A study on the effectiveness of different RNAi expression systems in arresting
*Drosophila melanogaster* embryos during early development

Honors Thesis
Presented to the College of Arts and Sciences
Cornell University
In partial fulfillment of the requirements for the Biology Honors Program

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May 2021
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ABSTRACT

The technique to arrest *Drosophila melanogaster* embryos at a specific developmental stage is desirable to various applications that require a population with relatively uniform amount of DNA. One example is to measure the allelic frequencies using pooled sequencing (Wei et al. 2017). To achieve this, RNAi constructs activated by Gal4 proteins were used to knock down genes essential to *D. melanogaster* embryonic development. I tested the arresting efficiencies of six different RNAi constructs. Four of which had an arrest rate greater than 97.3% (*trk*-pNP, *dl*-pNP, *dl*-UASz, *tor*-UASz; the names are defined in Method section). I also found strong evidence indicating that the Gal4 gene locus was affecting the arrest rate. Several constructs also showed evidence that the age of the female expressing them could be an affecting factor. In spite of this, there was sometimes high variations between results from different replicas of the same RNAi constructs, which could be either due to random environmental factors or polymorphisms within the same fly lines. Further studies would therefore be needed to better address this internal variation, and to generalize the technique for *D. melanogaster* strains with different genetic backgrounds.

INTRODUCTION

Pooled whole genome sequencing is a powerful approach to measure the allelic frequencies in a population. It has been proposed as an efficient method to identify meiotic drivers in *D. melanogaster* genomes (Wei et al. 2017). The current project serves as a follow up to address two possible biases in the proposed method. Though specifically designed to improve the application of pooled sequencing in meiotic driver identification, the outcome of this project sees its potential in being generalized to other experimental uses (such as to help observe the phenotypes of embryos at specific stages) as well.

Wei et al. 2017 focused on identifying meiotic drivers in female *D. melanogaster*. In normal Mendelian inheritance in a diploid organism, the two alleles of a certain
gene each has a 50% chance of being inherited by an individual offspring. Meiotic drivers, however, are selfish alleles that shift this probability to favor their own transmission. Nevertheless, such shifts can be so subtle that a large population needs to be scored to obtain statistically significant evidence. In addition, drivers are usually not associated with other visible phenotypes, making it further challenging to detect weak drivers.

To achieve efficient detection, Wei et al. 2017 has proposed to apply pooled whole genome sequencing to test the allelic frequencies in a large, carefully constructed population (Figure 1). Such a population can be created by first crossing two true breeding lines P1 and P2 that carry known single-nucleotide polymorphisms (SNPs) to generate a heterozygous F1 generation. The F1 flies will all be 50:50 hybrids of P1 and P2 genomes. The F1 females can then be backcrossed to one of the parental lines, say P2, to produce a BC1 generation. In Mendelian model, the genomic composition of this BC1 generation should be overall 25:75 between P1 and P2 alleles, which is the expected result when subjected to pooled sequencing for allelic frequencies of the SNPs. Therefore, for any given SNP, deviation from this ratio can potentially indicate that a meiotic driver is linked to it.

Although the proposed method has greatly increased the efficiency in detecting potential drivers, two major concerns still need to be addressed. First, an assumption for the frequency test to be accurate is that each individual fly produces the same amount of DNA, one that is not necessarily true in most cases. Larger individuals can produce more DNA simply because they have more cells. In addition, individuals carrying certain allele may have lower viability than others, which leads to an underrepresentation of these individuals (as well as their alleles) in the adult sample. Such non-driver effects can cause shifts in allelic frequencies in the population and therefore must be excluded.
Figure 1. Cross scheme for preparing sample population for detecting meiotic drivers using pooled sequencing (Wei et al. 2017). The parental lines P1 and P2 are homozygous with many known SNPs between the two genomes. The F1 generation will all inherit half of their chromosomes from each parent. Backcrossing the F1 with parental line P2 will generate BC1 embryos. For each SNP, half of the BC1 are expected to carry two P2 alleles, and half to carry one allele from each parental line. Meiotic drivers can shift this ratio.

The current project attempts to solve the problem through arresting all the BC1 embryos at a specific developmental stage. The arrested embryos can serve as an unbiased sample for the pooled sequencing test: since all the embryos stop their development at the same stage, each embryo is expected to contain the same number of cells and produce the same amount of DNA; in addition, the viability bias is reduced since regardless of whether each embryo would otherwise be able to develop into an adult, it is now included in the sample as a dead embryo. Therefore, both concerns mentioned above can be addressed.

Embryo arrest can be achieved by knocking down the expression of genes essential to embryonic development using RNA interference (Staller et al. 2013). This system uses a maternally expressed Gal4 protein to bind an upstream regulatory sequence (UAS), which then activates the expression of a short-hairpin RNA
(shRNA) sequence downstream of it. This shRNA has sequence complementation to an essential gene, and can therefore bind and deplete its mRNA (Figure 2). The design of this system allows two levels of regulation. First, the Gal4 gene and UAS-shRNA constructs can be maintained in two separate lines so that arresting does not happen until after the two lines are crossed; also, a specific promoter can be selected for Gal4 to control the time and location of shRNA expression.

Figure 2. Mechanism of Gal4>>UAS-shRNA system that uses RNAi to deplete the target mRNA (Staller et al. 2013). In the particular version used in this project, the mat-Gal4 protein (blue dots) are expressed in the maternal germline, which binds the UAS sequence and activates the transcription of the shRNA (red hairpin). The shRNA is then deposited into the oocyte and can be processed into single strands. When these products meet the mRNA of the target gene in the embryos (green; which are also maternally transcribed and deposited, and have sequence complementation to the shRNA), they deplete the mRNA through binding and cleavage.

