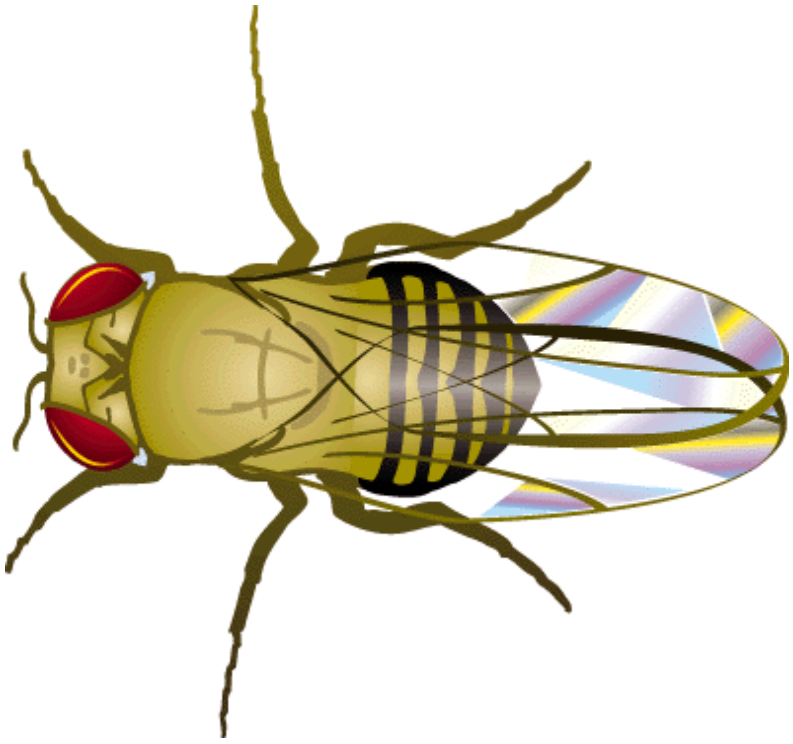
Developing *Drosophila melanogaster* as a model for aminoglycoside antibiotic toxicity



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Abstract

Pseudomonas aeruginosa bacterial infections are a common problem in immune-deficient patients, such as burn victims, cancer patients, and cystic fibrosis patients. Treating these infections generally involves using an aminoglycosidic antibiotic, such as neomycin, which functions by blocking bacterial protein synthesis. Unfortunately, the range between effective treatment and toxicity is narrow. Patients treated long-term for *P. aeruginosa* infections often experience renal failure and deafness. The goal of this project is to develop the fruit fly, *Drosophila melanogaster*, as a model to understand the effects of G418 treatment, an aminoglycoside, and to identify genes whose altered expression can help the fruit fly cope with the side effects of this type of drug. In order to develop this model, I first determined the ability of the flies to cope with the drug by establishing a dose-response curve of G418 concentration to survivorship. I then used two methods to test which tissues are most affected by the drug: a labeling method that showed that G418 concentrates in the gut and a tissue-specific expression system that let me determine which tissues are affected by high doses of neomycin treatment. Finally, I performed a genetic screen using RNA interference to reduce expression of candidate genes that might contribute to aminoglycoside toxicity. Knowledge gained from this type of study may help ameliorate the side effects of aminoglycosides or facilitate development of a drug that targets *P. aeruginosa* infections with the same specificity but without the long-term side effects.

Project Definition

Pseudomonas aeruginosa is a bacterium that infects mainly immune-compromised patients, such as cystic fibrosis, tuberculosis, cancer, and burn patients. Doctors usually prescribe aminoglycoside antibiotics to treat infections caused by this and other gram-negative, aerobic bacteria because of their efficacy and low cost. Unfortunately, aminoglycoside toxicity irreversibly affects up to 25% of the millions of patients treated annually with this drug (Moore et al. 1984). The goal of this project is to develop *Drosophila melanogaster*, the fruit fly, as a model for this toxicity. Three lines of evidence suggest that the fruit fly could serve as a good model for understanding aminoglycoside toxicity. First, there is a genetic basis to aminoglycoside toxicity, as evidenced by the fact that patas and macaque monkeys, which are in the same subfamily, are highly resistant and sensitive to aminoglycoside toxicity, respectively (Hawkins et al. 1977). Second, fruit flies have homologous tissues to those most affected in humans--a Malpighian tubule and vibration-sensing bristles homologous in function to the kidney and ear hair cells. In recent studies, researchers have shown that Malpighian tubules arise from ectodermal epithelial buds and surrounding mesenchymal mesoderm, which is more similar to vertebrate nephrogenesis than previously thought (Denholm et al. 2003). Finally, other researchers have already designed a tool that will be helpful in this project. They isolated a neomycin resistance gene, *neo*, from bacteria and put it into the fruit fly genome such that it expresses universally and confers resistance to aminoglycosides (Steller & Pirrotta 1985).

Drosophila melanogaster is one of the most well-developed model organisms for genetic studies. The fruit fly is also sensitive to high doses of aminoglycosides, indicating that it may be useful for understanding the genetic basis of toxicity (Steller & Pirrotta 1985). Developing a new genetic model for aminoglycoside toxicity brings about several questions. Is this the most

appropriate model organism for understanding drug toxicity? How applicable will these results be for a human understanding of the problem? Is this a cost effective model, or can another give the same results for a lower price? While researchers have already used other model organisms to understand some aspects of toxicity, few genes have been identified that increase tolerance to aminoglycoside toxicity, largely due to an inability to address one of the previous questions. For example, while researchers have made large advances in understanding hair cell toxicity using zebrafish, identifying genetic modifiers of toxicity is hindered by the fact that currently more than 20% of the genome has not been sequenced, according to the *Danio rerio* Sequencing Project. In contrast, the *Drosophila melanogaster* genome was sequenced nearly a decade ago (Adams et al. 2000), and has since been used to identify genes that underlie human diseases such as autism spectrum disorder, epilepsy, and schitzophrenia (Zweier et al. 2009).

