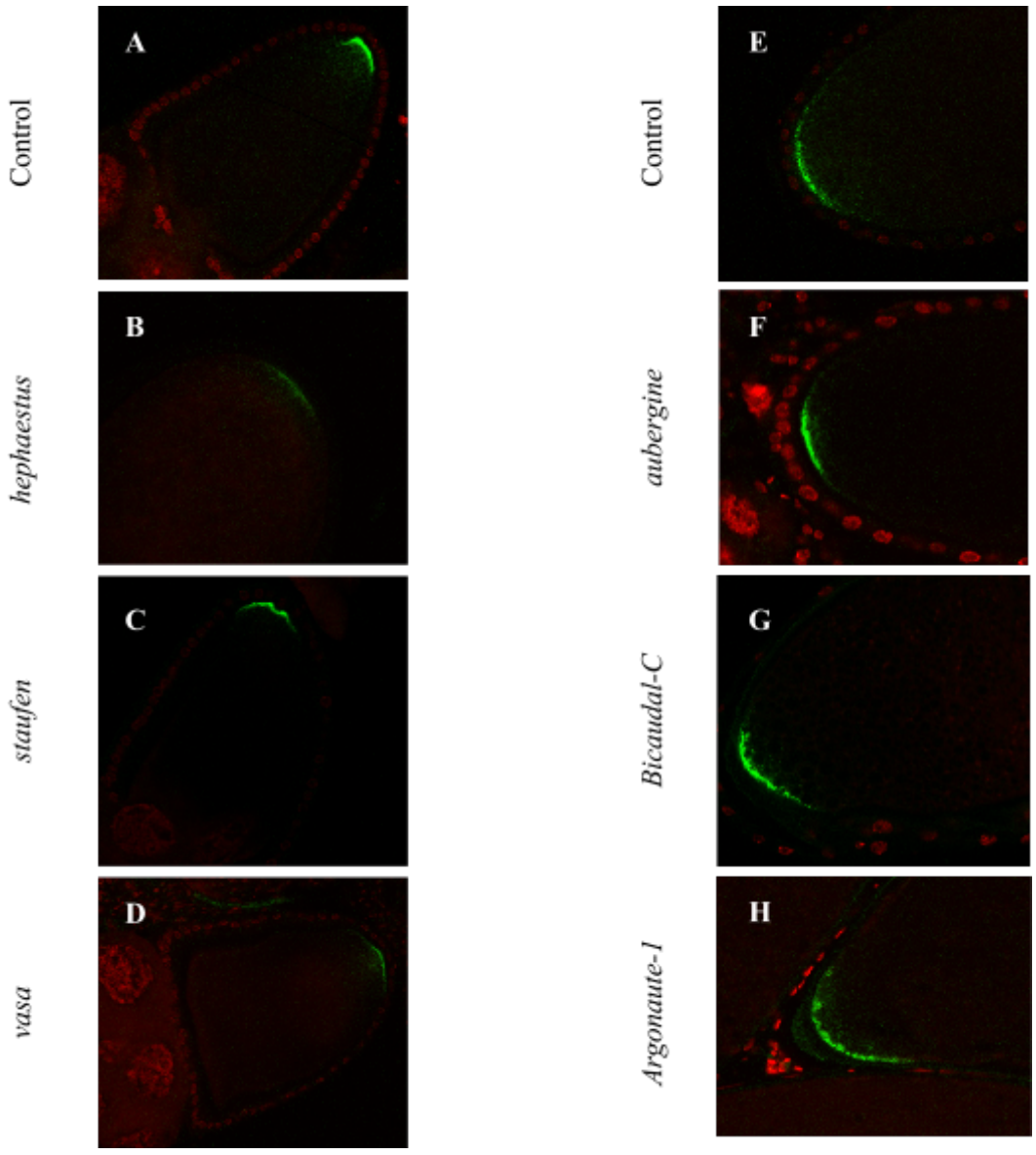

Figure 3. A-D are at Stage 10B of oocyte development; E-H are at Stage 11 of development. Both A and E are *osk::GFP MAT3* recombinants with no RNAi activated and no knockdowns performed. B-D and F-H all contain both the *osk::GFP MAT3* gene and the RNAi gene to drive the desired knockdown of expression.

Both the *vasa* and *hephaestus* knockdowns exhibit lower expression than that of the control. The *staufen* knockdown displays a slight decrease of Osk::GFP expression in comparison to that of the control. The *aubergine* knockdown appears to an increase in strength

and specificity of posterior localization of Oskar protein compared to Osk::GFP expression of the control, while that of the *Bicaudal-C* and *Argonaute-1* both appear to have similar localization patterns as the control, but slightly greater expression.