The particular version of the RNAi system in the current project uses a maternal-alpha-tubulin-Gal4 protein that is expressed in the maternal germline. Because of this, the shRNA will be maternally expressed and deposited into the embryos. Three genes, trunk, dorsal and torso, have been selected as separate targets, to get a better chance that one of them will arrest the embryos at a high rate. Each gene has been reported as an essential gene for early embryonic development, which meets the purpose of the project. Staller et al. 2013 has also reported that 100% arrest rate
could be achieved with constructs targeting *dorsal* and *torso*, proving that they are
good candidates for the target. Moreover, each of the three genes are also
maternally transcribed and deposited just like the shRNA (Schupbach and
Wieschaus, 1986). The selected RNAi system is therefore able to knock down their
translation in the embryos.

Previous work in this lab used two different types of plasmid vectors to integrate
the UAS-shRNA construct into *D. melanogaster* X-chromosome: UASp and UASz,
both improved from a UASt vector constructed in 1993 that used *Hsp70*-derived
promoter (DeLuca and Spradling, 2018). The UASp vector enhanced the shRNA
eexpression in the female germline, whereas the UASz vector improved from UASp
such that it minimized the negative effect of the piRNA *Hsp70* on the expression of
the shRNA, by further trimming the 5’-UTR sequence that is homologous *Hsp70*. The
performances of these constructs were tested with a Gal4 gene on the same X-
chromosome. These tests indicated that the efficiency of the shRNA was highly
affected by the genetic background in which it was expressed. A high arresting rate
in one strain of *D. melanogaster* did not imply the same in other strains, yet high
efficiency in all genetic backgrounds would be required for it to serve the meiotic
drive detection method. Similarly, the integration site of the constructs may also
affect shRNA expression.

Further studies must be carried out to find a suitable construct that
accommodates all of these findings and challenges. Here, six different UAS-shRNA
constructs were subjected to efficiency tests though crosses with different Gal4 lines.
The efficiency of each construct was quantified as the percentage of arrested
embryos in the targeted generation. Besides the type of the vector backbone and the
gene being targeted, two other factors that could potentially affect the efficiencies
were also tested: the location of the Gal4 gene in the genome and the age of the
female fly expressing the shRNA. The results covered in the scope of this report
indicated that four of these constructs had high arresting efficiency given the genetic
environment they were in. Maternal age and the Gal4 location did affect the
efficiencies, but did not show a clear trend across different crosses. There were also
evidence indicating that the efficiencies of these constructs were highly sensitive to environmental or random variabilities. However, to see whether the integration sites of the UAS-shRNA constructs would affect their ability to arrest, the constructs were integrated into a different site than in previous work. Further studies are needed after the selected constructs are integrated into their desired loci.

MATERIALS AND METHODS

Sequences of shRNA and vector plasmid

The sequences of the shRNA genes targeting each of the three genes are included in Appendix A (From the thesis by Hyunho Lee '19). Each sequence targets the corresponding gene in an exon region to silence the gene, and has sticky ends (ctag and aatt at the beginning of the two strands) to help them ligate with Nhe1 and EcoR1 restriction sites in the vector backbones. Moreover, the sequences highlighted in capital letters are complementary to each other to help produce the hair-pin loop in the shRNA product.

The structure of the UASz plasmid vector is included below (Figure 3) (DeLuca and Spradling, 2018). The grey region in the figure is flanked by the restriction sites of Nhe1 and EcoR1 enzymes. When subjected to restriction digestion of both enzymes simultaneously, this region will be excised and the major part of the plasmid will be linearized with sticky ends. The shRNA genes can then be ligated into this restricted region to form a circular plasmid again, which will be integrated into the fly genome. Upstream of this grey region is the promoter for the shRNA expression (indicated in dark blue), and further up is the UAS sequence (pink) that activates the process when bound by the Gal4 protein. The attB site (light blue) is where the plasmid recombines with the desired attP site in the chromosome of the host fly to integrate the whole vector into the host genome. This process will be facilitated by a phiC31 integrase gene in the host (Groth et al. 2004). Finally, the vector also carries

1 The sequences are from https://fgr.hms.harvard.edu/fly-in-vivo-rnai
a Mini white marker (shown in brown), which gives a \( w^+ \) phenotype when the plasmid is successfully integrated into the white-eyed host. Details will be covered below.

**Figure 3. The UASz vector (DeLuca and Spradling. 2018).** This is one of the vectors used to carry the shRNA sequence. It contains Nhe1 and EcoR1 restriction sites where the shRNA sequence could be integrated. The upstream Hsp70 promoter associated with the UAS sequence has been improved from previous UASp vector such that it experiences less negative effect from the \( hsp70 \) piRNA. The vector also contains an attB site that helps integration into the attP site in the fly genome, and a mini white (\( w^+ \)) marker that facilitates selection for individuals with successful integration.

The structure of the pNP vector is as shown below (Figure 4) (Qiao et al. 2018). Similar to the UASz vector, it also has an attB site (light green) to facilitate the integration process, and a vermilion marker (shown in vermilion) to indicate successful integration by producing a \( v^+ \) phenotype in the \( v \) host background. It also contains a UAS sequence followed by the DSCP sequence as a promoter. The downstream MCS1 region (green) is too flanked by EcoRI and Nhel restriction sites, which can be excised and used to include the shRNA sequence. Notably different from the UASz vector is that, the pNP vector also contains a MCS2 region (green) which has similar function to the MCS1 region. The purpose of this region is to give this system the potential to include different shRNA sequences at the same time to
target multiple genes. This function has not been explored in the scope of this report, but can be potentially useful in the future.