A final, harder to predict question after choosing *Drosophila* as my model organism is: What kinds of results will tell us the most information about aminoglycoside toxicity? I will address this question during this project by addressing the following issues:

- Over what range of drug concentration does G418 treatment have an effect on but not kill flies? I established a dose-response curve of G418 concentration to survivorship.
- Do wild-type flies treated with high doses of G418 experience damage similar to that in humans? I tagged G418 with a fluorescent molecule and showed visually that it concentrates in the gut. I also used a tissue-specific expression system to determine which tissues require the neo^R gene and are therefore sensitive to aminoglycosides.
- What tissues or genes aid neomycin toxicity, resulting in fly death? I performed a screen of candidate genes to determine which genes allow for increased survivorship when their expression is reduced.

Medical Significance

Pseudomonas aeruginosa is a pathogenic bacterium found abundantly in nature in soil, marshes, coastal marine habitats, and in both plant and animal tissues. Most people are unaffected by its virulence because the human immune system is strong enough to ward off these bacterial infections. However, individuals with compromised immune systems such as people with cystic fibrosis, tuberculosis, burn victims, cancer patients, and long-term intensive-care patients, are highly vulnerable to infections by these bacteria (Driscoll et al. 2007).

Aminoglycosides, such as tobramycin and neomycin, are the most common antibiotics used worldwide, mainly due to their high efficacy and low cost (Forge & Schacht 2000). They are used to treat patients infected with *P. aeruginosa* and other aerobic gram-negative bacteria (Fischel-Ghodsian 2005). In the United States during 1986, approximately 4 million people were treated with aminoglycosides (Price 1986). This class of antibiotics binds to the 30S ribosomal subunit, which blocks initiation of protein synthesis, causes misreading of the mRNA, or facilitates premature termination of ongoing translation of mRNA template (Davies & Smith 1978). A major problem with this class of antibiotics is that small changes in drug concentration lead to decreased effectiveness or increased side effects. The maximal recommended serum concentration for serious infections is 6-8 mg/L with a minimal inhibitory concentration (MIC) of 0.5-1 mg/L in the serum (McCormack & Jewesson 1992). When serum levels are within this range, studies have shown that 88% of patients with urinary tract infections are cured, while only 35% are cured when serum levels lower than the MIC (Noone et al. 1974). Clearly, patients benefit from closely monitored serum levels. Furthermore, prolonged use frequently causes kidney failure and hearing loss. Ten percent of patients develop hearing loss in a 3 month daily dosing period (Singer et al. 1996).

While researchers speculate that nephrotoxicity induced by aminoglycoside toxicity is probably always reversible under rational doses (Lietman 1977), ototoxicity and vestibular toxicity is usually irreversible. More than 20% of hospitalized patients with bacterial infections develop hearing loss within 9 days of being administered 2 mg of gentamicin or tobramycin per kg body weight every 8 hours (Moore et al. 1984). Although nephrotoxicity is reversible, most patients taking these antibiotics are extremely vulnerable to infection. Cystic fibrosis, for example, is a lifelong genetic disease that causes unusually thick mucus production in the lungs, which hinders bacterial clearance and leads to chronic respiratory infections (Touw 1998). In these types of patients where bacteria reside in thick biofilms, cyclic dosing of antibiotics to allow the kidneys to repair themselves can lead to antibiotic resistance. Researchers have shown that aminoglycoside resistance due to enzymatic inactivation of these drugs is a global issue with differences in resistance mechanisms human patients in East Asia, Chile, and the US (Mayer 1986).

While several studies have analyzed genetic risk factors for sensitivity (Fischel-Ghodsian 2005) (Guan 2006) (Mingeot-Leclercq & Tulkens 1999a), few have looked *in vivo* for genes that can be knocked out to increase resistance to toxicity (Owens et al. 2008). Information from both types of studies could be clinically useful. While patients that are sensitive to aminoglycosides need to be more cautious about exposure, patients that are resistant can help us elucidate a mechanism for the sensitivity in humans or develop better therapies.

Social, Ethical, and Economical Issues

Using human patients to understand aminoglycoside toxicity has several limitations. First and foremost, risk factors that are identified in lifestyle and patient genomics are correlative, not causal. Humans cannot be genetically manipulated to understand underlying mechanisms of

sensitivity for social and ethical reasons. Primates are very similar to humans in many ways, but are also not a good model for genetics due to the lack of genetic tools, the complexity of the genome, and the cost of maintenance. Mice are the closest relatives that have been well-established as a model organism. However, they are far more costly to maintain, have a longer life cycle, and have less accessible ear hair cells than fruit flies. Other organisms, including zebrafish and chickens, have been studied to understand the regenerative properties of hair cells, but lack a fully sequenced genome. For the purpose of this study, the fruit fly is an excellent model because it is easy to genetically manipulate, has a homolog to 75% of human disease genes (Reiter et al. 2001), and has none of the social, ethical, and economical limitations associated with vertebrates. For this reason, there are minimal issues with fruit fly research. However, future uses of this study could involve some of social, ethical, and economical issues.