Discussion

The aim of this project was to create a new tool to look for new factors involved in *osk* translational control by monitoring Osk::GFP expression and have the capability of driving RNAi function. The creation of a recombinant capable of this, containing both *osk::GFP* and *MAT3* genes, was accomplished using a series of genetic crosses to not only develop and identify recombinants, but to cross these recombinants to balancers that prevent any ~~no~~ loss of the recombinant chromosome. PCR was used to enhance the target identifying sequences of both *osk::GFP* and *MAT3* in the potential recombinants. Gel electrophoresis separated the target DNA fragments and compare the resulting bands of DNA to the control to ensure that the potential recombinants chosen do have both genes. Using these techniques, four of the five potential recombinants were confirmed to have both *MAT3* and *osk::GFP*. After this, one of the confirmed recombinants was crossed to TRiP lines targeting *hephaestus, vasa, staufen, aubergine, Argonaute-1* and *Bicaudal-C* to drive RNAi activity of the target gene of each cross.

These knockdowns were then visualized using confocal microscopy and it was found that the *Bicaudal-C, vasa,* and *Argonaute-1* knockdowns performed as expected for a partial decrease in expression of these *osk* repressors, but *hephaestus* and *aubergine* both showed abnormal expression in these knockdowns. *Bicaudal-C, vasa* and *Argonaute-1* are involved in the translational repression, so the decreased expression of these repressors should increase *osk* expression and did, as seen in Figure 3. The knockdown of *staufen* produced a slight decrease in *osk* mRNA, which is expected as *stau* acts as an activator in *osk* translational control.

The *hephaestus* and *aubergine* knockdowns responded abnormally, with the knockdown of *hephaestus* displaying decreased *osk* expression. As a repressor of *osk* mRNA, the partial or complete knockdown of these genes should result in increased expression of Osk, not less. In addition, as *aubergine* is an activator of *osk* mRNA, its knockdown should have yielded less expression of Osk::GFP, but the results showed greater expression in Figure 3. This abnormal expression of *osk* in the *hephaestus* and *aubergine* knockdowns could potentially be due to equipment use and external factors. The *hephaestus* knockdown was visualized later than desired due to complications with the confocal microscope that restricted its use and this may have lead to decreased fluorescence of the samples. In the future, the *hephaestus* and *aubergine* knockdowns should be repeated in order to ensure that proper visualization is taken. In addition, knockdowns of other important genes involved in translational control such as *tudor* or *bruno* would be helpful in determining whether the *osk::GFP MAT3* recombinant has any potential interactions with Tudor, Orb, or Bruno which change *osk* expression. The availability of both a GAL4 driver and Osk::GFP fusion protein will also be useful in future experiments for discovering other genes involved in *osk* translational control by using the wide range of TRiP lines available to test possible regulators.

Materials and Methods

Fly stocks

The fly populations which were crossed to the GFP-tagged Osk and driver were from the Transgenic RNAi Project (TRiP) and contained TRiP lines which, when combined with a driver, would result in a partial or complete knockdown of one of the following genes: *vasa, cup, tudor, staufen, aubergine, hephaestus, argonaute,* and *bicaudal c*. These knockdowns used RNA interference to selectively reduce the expression of a gene either partially or completely. In doing

so, the effects of the loss of protein can be observed in mutants wherein a knockout, which would completely eliminate the expression of a gene, would be fatal. These flies were raised on standard cornmeal-agar medium at 25° C to maintain a faster generation time.

Polymerase Chain Reaction (PCR)

A sample male fly was mashed and added to 50 µL of squishing buffer with a 1/100 dilution of Proteinase K (20mg/ml). It was then incubated at 37° C for 25 minutes and then the Proteinase K was inactivated by heating the sample to 95° C for 2 minutes. 23µL of Platinum PCR mix was added to 1 µL of the sample and squish buffer and 1µL of oligonucleotides. The oligonucleotides used to identify the GAL4 driver (MAT3), were 5255/5256 and those used to identify 9173 were 5290/5099. Once this was complete, they were placed in a T100 Thermal cycler to undergo amplification with initialization at 95° C at 5 minutes, denaturation at 95° C for 30 seconds, annealing at 55° C for 30 seconds, elongation at 72° C for 45 seconds and final elongation at 72° C for 7 minutes, with the denaturation through elongation steps repeated 34 times to ensure an amplified yield of the target sequences.

Gel Electrophoresis

Using a 1.2% agarose gel submerged in TAE buffer, 20µLof sample and loading buffer are loaded into the wells and electrophoresed at 100V with the sample running towards the anode for 1 hour. A Lambda DNA ladder whose known separated bands help to determine the size of the fragments in the bands.

Ovary Dissection

Flies collected for dissection were placed in a fattening vial for 3 days. The flies were dissected with ovaries being placed in phosphate buffered saline (PBS) before being fixed in a solution of 0.01% formaldehyde for 20 minutes. The PBS and formaldehyde was removed and washed with

a solution of phosphate buffered saline with 0.5% triton (PBT) over the course of an hour prior to blocking for 2 hours with a solution of PBT+ 5% goat serum. The ovaries were then washed with PBT for 2 hours before being transferred to a labeled microscope slide and mounting medium (Vectastain for fluorescence) was added.

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