![Diagram of the pNP vector](image)

**Figure 4. The pNP vector (Qiao et al. 2018).** This vector is similar to the UASz vector and has been used to carry the shRNA sequence. It is different from the UASz vector such that it has two regions that can be excised by restriction enzymes (green, MCS1 and MCS2), which gives it the ability to carry multiple shRNA sequences and express them at the same time. The vector also has different promoter and UAS sequences. It also has a vermillion marker (v+, shown in vermilion) that facilitates selection, instead of a w+ marker.

**Fly lines**

The majority of this study involved crosses between two groups of *D. melanogaster* lines. One group was homozygous with the Gal4 gene and the other homozygous with the UAS-shRNA construct (including a null negative control; subsequently referred to as the UAS lines).

Two homozygous Gal4 lines were used in this study. They both contain the same mat-alpha-tubulin-Gal4 gene that is expressed in the maternal germline. The Gal4 genes have been integrated on different chromosomes for the two lines. Line
“92m6” is a lab strain created by Dr. Sarah Lower and has the Gal4 gene on the X-chromosomes (hence referred to as Gal4-X below). The other Gal4 line (referred to as Gal4-III) is from Bloomington Drosophila Stock Center\(^3\), with Gal4 on the third chromosome. The purpose of including the two different lines was to test whether the genomic location of Gal4 would affect the efficiency of the arresting mechanism (by affecting its own expression and therefore the expression of the shRNA).

The UAS lines contained experimental lines and control lines. There were 6 different experimental lines, 3 carrying the UASz vector and 3 with the pNP vector. Among the lines with the same vector, each of the three lines carries the shRNA sequence targeting the trunk, dorsal, and torso genes respectively, as mentioned earlier (the lines will be referred to as “target gene abbreviation-plasmid name” below, e.g., tor-UASz, dl-pNP, etc.). Parental to the experimental lines are two lines that are otherwise wildtype: y w F10 has been used to construct the UASz lines and y v for the pNP lines. The y w F10 line is a highly inbred lab strain with yellow body and white eye phenotypes, in which the white eye phenotype could facilitate the selection for the mini white (w\(^+\)) marker on the UASz plasmid. The y v line works in a similar way such that it could help select for the v\(^+\) marker on the pNP plasmid. All of the plasmids had been integrated into the attP40 site on the second chromosome, which could be used to compare with previous work in the lab when the constructs were on the X-chromosome to see potential site-specific effects.

The three positive controls used in this experiment were all from Bloomington Stock Center. All three lines use a VALIUM20 vector to carry the shRNA sequence. Line dl+ctrlC3\(^4\) targeted dorsal, and the vector has been integrated into the attP2 site on chromosome 3; line tor+ctrlC3\(^5\) has torso-targeting shRNA integrated into the attP2 site; line tor+ctrlC2\(^6\) has a different torso-targeting shRNA integrated into the attP40 site on chromosome 2. The first two RNAi constructs had previously been

\(^2\) Genotype y w {RFP.attP}w\(\cdot\)Gal4; {vas-int GFP/RFP}

\(^3\) Stock number 7063. Genotype w\(^{[\cdot]}\); P[w\(+\cdot mC\)=matalpha4-GAL-VP16]V37

\(^4\) Bloomington stock number 32934. Genotype y\([1]\) sc\([\cdot]\) v\([1]\) sev\([21]\); P(y\([\cdot\cdot t7.7]\)

\(^5\) Bloomington stock number 33627. Genotype y\([1]\) v\([1]\); P[y\([\cdot\cdot t7.7]\) v\([\cdot\cdot t1.8]\)=TRiP.HMS00021]attP2

\(^6\) Bloomington stock number 58312. Genotype y\([1]\) v\([1]\); P[y\([\cdot\cdot t7.7]\) v\([\cdot\cdot t1.8]\)=TRiP.HMJ22419]attP40
reported to arrest the embryos at a high efficiency in a specific genetic background (Staller et al. 2013). However, due to the difference in the genetic backgrounds of these lines as well as the Gal4 lines, the positive controls were not guaranteed to give the same good performance as reported. In addition to the positive controls, y w F10 mentioned above with no RNAi construct in the genome was used as a negative control in this study.

Homozygosity was required for both the Gal4 and UAS lines. The positive controls and Gal4 lines were available in homozygous, but the UAS lines must be processed after injecting the vectors into the host embryos. Previous work had been done in the lab to select UASz and pNP individuals expressing the positive markers on the plasmid (w+ and v+, respectively), which indicated successful integration of the corresponding vector. The phiC31 gene in their genomes had been subsequently removed through crossing, so that the integrations of the plasmids were irreversible. The current project started with crossing the resulting progenies and selecting for the homozygous w+/w+ and v+/v+ phenotypes respectively, which would indicate that they were homozygous for the UAS-shRNA constructs. The w+/w+ phenotype was very different from the heterozygous w+/w phenotype, so the individuals could directly be selected to establish the lines. However, the v+/v+ and v+/v individuals were not visually differentiable. Therefore, multiple single-pair crosses were performed between males and virgin females expressing the v+ phenotype (which might be v+/v+ or v+/v). The F1 progenies produced from each cross were again crossed to produce an F2 generation. If one of the parents was heterozygous, the F1 generation should have many heterozygous individuals, resulting in phenotypically vermillion flies in the F2 generation. Therefore, lines that did not show vermillion phenotype in any of the F2 progenies were kept for this experiment. Each line was kept for a few more generations to make sure there was no vermillion individual appearing in the population. Before starting the experiment, PCR test across part of the inserted sequence was performed on 5 randomly chosen individuals in each line to confirm their homozygosity, which all showed positive results.
Crossing scheme for the efficiency test