The most ideal outcome of information identified by this project would be an application to human patients. This would likely come in the form of personalized medicine, in which doctors use clinical, genetic, and environmental information specific to the patient to make recommendations about the optimal therapeutic route. Specifically, patients' DNA could be screened for genes that render them tolerant or sensitive to aminoglycosides toxicity. In a clinical setting, this could be quickly accomplished by designing a small microarray, in which short pieces of single-stranded DNA containing pieces of genes of interest are fixed to a solid support. The patient's DNA could then be made single-stranded and labeled with a fluorophore, then washed over the DNA on the array so that the pieces that bind tightly (indicated by high fluorescent intensity) indicate full complimentarity. Regions that do not fluoresce indicate a region containing a mutation. This method can be used to determine which genes patients are mutant for. Patients with mutations in genes that render them more tolerant could be given

aminoglycosides with less fear of toxic side effects, while those who are genetically predisposed for sensitivity could be given another class of antibiotic. Although the other drugs may be less effective or more costly, the patients would not have to deal with the toxic side effects of aminoglycosides. In addition, sensitive patients could be given personalized therapy to decrease the expression of genes that sensitize them to toxicity.

Before genetic applications are available in the clinic, an animal model more genetically similar to humans (i.e. mice or monkeys) would need to be investigated to ensure preservation of gene function in vertebrate models. One major issue with investigating aminoglycoside toxicity in a higher order model is the damaging effects of the drugs. The gain of knowledge that will impact human patients in the future will need to be weighed against the harmful effects against higher order animal models. The Institutional Animal Care and Use Committee (IACUC) will be a great resource for these studies.

A major social issue associated with this study is dealing with patient genetics. While health care practitioners should be given every piece of economical information to make an informed therapeutic decision, the availability of this personal information has been a controversial issue in the past. Patients should be informed of laws that protect their genetic information, as well as any potential loopholes. Currently in Washington State, health care providers may not disclose genetic information without informed consent. The Genetics Privacy Act is a federal act based on the premise that genetic information is different from other types of personal information in ways that require special protection. It protects individual privacy while permitting medical uses of genetic analysis, legitimate research in genetics, and genetic analysis for identification purposes. The Genetics and Health Insurance State Anti-Discrimination Laws restrict health insurers from using genetic information to deny coverage or set premiums.

While insurance companies may not want to pay for additional testing to determine the optimal treatment route, they should also be aware of the cost of treating a patient for the toxic side effects of aminoglycosides. While only 3/135 (2%) patients experienced a loss of ≥50 dB resulting from aminoglycoside treatment every 8 hours for 9 days (Moore et al. 2010), understanding individual patient sensitivity may have prevented this loss. Cochlear implants are surgically available implant devices that allow severely hearing impaired individuals to hear. These devices cost $45,000 to $105,000 for the evaluation, surgery, hardware, hospitalization, and rehabilitation. For less drastic hearing loss, hearing aids are available, which cost between $500 to $6,000 for one. In contrast, the Duke Microarray Core Facility can currently design a microarray chip, check and label the sample, hybridize, scan, and analyze for $300. Additionally, since the chips can be reused, subsequent rounds should only cost $200.

A potential economic issue in future studies using this information comes from the high cost of personalized medicine. The cost of sequencing has dropped drastically over the last decade, but acquiring patients' genetic information would raise the cost of health care. Nevertheless, the 25% of patients experiencing some hearing loss when exposed to aminoglycosides need to be considered when contemplating increased health care costs. Efforts have been made to determine which sequencing strategies associated with personalized medicine maximize accuracy and minimize cost (Du et al. 2009). Some argue that even though sequencing costs have plummeted, the long and short-term costs of personalized medicine outweigh the savings of the "one drug fits all" approach (Dean 2009).

Technical Background

I will use *Drosophila melanogaster* in order to identify key genes and tissues involved in aminoglycoside sensitivity. One major advantage to using the fruit fly as a model is the rapid

generation time; they take only 10 days to reach full maturity. Furthermore, a single female can lay hundreds of eggs, making progeny extremely abundant. Finally, the genetic tools available in fruit flies are unsurpassed by any model organism of equal complexity. Two genetic tools are available in *D. melanogaster* that stand out for this study: the GAL4-UAS system and RNA interference (RNAi).

The GAL4-UAS system is used to overexpress genes. Researchers took advantage of a transcription factor, GAL4, in yeast. This transcription factor binds to an upstream activating sequence (UAS), enabling RNA polymerase to bind and transcription to begin. To utilize this system, the researchers put a fly promoter, Adh, in front of GAL4 and inserted this piece of DNA into the fly genome, such that the GAL4 protein was transcribed only when Adh was normally active. Then, they put the lacz gene directly downstream of the UAS sequence. They discovered that the lacz gene was transcribed in the expression pattern of Adh (Fischer et al. 1988). In essence, the GAL4 regulatory region targets a specific tissue, determining the "where" and "when" of expression, and the UAS insertion can be varied to express any gene of interest, determining the "what." Thanks to the hard work of many researchers, there are thousands of GAL4 drivers that enable tissue-specific expression patterns, and there are a large number of UAS reporters available at stock centers worldwide. Figure 1 diagrams how this system works.