To examine the efficiency of each Gal4>>UAS-shRNA system, I performed different variations of the following crosses in each experiment. The parental crosses were between one UAS line and one Gal4 line. Virgin females were collected for whichever line chosen to be the female in a specific experiment, but the system was expected to work either way. The virgin females were kept in vials for ~3 days to age, and the vials were incubated for another 5 days and examined for any existing larvae. If larvae appeared in a vial, all females that had been kept in that vial would be discarded since some of them were not virgin. The F1 females produced by crossing the males and virgin females carried one Gal4 gene and one UAS-shRNA construct (except for the negative control). Since the Gal4 gene were only expressed in the female germline and so would the shRNA, the male genotypes would not contribute to arresting. Moreover, no matter what males these F1 females were crossed with, the F2 embryos were expected to be arrested since the maternally deposited shRNA would in theory knock down the targeted essential gene. Therefore, for convenience, brother-sister crosses (did not require virgins) were performed in the F1 generation to produce the F2 embryos. The F2 generation were then examined for the percentage of arrested embryo to quantify the efficiency of each construct under the specific experimental condition. The detailed experimental process was different in each round, as described below.

Preliminary examination with the UASz lines

The UASz lines were created earlier than the pNP lines. The UASz lines were therefore tested for efficiency before the pNP lines were ready. The test included the 3 UASz lines, all 3 positive controls and the y w F10 negative control for the UAS lines, and crossed them reciprocally with the Gal4-III line. Since Stellar et al. 2013 reported 100% efficiencies for two of the positive controls, the original plan was to record a 0/1 score for whether each cross had successfully arrested all F2 embryos. However, almost all crosses produced viable F2 larvae. Therefore, each cross was instead estimated for the number of viable larvae in the F2 generation by the amount
of trace they left behind. This experiment was therefore considered preliminary. The detailed procedure was as following.

For each UAS line, 3 replica crosses were set up between 5 virgin UAS females and 5 Gal4-III males. Due to the limited number of Gal4-III virgin females collected, only one replica was set up for the reciprocal cross for each UAS line, also with 5 males and 5 females. Multiple F1 crosses were performed in vials with 5 males and 5 females for each parental replica (for each parental replica aimed for 3 but no more than that; some parental replica did not produce the required number of flies). The amount of trace left behind by the F2 larvae that survived the arresting mechanism was estimated for each F1 replica to represent their number. The result was qualitatively informative and helped designing the details of the next two experiments.

**Quantifying the efficiency for all UASz and pNP lines**

The preliminary experiment showed that most of the UASz lines were not able to completely knock down their target genes. Therefore, I performed further studies to quantify the efficiencies of the constructs. Moreover, all lines mentioned in the Fly lines section were included in the subsequent experiments to offer comparison between different Gal4 loci and different vector backbones (except for tor+ctrlC3, which had unexpectedly low rate of arrest in the preliminary experiment). Factors such as the ages of the F1 females and the environmental temperature were also better controlled. Finally, the embryos produced by the F1 cross were collected and scored to quantify the efficiency of each construct. The resulting data was analyzed for affecting conditions including the location of the Gal4 gene, the nature of the vector + shRNA combination, as well as the maternal ages.

In this experiment, the crosses were performed as mentioned in the Crossing scheme section, using all 9 UAS lines (3 UASz, 3 pNP, 2 positive control, 1 negative control) and 2 Gal4 lines. All the flies including the progenies and embryos were incubated in a 25 °C incubator. All of the crosses used the UAS lines as the female parent and Gal4 as the male parent. This was because the preliminary test did not
demonstrate much difference between the reciprocal crosses, and also to collect virgin Gal4 females for all their crosses was less practical. Each parental cross contained 3 replicas, and at least 10 males and 10 females (and at most 15 x 15) were used for each replica, to produce enough F1 flies for egg collection. Because of the increased number of individuals in each cross, the vials were flipped every 2 days to reduce density of eggs laid.

To set up the collections, the F1 progenies were transferred into egg-collection bottles on the 4th day of their first eclosion. This would keep the age of the F1 flies below 4 days. Ideally, about 50 males and 50 females from each parental replica would be used for every collection to get a significant number of embryos. However, since some parental replicas did not produce enough progenies, some crosses used F1 flies from one parental replica (that produced more than 100 males and 100 females) for multiple collections, and some collections used all the F1 flies from a parental replica even though there were not enough flies. Each collection bottle was capped with a grape juice gel plate covered with a thin layer of yeast paste and set upside down so that the flies could be attracted to the plate and lay eggs on it. These plates were labeled plates 1.

The first round of the collection started at time 0 and went on for about 30 hours. The ambiguity in the collection time was a result of precisely counting the number of F1 flies used in each bottle, which took hours to set all collections up and the average collection time was used instead. At time 30h, the plates 1 were replaced with a new set of plates (labeled plates 2). The plates 1 were capped and kept in the incubator. The collections with plates 2 went on for 24h. At time 54h, the flies from each bottle were transferred into vials containing fly food. The plates 2 were kept in the incubator with the plates 1. Since previous work in the lab had reported late arresting phenotypes (F2 progenies die at larval stage instead of embryo stages as expected; Hyunho Lee’s thesis), the plates were scored for number of flat and plump eggs (eggs that hatched and did not hatch, respectively), as well as live and dead larvae. The plates were kept for five days to allow larvae to be potentially hatched and die.
The scoring of plates 1 and 2 took place on days 7 and 8 since time 0, respectively. The embryos and larvae were directly scored on the plates under the microscope. However, it turned out that the embryos started to degrade, and were disturbed by large amount of larval activity. These factors caused great uncertainty in the embryo scoring and they were eventually estimated on a single quadrant of the plate. Considering that the first two sets of embryos were collected at roughly the same time, only the less degraded plate of the two was scored for each replica, to save time and prevent further degradation. In addition, contradicting to the previous findings, there was not a large number of dead larvae observed in any of the plates. As a result, plates 3 collected on day 7 were only incubated for 2 days after collection was finished, and number of dead larvae were not scored.