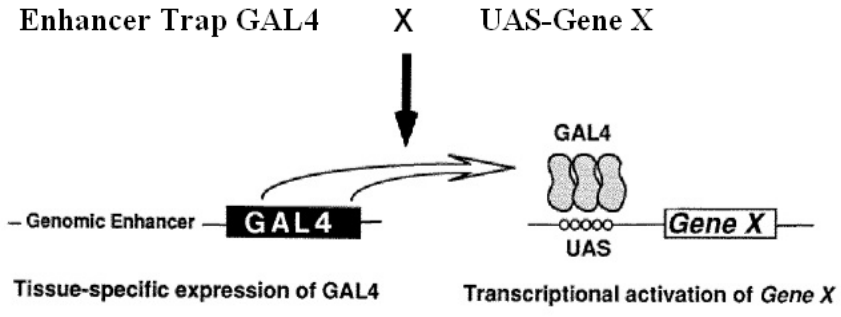


Figure 1 - The GAL4-UAS system.

Several methods exist for reducing gene expression or shutting off transcription of a gene altogether, including UV radiation, ethylmethane sulfonate (EMS) mutagenesis, and P-element mutagenesis. However, these methods affect random genes and can sometimes reduce or knock out the expression of more than one gene. In contrast, RNAi is used to reduce the expression of a specific gene. Researchers commonly silence *Drosophila* genes by inserting an inverted repeat sequence of a gene already found in the genome into a UAS construct. This allows genes to be knocked down only in specific tissues, which is helpful when the gene is essential or important for survival. One limitation of RNAi is that it does not turn off gene expression completely. However, for the purposes of this project, a null mutation is not necessary, as long as gene expression is reduced enough to show a phenotype.

Researchers in the Mello Lab originally discovered that introducing double stranded RNA (dsRNA) into cells reduced gene expression beyond single stranded antisense RNA (Fire et al. 1998). Once dsRNA is in the cell, the enzyme Dicer cuts it up into 21-23 base pair sequences called small interference RNA (siRNA) (Ketting et al. 2001). Then, the siRNA is separated into single stranded RNA and incorporated into the RNA-induced silencing complex (RISC). The RISC targets its homologous sequence by base pairing, and then cleaves the mRNA, preventing translation from taking place. Through the significant time and effort of researchers, there is a genome-wide library for *Drosophila* RNAi screening (Dietzl et al. 2007).

During this project, I developed a tool using the GAL4-UAS system that allows me to systematically express the neo^R gene in specific tissues. At the beginning of this project, I did not know which tissues require this gene and are therefore more sensitive to aminoglycoside toxicity. Thus, the ideal construct would have high expression in all tissues. Researchers have found that tissue-specific expression levels are dependent on position in the genome (Levis et al.

1985). Integration of pieces of DNA into the fly genome via P-element transposition has long been possible (Spradling & Rubin 1982), though the insertion sites are random and therefore lead to variable expression levels. Fortunately, the recently developed φC31 integration system enables researchers to control where the DNA is inserted (Bischof et al. 2007). This system allowed me to choose insertion sites with high expression in most tissues.

This site-specific integration system takes advantage of an enzyme called serine integrase and produced by the φC31 bacteriophage, which mediates sequence-directed recombination between a bacterial attachment (attB) and phage attachment (attP) site. Bischof et al. (2007) randomly inserted attP sites into the *Drosophila* genome to serve as "landing sites" using transposable elements (Coates et al. 1997). In order to use this tool for cloning, scientists use plasmids containing an attB sequence to recombine their gene of interest into these landing sites. Markstein et al. (2008) quantitatively characterized how much gene expression depends on the attP position within the genome by integrating UAS-luciferase, a firefly protein, into 25 different sites. They measured the basal and induced level of expression of luciferase present normalized to total protein in larvae in UAS-luciferase alone and UAS-luciferase x GAL4 drivers specific to the muscle, fat body, and nervous system. Of the 20 attP sites they analyzed, attP40 and attP2 had the best combination of low basal levels and high inducible levels of expression in the tissues they analyzed.

Theory

Before I can explored which tissues require the neo^R gene, I needed to know how much G418 is required to have an effect on wild-type flies. In humans, clinical trials require pharmacology studies to determine the therapeutic window of a new drug. Pharmacokinetics and pharmacodynamics are fields that explain the fate of a substance delivered usually to humans.

However, this area of pharmacology has also been studied in monkeys (Hemeryck et al. 2006), mice (Singh et al. 2008), chickens (Sriranjani et al. 2006), zebrafish (Nyholm et al. 2009), and other organisms, suggesting that it is useful for understanding drug trafficking in more basic organisms as well. Pharmacokinetics determines what the body does to the drug, while pharmacodynamics explores what the drug does to the body.

Pharmacokinetics uses several characteristics to understand what the body does to the drug: liberation, absorption, distribution, metabolism, and excretion. (Ruiz-Garcia et al. 2008). Liberation is the process by which the drug enters the system from formulation. Absorption is when the drug enters the circulatory system. Distribution is when the drug is sent to different tissues in the body. Metabolism is the irreversible transformation of the drug into metabolites. Excretion is the elimination of the substance from the body. Compartmental analysis of the drug is a model that predicts the concentration-time curve. For the purposes of this study, I will fit a model to the survivorship-time curve at a several given concentrations.

Pharmacodynamics attempts to explain what the drug does to the body, generally in the form of dose response curves, which is determined by fraction bound vs. concentration. The therapeutic window of a drug is a key measurement within which clinicians try to keep patients to avoid toxicity issues. When a drug or ligand binds to a receptor, it forms a complex, which can be represented as:

$$L + R \leftrightarrow L \cdot R \qquad [1]$$

where L is ligand or drug and R is receptor or drug target. The equilibrium dissociation constant, K_d, is:

$$K_d = \frac{[L][R]}{[L \cdot R]} \qquad [2]$$

where brackets indicate concentration. The fraction bound is:

$$\frac{[L \cdot R]}{[L \cdot R]+[L]} \quad [3]$$

which reduces to:

$$Fraction\ bound = \frac{1}{1+\frac{K_d}{[L]}} \quad [4]$$

The fraction bound curve is a second-order equation and thus sigmoidal because it is dependent on both the drug and drug target concentrations.

Review of Literature

Genetic basis to toxicity

Researchers trained patas and macaque monkeys to respond to sounds from headphones using positive reinforcement training by giving them banana-flavored food pellets. They injected 20 mg/kg/day dihydrostreptomycin (DHSM) intramuscularly to these trained monkeys and determined that macaque monkeys are highly sensitive to DSHM toxicity, while patas monkeys are highly susceptible to the drug toxicity (Hawkins et al. 1977), despite being in the same subfamily. Analysis of monkeys' cochlea at the end of the study was consistent with this finding. This difference between species indicates that there is a genetic component to both aminoglycoside toxicity susceptibility and resistance. One way to determine which genes are responsible for this difference is to identify genes that are different between these species and explore their function in lower order organisms such as the fruit fly.

Other researchers interested in the genetic basis to aminoglycoside toxicity sequenced the mitochondrial DNA of patients with maternally-inherited susceptibility to aminoglycoside toxicity where its use is widespread, including China and Isreal (Prezant et al. 1993). In Shanghai, 22% of all deaf mutes are deaf because of exposure to aminoglycosides (Guan 2006). Sequencing analysis revealed a mutation in the mitochondrial 12S ribosomal RNA gene common to all four families with susceptibility, the A1555G mutation. This mutation makes the rRNA in

this mitochondrial gene more similar in structure to bacterial rRNA (Forge & Schacht 2000). While identifying this mutation was informative, the sequencing information alone is not causal evidence. Furthermore, this gene was identified from the sequence of mitochondrial DNA alone and does not explain any nephrotoxic susceptibility. There are likely more genes involved in the interaction with aminoglycosides, some of which may be chromosomal.

Ototoxicity

Interestingly, ear hair cell sensitivity in humans does not occur because aminoglycosides concentrate in these tissues. The concentration of these drugs in the inner ear does not exceed serum levels (Henley & Schacht 1988). In addition, although the concentration of the aminoglycosides gentamicin, netilmicin, dibekacin, and amikacin in the renal cortex is higher than in the serum, concentration does not correlate with nephrotoxicity (Brion et al. 1984). While the serum half-life of aminoglycosides is only several hours, the renal tissue half-life is several hundred hours (Appel 1990). Similarly, aminoglycosides persist in the inner ear for 6 months or longer (Dulon et al. 1993). This finding suggests that prolonged exposure is required for toxicity.

Studies examining the ototoxicity due to aminoglycoside treatment in mice showed that dihydrostreptomycin blocks mechano-electrical transducer channels and also enter outer hair cells through these channels (Marcotti et al. 2005). In the first step of the proposed mechanism in ear hair cells, the positively charged aminoglycoside is attracted to the glycocalyx on the apical surface of the cells and binds to the stereocilia. Once the aminoglycoside is bound, it competes with Ca^{2+} to reversibly interfere with transduction channels (Fischel-Ghodsian 2005). In the second step, the drug enters the cell, potentially from the basal side. Once inside the cell, aminoglycosides form a complex with iron, which catalyzes reactive oxygen species (ROS) to be produced from unsaturated fatty acids (Rybak & Ramkumar 2007). These ROS can then promote

necrotic cell death as well as signal transduction through the c-Jun N-terminal kinase (JNK) pathway, which regulates cell proliferation and in this case apoptosis (Davis 2000). This mechanism is shown in Figure 2.

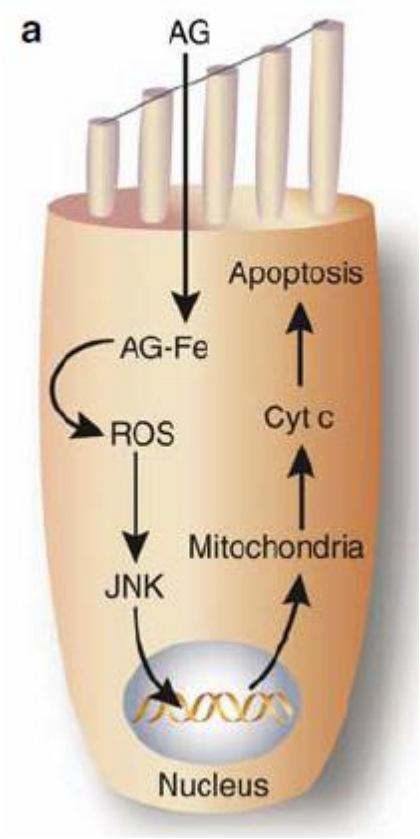


Figure 2 - Mechanism of hair cell death as a result of aminoglycoside (AG) exposure. (Rybak & Ramkumar 2007)

Nephrotoxicity

Aminoglycosides are mainly removed from the blood stream by glomerular filtration through the kidney, a process by which blood courses through the glomeruli. Within the kidney, useful chemicals and wastes are selectively passed through a membrane through diffusion and osmosis and then flow into the Bowman's capsule (Appel 1990). Once aminoglycosides have been filtered, the cationic region binds to acidic phospholipid receptors on the brush-border of

cells of the proximal convoluted tubule and pars recta in a kidney nephron, shown pictorially in Figure 3.