On day 7, the flies were transferred back to a collection bottle, and the third collection was set up with plates 3. The collection went on for 36 hours to compensate for the loss of F1 flies during the 7 days. After that, the plates 3 were incubated and scored as indicated above.

As a summary to this stage of experiment, factors such as incubation time could greatly affect the quality of the data collected. Moreover, the number of embryos collected in 24 hours for each replica was more than enough to achieve the desired precision if the embryos were preserved properly. The durations of collections also needed to be better controlled, and the dates on which collections take place needed to be evenly spaced out to better reflect the maternal age effect. Finally, the flies should be kept in a larger chamber (for example, a bottle instead of a vial) between collections to prevent overcrowding. As a result, the same experiment was repeated with following changes:

First, based on the result from the first round, some UAS lines with extremely low efficiencies were excluded. Only 6 UAS lines were crossed with the two Gal4 lines: trk-pNP, dl-pNP, dl-UASz, tor-UASz, dl+ctrlC3 and y w F10 (negative control). The relatively less efficient dl-pNP line was also included so that there was a line using each of the three vectors targeting dorsal. Line tor+ctrlC2 was excluded for its less promising performance.
Second, the collection schedule was quite different. To gain a better control of experimental time, the number of F1 flies used for each collection was not counted explicitly, but all of the available F1 flies from each parental replica (without mixing within the cross) were used to set up individual collections. Before each collection, the flies were acclimatized in their collection bottles capped with a grape juice gel plate with a thick layer of yeast on it (to feed the flies so that they could produce eggs at high frequency), for 24 hours. After acclimatization, the grape plates were discarded, and the bottles were capped with new plates containing a thin layer of yeast for embryo collection. The duration of each collection was reduced to 4 hours so that the number of embryos on the plate would still generate statistically significant result while being easier to score. A total of three collections were carried out. The acclimatization for each round of collection took place on day 1 (time 0), 4 and 7 so that the maternal ages were better interspaced. The flies were kept in bottles with food after each collection, so that they did not get overcrowded.

Finally, the precision of scoring was improved. Each collection plate was incubated for 2 days, which gave all the embryos plenty of time to potentially hatch. After the incubation, each plate was precisely counted for the total number of eggs and number of flat eggs, as well as number of live larvae. The number of dead larvae was not scored.

**RESULTS**

*Preliminary test*

For the preliminary test (UASz x Gal4-III), the efficiency of each construct in arresting the F2 embryos was estimated using the amount of trace left in the food by the larvae that escaped the arresting. The vials containing F2 flies were assigned scores 0, 1, 2 or 3. The “0” vials contained absolutely no larvae, and the number increased with the amount of larval trace observed in each vial. The mean score was calculated for each cross to represent the overall efficiency of the construct. Figure 5 demonstrates the results for crosses using the UAS lines as parental females. The
results for the reciprocal were not shown since there were not enough replications for them, and they generally showed similar trend as those with UAS females (as expected). Crosses with tor-UASz and dl-UASz had similarly low mean scores (about 1.00) as two positive control lines tor+ctrlC2 and dl+ctrlC3 (shown in blue). However, tor-UASz and tor+ctrlC3 (unexpectedly) exhibited high mean score (close to 3.00) like the negative control did (orange), which indicated that they probably had low efficiencies in arresting. While the two positive controls targeting torso might seem to be suggesting that the genetic environment of the constructs were affecting the RNAi efficiency, they did use different shRNA sequences to target the same gene, so the conclusion could not be drawn. The results were also an indication that most
constructs would not arrest the embryos at 100% rate, making a quantitative examination of the efficiencies necessary.

**Quantitative analysis (first round)**

Several crosses produced large number of live larvae (>15%, see Appendix B for example) that was comparable to the negative controls. These crosses (including the negative control) were not scored due to their low efficiencies (which were considered as failed regarding the purpose of the project) and great amount of disturbance of the embryos by larval activity, and were labeled N/A in the table.

The crosses not scored included *dl-pNP* and *tor-pNP* crosses with Gal4-X, as well as *tor-ctrlC2* and *trk-UASz* crosses with Gal4-III. In addition, crosses *tor-ctrlC2* and *trk-UASz* with Gal4-X, and *dl-UASz* with Gal4A were scored but excluded from the analysis. The latter crosses demonstrated great inconsistencies among different replicas such that some replicas gave large number of larvae whereas others did not. Comparing the crosses with the same UAS-shRNA constructs but different Gal4 loci, these observations also provided evidence supporting the hypothesis that Gal4 locus would affect the arresting efficiency of the construct. However, they did not show a general trend as which Gal4 locus would give higher efficiency. This effect was therefore considered specific to individual constructs.

The rest of the crosses were scored as mentioned in the *Materials and Methods* section. Unfortunately, the numbers of flat eggs were initially greatly overestimated because of being unfamiliar with their appearance, and therefore the numbers were not used. The large number of dead larvae suggested by previous work was also not observed. As a result, the percentage arrest for each plate was calculated by one minus the percentage of live larvae counted in the total estimated number of embryos.

Scatter plots for the percentage of arresting was created for these crosses (Figure 6). Variation in sample numbers (number of data points) in different collections of the same cross was either because certain collections did not produce
Figure 6. Each data point represents the percentage of arrested embryos on each individual plate. This was calculated by one minus the percentage of counted larvae in estimated total embryos. In both panels, each column represents a genotype of the female parent, whereas the genotype of the male parent is represented by the shape of data points (circle for Gal4-III and triangle for Gal4-X). Both panels include the dl+ctrlC3 crosses as a reference. The color of the data points represents the collection that specific plate came from, which was directly correlated with the age of the F1 mother. The size of data points represents the sample size of each plate. (A) The percentage of arrested embryos for crosses with pNP females. (B) The percentage of arrested embryos for crosses with UASz females.

Any embryos or they were greatly disturbed and could not be scored (e.g., dl-pNP x Gal4-III did not have plates3). All of the samples included here had more than 90% arrest rate. These UAS lines were therefore considered as candidates for further experiments. In addition, for most crosses, the collections with older F1 females (i.e., plates 3) seemed to have higher rate of arrest. However, the difference between plates was small and the statistical significance should be tested with data of higher
precision. Moreover, there were exceptions to this observation (e.g., tor-UASz x Gal4-X), and therefore the maternal age effect could also be cross-specific.

**Quantitative analysis (second round)**

Similar scatter plots were created for the data from the second round of quantitative analysis (Figure 7). There were a number of differences from the first round of experiment. First, the sample size in each plate was generally smaller in this experiment due to shorter collection time. Second, all plates were counted precisely for the number of total embryos, flat eggs (hatched), and larvae. Since the numbers of flat eggs and larvae were mostly consistent, the percentage arrest was calculated in the same way as in round 1. And finally, since every plate gave high arrest rate in this experiment (except for the negative control as expected), all of them were scored and included in the analysis.

As shown in Figure 7, every sample had >85% arrest rate, and most of them were >90%. This was because most of the UAS lines selected for this experiment gave high arrest rate in the first round. One exception was the cross dl-UASz x Gal4-III, which did not have promising result in the first round but had 100% arrest rate in all samples in this round. It was therefore another example of inconsistencies between replicas of the same cross.

Two additional scatter plots could be found in Appendix C. The first plot aggregated all the replica for each collection of every cross. The resulting data was subject to a t-test to compare whether there was statistical difference between different collections of each cross, hence finding out whether the age of the F1 female was affecting the rate of arrest. For each individual collection, every single embryo was assigned to 0 or 1 based on if they were hatched or arrested, respectively. Two-tailed t-tests were then carried out for each cross between plates 1 and 2 (for p value p1), and plates 2 and 3 (for p2), with a significance level of 0.05. In cross trk-pNP x Gal4-III, there was statistically significant evidence supporting that the arrest rate increased as the F1 female age increased (p1 = 0.0174, p2 = 2.85 x 10^{-5}). The same pattern was shown in cross dl+ctrlC3 x Gal4-III, with p1 = 0.0451.
and $p_2 = 0.0213$. In cross $dl$-pNP x Gal4-X, there was a decrease in arrest rate from plates 2 to plates 3 ($p_2 = 0.0452$). In tor-UASz x Gal4-III, there was a decrease in arrest rate in plates 2, whereas the other two plates both showed 100% arrest rate ($p_1 = p_2 = 0.00431$). The other crosses all showed constant arrest rates across the three collections.

**Figure 7.** The percentage of arrested embryos in the second round of experiment, calculated and presented in the same way as in Figure 6. For both panels, each column represents a female parental genotype, with results from crosses with the two different male genotypes separated on the two sides of the column and differentiated with the shape of the data points. The results for $dl$+ctrlC3 are again in both panels as a reference. The size of each data point represents its sample size and the color represents the collection or F1 female age (plates 1 was collected on day 2, plates 2 on day 5 and plates 3 on day 8). (A) The percentage arrest with pNP females. (B) The percentage arrest with UASz females.

The second plot gave the aggregated arrest rate for each cross by summing up all the collections containing all replicas for the cross. This provided comparison...
between crosses with the same female parent but different male Gal4 parents. Two-tailed t-test was also performed for each female genotype, and statistically significant differences were found between the two Gal4 loci in crosses with trk-pNP, dl+ctrlC3 and tor-UASz (p = 1.09 x 10^{-9}, 3.70 x 10^{-5} and 4.53 x 10^{-3}, respectively). It was also worth noting that all crosses in this round of experiment had overall arrest rate >97.3% (see Appendix D for raw data).

**Discussion**

The most fundamental finding of this project was that it tested and confirmed the high efficiency in arresting F2 embryos through RNAi, when the selected UAS lines were crossed with the two Gal4 lines to assemble the Gal4>>UAS-shRNA constructs in the F1 females. These UAS lines included trk-pNP, dl-pNP, dl-UASz, and tor-UASz, when the construct was integrated into the attP40 site on the second chromosome, as well as the positive control dl+ctrlC3 with the construct in the attP2 site on the third chromosome. The two Gal4 lines could either have the Gal4 gene on the third chromosome or on the X chromosome, and both types of crosses produced high yet different arrest rates. These findings would serve as the basis for future studies in constructing the embryonic arresting mechanism, when the constructs would need to be integrated into different sites.

The results also supported a number of additional findings. Firstly, the genomic location of the Gal4 gene did affect the efficiency of the RNAi mechanism. Some UAS constructs had drastically different outcomes when the Gal4 gene was at different loci. For example, dl-UASz had consistently high arrest rate when crossed with Gal4-X, but one replica in crosses with Gal4-III produced large number of larvae in the first round of quantitative experiment. Even in the second round of the experiment, in which all the crosses produced a high rate of arrest, t-tests showed that statistically significant differences existed between crosses with the same female genotype and different male Gal4 genotypes. These differences could be due to the effect of cis-regulating elements around the locus of Gal4. Despite the strong
evidence supporting existence of the Gal4 locus effect, however, there was no clear trend as which Gal4 locus would lead to higher arresting rate.