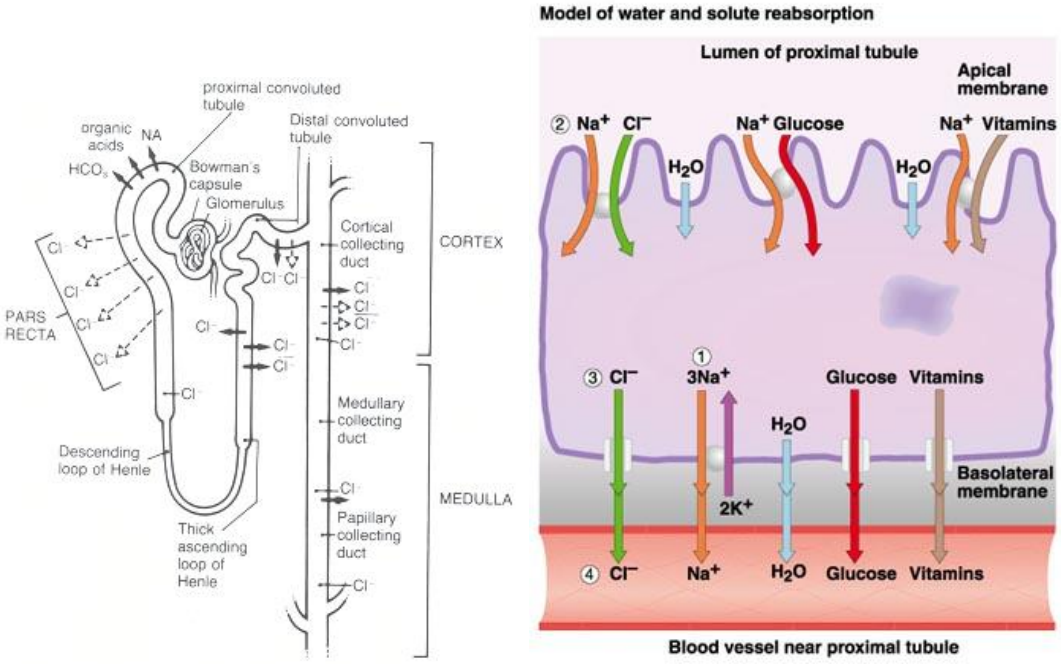


Figure 3 - (Left) A typical nephron, the functional unit of the kidney. Each nephron is composed of a capillary bed for filtration, called the glomerulus, and tubule segments located in the cortex and medulla of the kidney. (Right) A brush border cell within the proximal convoluted tubule.

The drug is reabsorbed largely through pinocytosis ("cell drinking"), in which cells endocytose particles into the cell in small vesicles, which then fuse with lysosomes to degrade the particles. This results in recurrent aminoglycoside concentration within the renal cortex (Appel 1990). However, other researchers found that high concentrations of aminoglycosides do not correlate well with nephrotoxicity in animal models, suggesting that toxicity may instead be due to the intrinsic toxicity mechanism of the renal proximal tubule, as described below (Brier et al. 1985).

Aminoglycosides alter plasma membrane, mitochondria, and lysosome structure and function in the proximal convoluted tubule (Mingeot-Leclercq & Tulkens 1999b). While aminoglycosides bind to phospholipids throughout the kidney, megalin, also known as

glycoprotein 330 (GP330), acts as an endocytic receptor and primary transport point concentrated in the renal proximal tubule, retinal, and inner ear epithelia. Megalin is a receptor for Ca^{2+}, plasminogen, lipoprotein lipase, and 39-40 kD receptor-associated proteins (Nagai & Takano 2004). In addition, it brings polybasic drugs, including aminoglycosides, into the cell. Once inside the cell, aminoglycosides damage mitochondria and lysosomes. While mitochondria are generally viewed as the "power house" of the cell, they also have an important role in cell life/death decisions and are harmed by aminoglycosides (Kroemer & Reed 2007). Mitochondria can induce cell death by producing apoptic signaling molecules and specifically ROS when exposed to gentamicin (Zorov 2010). ROS attack numerous cell targets, including DNA, to induce breaks. Structurally, cell death occurs when mitochondrial inner membranes, which are normally impermeable to protons, become leaky for compounds up to 1.5 kDa. This transition is termed the mitochondrial permeability transition (Zorov et al. 2009).

Aminoglycosides also induce significant structural changes to lysosomes in the renal proximal convoluted tubule, likely as a result of their prolonged presence in these tissues. Again, lysosomes and other organelles in the renal proximal convoluted tubule are likely more affected than other tissues because more aminoglycosides are let into these tissues than others because of site-specific receptors such as megalin. Because unrelated metabolic changes take place during aminoglycoside toxicity, one hypothesis is that these drugs become more toxic when they are released from lysosomes, which takes place when a threshold of accumulation in the organelle has been reached. The rapid release would be capable of inducing cell death (Mingeot-Leclercq & Tulkens 1999a).

The neo^R gene

One way to determine which tissues are most sensitive to aminoglycosides is to express a gene that can get rid of these drugs and determine how flies survive best. G418 and neomycin are both aminoglycosides that are inactivated by a gene, neomycin phosphotransferase II (*neo*), encoded by the bacterial transposon, Tn5 (Davies & Smith 1978). The *neo* gene is commonly found in *P. aeruginosa*, and phosphorylates the 3' hydroxyl group of aminoglycosides. This gene was excised from bacteria and inserted into a plasmid (pAG50) under control of the hsp70 promoter, which is on at low levels in all cells in *Drosophila*. It was then inserted into the fly genome via P-element transposition in order to confer neomycin resistance (*neo^R*) universally in flies (Steller & Pirrotta 1985). Since the strain of flies containing this insertion was developed, the *neo^R* gene has been used in *Drosophila* as a selectable marker, never in aminoglycoside studies. Furthermore, a dose response curve of aminoglycosides in wild-type flies has never been established because using the *neo^R* gene only requires that researchers know a dose that kills all flies lacking the *neo^R* gene.

Regeneration studies

While knowing that Macaque and Patas monkeys are affected differently by aminoglycosides gives hope that there is a genetic basis to its toxic effects, this information alone does not tell what kind of effects may be present or whether or not there is a genetic basis to toxicity in lower order organisms. Fortunately, other researchers have dosed organisms such as zebrafish and chickens to determine that aminoglycosides had ototoxic effects on these organisms (Williams & Holder 2000) (Roberson & Rubel 1993). Because they can regenerate ear hair cells, unlike mammals, genes in these organisms have been mostly studied for their regenerative function (Brignull et al. 2009). These studies are promising because they shows that

the same tissue affected in humans are affected in these lower order organisms, indicating that a similar mechanism for toxicity may be taking place. Research in zebrafish also warned, however, that the effects of aminoglycosides are highly dependent on the specific drug used in studies (Owens et al. 2009).

Fly dosing methods

In order develop *Drosophila* as a model to understand the genetic basis of aminoglycoside tolerance, I will need to first dose flies to determine a range of concentrations that has an effect but does not kill them. There are two main ways to dose fruit flies: dosing larvae by mixing the drug into the food, or starving adults and then allowing them to consume the drug only. Both have advantages and disadvantages. Dosing larvae was a method designed specifically for G418 antibiotic dosage in order to use the neo^R gene as a selectable marker that produced only adults containing the neo^R gene. At 0.5 mg/mL of G418, all flies die without the neo^R gene, while flies with the gene survive (Steller & Pirrotta 1985). To dose the larvae, these researchers melted the fly food in a microwave, cooled to 40 °C, and added G418 at final concentrations of 50-1000 μg/mL..

Dosing larvae with G418 is already an established method. Dosing adults is also advantageous, however, because genes expressed in adult fly tissues are likely to be more similar to those in adult patients, whereas dosing larvae is analogous to dosing babies or embryos. Thus, more relevant genetic modifiers of toxicity may be identified by using adult flies rather than larvae. I took advantage of another method of dosing flies involving adults instead of larvae during this study that was originally developed for the purpose of inducing DNA mutations. Ethylmethane sulfonate (EMS) became the mutation-inducer of choice upon the discovery that it induces mutations randomly throughout the genome proportionally to EMS concentration

because it does not cause as much DNA damage as other methods such as UV and x-ray mutagenesis (Ohnishi 1977). To dose adults, flies are starved for 8 hours with water to prevent dehydration. Then, they are kept on EMS mixed with 1% sucrose solution for approximately 18 hours, and then allowed to mate with females (Roberts 1998). A limitation of this method is that adults eat less than larvae (Zinke et al. 2002), which might make the larvae dosing method less variable.

Previous relevant work in the Berg Lab

The Berg lab studies dorsal appendage formation during oogenesis, which is unrelated to aminoglycoside toxicity studies. However, we all use *Drosophila melanogaster* as a model organism and therefore have extensive experience in fly culture techniques and genetic analysis. The lab has experience crossing flies, using the GAL4-UAS system, RNAi, and the φC31 system. Our lab has also done EMS screens in the past, and therefore has experience dosing adult flies. Some researchers have also selected for flies with the neo^R gene, and therefore have experience administering a lethal dose of G418 to flies. Thus, while I can easily find help for some methods, no one in the lab has experience establishing a model for drug toxicity, meaning I will need to look beyond our lab for answers to many questions.

Outstanding technical issues at the outset of the project

Because *Drosophila* has never been developed as a model for drug toxicity, I faced technical challenges for all of my project goals. First, other researchers have identified a lethal dose of G418 for wild type flies, but have not determined a range over which there is some decrease in survivorship. Therefore, I needed to establish this curve so that I could reliably study the effect of G418 toxicity in wild type flies without killing all of them. I also needed to look at

survival trends from FRT82B flies, the starting neo^{R} strain of flies that express this resistance gene universally under the control of the hsp70 promoter.

The second major technical challenge I faced involved a method of visualizing G418 and feeding it to larvae. While aminoglycosides other than G418 have been traced through zebrafish (Wang & Steyger 2009) and cultured cells (Sandoval et al. 1998) by conjugating a fluorescent tag, Texas Red, to gentamicin, G418 has not been traced previously in any organism. Therefore, I needed to adapt their methods to G418 to perform pulse-chase experiments. Because gentamicin and G418 are structurally similar, I only needed to adapt the protocol to the molecular weight of G418 because it should react similarly.

The third major challenge I faced involved designing a piece of the tissue-specific expression system. Because a UAS-neo^{R} construct did not exist at the outset of my project, I needed to create one to determine which tissues are most sensitive to aminoglycosides. To do this, I came up with a cloning strategy that took advantage of the φC31 system. After cloning this construct and selecting for flies that had this insertion, I crossed it to 16 GAL4 drivers, which revealed tissues most sensitive to the drug.

The final outstanding technical issue was determining which genes could be knocked out to increase survivorship. Given my 9-month capstone project timeline, it was not possible to screen the entire genome for genes that could be knocked out to increase tolerance to G418. Therefore, choosing candidate genes carefully was nontrivial. Furthermore, I needed to be able to sort out when G418 was decreasing survivorship versus when the knockout flies were sick simply due to their genotype. The variability in the quality of fly food may have contributed to the complexity of this issue.