Secondly, as shown above in Figure 6 for the first round of experiment, maternal age seemed to be affecting the arresting efficiency, such that embryos produced by older F1 females had higher arresting rate. The t-tests carried out for the data from the second round confirmed that hypothesis at 5% level of significance for trk-pNP x Gal4-III and dl+ctrlC3 x Gal4-III. However, the same tests also showed crosses with different correlations between F1 female age and the F2 arrest rate (dl-pNP x Gal4-X; tor-UASz x Gal4-III). More importantly, most of the crosses did not show statistically significant change in arrest rate between samples collected at different time (with different maternal ages). In addition, since the total sample sizes of both crosses involving tor-UASz was small (347 with Gal4-III, 130 with Gal4-X), further studies would always be helpful to obtaining stronger evidence.

In both cases above, there were variations in the results generated by each cross with respect to both Gal4 locus and F1 female age effects. However, there was no general consistent trend in either case as which Gal4 locus or what maternal age would produce embryos with high arrest rate. These were indications that the Gal4>>UAS-shRNA system was sensitive to various factors including the genetic environment of the construct and the age of the female expressing it. However, further studies were needed to find out the factor that was deciding the arrest rate.

Lastly, there were great differences between replicas in certain crosses. Examples included results of cross dl-UASz x Gal4-III in the two rounds of experiment, as well as the crosses that showed inconsistent results in the first round as mentioned in the Results part. There were two possible explanations to this observation. The first idea was that this could be due to random genetic polymorphisms between individual parents from the same line, which gave the Gal4 gene or the UAS-shRNA construct variable genetic background and affected their efficiencies. If this hypothesis were true, it would largely affect the reliability of statistical tests for the Gal4 locus effect and maternal age effect, as different replicas were aggregated for the purpose of the test. This problem could be addressed by
further inbreeding each parental line. An alternative idea was that, the Gal4>>UAS-shRNA system could be sensitive to environmental changes such as the fly food or temperature, and the inconsistencies between replica could be random. In this case, the statistical tests on aggregated data would be more reliable as it would take random environmental factors into account.

Several factors could be improved from the current experimental design to get a more accurate and reliable quantitative result on arrest rate. First, parental populations could be more inbred as an attempt to obtain consistent data across different replica for each cross. Second, since the age of the F1 female could have various effects on the arrest rate, sampling from evenly spaced time after the embryonic collection start is necessary, as performed in the second round of the experiment. Third, different crosses would produce different number of embryos at different times. To have a sufficiently large (but not too large to score) sample size, try 8 hours of collection time. And fourth, incubation time for around 2 days would be sufficient to reveal whether the embryos were arrested, and therefore should keep it this way to prevent degradation of the embryos. Last, embryos and larvae were scored after incubation in this study. Therefore, for those plates that produced large number of live larvae (such as the negative control), it could be hard to score the number of embryos due to larval disturbance, especially the negative control. A possible way to better quantify these groups is to score the embryos before incubation. However, since the scoring process would take a long period of time outside the incubator, and it would also cause a lot of disturbance on the embryos themselves, how these would affect the arrest rate should be examined before applying this method.

Future directions of this project can repeat similar efficiency tests on these candidate constructs, but with them integrated on the X-chromosome of male D. melanogaster. The purpose of this is to construct a maintainable line of flies that contain both the Gal4 gene and the UAS-shRNA construct in the same fly, so that these individuals can be directly crossed with other population and achieve arrest in the following generations. For example, in the cross demonstrated in Figure 1, the P1
females can be the test population with potential meiotic drivers, and the P2 males are carrying the Gal4>>UAS-shRNA system.

**Figure 1(Copy).** (Wei et al. 2017). Using a true breeding (except X-chromosome) line with Gal4>>UAS-shRNA on male X-chromosomes for P2 will introduce the system into F1 females, causing arrest in the F2 generation.

The P2 population can be created by integrating both Gal4 and UAS-shRNA onto the male *D. melanogaster* X chromosomes, while crossing them with XXY females carrying attached (physically connected, so always segregate together) X chromosomes. In this cross, the maternally expressed Gal4>>UAS-shRNA constructs are always passed on through male X chromosomes but never in the females. Therefore, they will not be expressed and the population will not be arrested. This system has been successful in previous work with other UAS constructs (Hyunho Lee’s thesis). A detailed explanation of the mechanism can be found in Appendix E. Repeated tests are necessary when integrating the constructs into different loci, due to unpredictable effects of genetic environment on the efficiency of each construct.

Finally, there are several possible approaches to improve the performance of the RNAi constructs. One would be designing RNAi constructs to target different
essential genes, which is always an option when the current constructs are not performing well in the desired genetic environment. Another approach is to utilize the ability of the pNP vector to carry multiple shRNA sequences and therefore targeting multiple genes simultaneously (Qiao et al. 2018). These different shRNA sequences could be made to target different genes essential for the same embryonic developmental stage, with the expectation that knocking down multiple essential genes at the same time would give a stronger arrest rate. The developmental stage in which the embryos are arrested could also be controlled in this way, as the targets would be functioning at the same stage of development.

ACKNOWLEDGEMENTS

I would like to thank Dr. Daniel A. Barbash, Shuqing Ji and all other lab members for providing generous support and guidance that helped me through my research process. I would also like to thank Dr. Dean M. Castillo for training me with various lab techniques during my early research experience.

In this unusual year of the COVID-19 pandemic, I would also like to thank everyone I know and do not know who have stood with us. Whether you are a medical worker battling at the front line, or an ordinary person like me working from home and complying with the regulations, I appreciate your effort to help contain the virus. Every single person and every bit of contribution matters.
Appendix

Appendix A. The shRNA sequence used for targeting each essential gene.

Dorsal
Top Strand:
tagcagTTGCTGCGATTGCTTTCAATaggtatatcagccataTTGAAAGCACAATCGCAACGAGcgc
Bottom Strand:
attgctTTGCTGCGATTGCTTTCAATaggtatatcagccataTTGAAAGCACAATCGCAACGAGctg

Torso
Top strand:
tagcagCTGCAACCGAGCAAAATTATaggtatatcagccataTTAATTTGGACTCGGTGCAGGcgc
Bottom strand:
attgctCTGCAACCGAGCAAAATTATaggtatatcagccataTTAATTTGGACTCGGTGCAGGctg

Trunk
Top strand:
tagcagTTGGAAGAAATAGCGGTAAAtagttatatcagccataTTAACCCTATTCTCTCCCCCCAGcgc
Bottom strand:
attgctTTGGAAGAAATAGCGGTAAAtagttatatcagccataTTAACCCTATTCTCTCCCCCCAGctg

Figure A.1. The shRNA sequence targeting each gene (Hyunho Lee’s thesis).
These three sequences each have sequence complementary to their target gene (capitalized), and the two capitalized regions for each sequence are complementary to each other to form the hairpin structure. They also have sequences on the two ends to facilitate their integration into the vector plasmid.
Appendix B. Example of plates that produced large number of larvae.
Appendix C. Aggregated scatter plots of data from quantitative analysis 2

Figure C.1. Rate of arrest from quantitative analysis 2 aggregated with replicas for each collection in every cross. This offers an alternative view to figure 7, in which all 3 replicas (or fewer, depending on how many actually produced results) for each collection are combined for arrest rate calculation, which represents the arrest rate for each cross with F1 females at each age. The notations are the same as in figure 7.
Appendix C2. Aggregated scatter plots from quantitative analysis 2

Figure C.2. Rate of arrest in quantitative analysis 2 with all data from each cross aggregated. This offers yet another view of figure 7. In this figure, all the data from each cross (including different collections, or plates) are aggregated to calculate one single arrest rate for that cross. This offers a clear comparison between crosses with the same female genotype but different Gal4 males. The notations are the same except that the color of the data points does not contain information.
Appendix D. Raw data for aggregated arrest rate for each cross in experiment 2

<table>
<thead>
<tr>
<th>Male Gal4</th>
<th>Female UAS</th>
<th>Total Egg</th>
<th>Larvae</th>
<th>Percent Arrested</th>
<th>Arrested</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gal4-III</td>
<td>d1:pNP</td>
<td>953</td>
<td>5</td>
<td>0.99376</td>
<td>947</td>
</tr>
<tr>
<td>Gal4-X</td>
<td>d1:pNP</td>
<td>545</td>
<td>7</td>
<td>0.98756</td>
<td>538</td>
</tr>
<tr>
<td>Gal4-III</td>
<td>d1:UASz</td>
<td>1716</td>
<td>0</td>
<td>1.0000000</td>
<td>1716</td>
</tr>
<tr>
<td>Gal4-X</td>
<td>d1:UASz</td>
<td>779</td>
<td>0</td>
<td>1.0000000</td>
<td>779</td>
</tr>
<tr>
<td>Gal4-X</td>
<td>d1:ctrlC3</td>
<td>6124</td>
<td>17</td>
<td>0.99722</td>
<td>6107</td>
</tr>
<tr>
<td>Gal4-X</td>
<td>d1:ctrlC3</td>
<td>3539</td>
<td>0</td>
<td>1.0000000</td>
<td>3539</td>
</tr>
<tr>
<td>Gal4-III</td>
<td>tor:UASz</td>
<td>347</td>
<td>8</td>
<td>0.97694</td>
<td>339</td>
</tr>
<tr>
<td>Gal4-X</td>
<td>tor:UASz</td>
<td>130</td>
<td>0</td>
<td>1.0000000</td>
<td>130</td>
</tr>
<tr>
<td>Gal4-III</td>
<td>trk:pNP</td>
<td>1563</td>
<td>41</td>
<td>0.99743</td>
<td>1522</td>
</tr>
<tr>
<td>Gal4-X</td>
<td>trk:pNP</td>
<td>4115</td>
<td>5</td>
<td>0.99976</td>
<td>4110</td>
</tr>
</tbody>
</table>

Figure D.1. Aggregated arrest rate for every cross in quantitative analysis round 2. Note that all crosses have arrested at rate greater than 97.3%. The TotalEgg column also shows the sample size for each cross, in which both crosses with tor-UASz had relatively small sizes.

Appendix E. The cross scheme for maintaining Gal4>>UAS-shRNA lines

<table>
<thead>
<tr>
<th>Female</th>
<th>Male</th>
<th>X_{sh}</th>
<th>Y</th>
</tr>
</thead>
<tbody>
<tr>
<td>XX</td>
<td>X_{sh}</td>
<td>XXX_{sh}</td>
<td>XXY</td>
</tr>
<tr>
<td>Y</td>
<td>X_{sh}Y</td>
<td>YY</td>
<td></td>
</tr>
</tbody>
</table>

Table E.1. The Punnett square demonstrating a cross between males containing both the Gal4 gene and the UAS-shRNA construct and XXY females with attached X chromosomes (Hyunho Lee’s thesis). Since the female X chromosomes are physically attached, they always segregate together in meiosis. They can form four types of embryos with males carrying both the Gal4 gene and the UAS-shRNA construct on their X chromosome (denoted as X_{sh}). The XXX_{sh} and YY embryos are not viable. The X_{sh}Y embryos produce males that again carry both genes on the X chromosome, and the XXY embryos produce females with attached X chromosomes. Therefore, the phenotypes of the progenies stay the same as their parents. Moreover, since the Gal4>>UAS-shRNA system is always passed on in the males but not females, the RNAi will not be expressed (the Gal4 gene used is only expressed in female germline).
REFERENCES