Initial Design of Experiments, Tools, and Devices

Materials and Methods

Larval Dosing

In order to determine a range of G418 concentrations that had an effect, I quantified survivorship for a range of doses. I redesigned my method twice to optimize G418 treatment. The following methods detail which parameters I changed to reduce variability in survivorship.

Method 1

I chose Canton S (wild type) and neomycin resistant (FRT82B) flies from bottles and vials on the basis of availability. This resulted in extremely variable number of progeny, however, because older flies do not produce as many offspring as young flies. I chose four females and three males as parents for each vial and set up 3 vials of each genotype to allow statistical inferences to be made from the results. I allowed them to lay eggs for two days, transferred them to a new vial twice, each after two days, and then discarded them. Because adult females can lay several hundred eggs, I reasoned that they would lay maximally with fresh food and that fecundity would not be significantly reduced in 6 days. I dissolved G418 (Clontech) in water (ddH_2O) such that the final concentration of the drug when mixed with 7.25 mL of food was 0, 0.001, 0.006, 0.01, 0.06, 0.1, and 0.6 mg/mL. I chose this logarithmic range up to 0.6 mg/mL to find a window that reduced survivorship, knowing from previous experience in the lab that 0.6 mg/mL was sufficient to kill all wild-type flies. From the experimental range I dosed, only 0.1 and 0.6 mg/mL G418 reduced survivorship. I dosed the food by poking holes in the food with a glass pipette, then adding 174 µL of drug to each vial and allowing the drug to settle in the food overnight. I counted dosed progeny after 12 days because flies require 10 days to eclose and I allowed parental flies to lay eggs for two days.

Method 2

In order to decrease the variability in the health of the parental flies, I aged adults to 8 to 24 hours of age. I also included more parental females so that the fly-to-fly variability in fecundity was less noticeable. I chose 8 females and 5 males per dose and allowed them to lay eggs for 5 days, then discarded them without transferring. However, this resulted in overcrowding, which stunted the development of the majority of flies. I also reduced the volume of each dose to 130.5 µL of drug per vial to reduce pooling. As mentioned previously, I also wanted to focus the dosing range between 0.1 and 0.6 mg/mL, so I used a final concentration of 0, 0.05, 0.15, 0.25, 0.35, and 0.45 mg/mL G418 in the food. I also increased the number of vials to N = 17.

Method 3

I decreased the number of parental adult flies per vial to 5 females and 3 males to decrease crowding. I collected the flies and separated males and females between 8 - 16 hours of age and placed the parental females in vials with wet yeast overnight to stimulate egg production. I transferred the parental flies to dosed food the following day. Because fecundity is high for at least the first four days of adulthood, I allowed them to lay eggs for two days, transferred them to a new vial, allowed them to lay eggs for another two days, then discarded them. In addition to the previous doses, I included a 0.1 mg/mL G418 dose to get a better survival curve. I also increased the number of vials of each dose to $N \geq 27$ to get more significant data.

Adult Dosing

Because adult flies eat less than larvae and do not burrow in the food, they would be far less exposed to the drug administered the drug via the larval dosing method. Because other researchers have already developed an adult dosing method to successfully deliver ethyl

methanesulfonate (EMS), which induces mutagenesis, I decided to modify this method to administer G418. Additionally, this method does not require diluting the drug in the food, meaning it should require less drug than dosing adults through food. My method of delivering G418 to adult flies has been adapted from EMS dosing methods discussed by TA Grigliatti's chapter on Mutagenesis in Roberts' book (Roberts 1998). I aged approximately 100 flies per vial (50 male, 50 female) two days and then starved them for 8 hours in a plastic bottle containing a moistened piece of Whatman filter paper. Then, I added 0.75 mL of 0, 0.1, 0.5, 5, and 50 mg/mL G418 in a 1% sucrose solution to the filter paper. I left flies on the paper for 18 hours, then counted for survivorship. While on the drug, I noticed that more wild-type flies congregated on the bottom of the vial compared to FRT82B flies and that males appeared more subject to dehydration than females, likely due to their smaller body mass.

Conjugating G418 to Texas Red

I adapted a protocol described previously (Sandoval et al. 1998) to conjugate G418 to Texas Red (TR), rather than gentamicin, which is a lower molecular weight aminoglycoside. I made a 100 mM buffer solution of K_2CO_3 at pH 8.5, then made a 10 mg/mL solution of G418 in the K_2CO_3 buffer solution. I added 2 mg of Texas Red to 100 µL of N,N-dimethylformamide. I then added a 30 molar excess of G418 slowly to the Texas Red and dimethylformamide solution on ice. Next, I melted one bottle of fly food and poured ~2 mL into small bacterial culture tubes and stirred in the G418-TR to a final concentration of 0.45 mg/mL G418. I allowed parental flies to lay eggs on the drugged food overnight, then discarded them. Thus, larvae were exposed to the drug for the duration of their life. At this lethal dose, however, most wild-type flies don't develop beyond the first or second instar larval stage. Before imaging the larvae at these stages, I put

them on wet yeast with blue food coloring for 4 hours to allow them to clear the residual drugged food from their gut.

Costs

Product	**Description**	**Quantity**	**Price**
G418	Antibiotic	5 g	$348
Texas Red	Texas Red-X, succinimidyl ester *single isomer*	2 mg	$181
N, N-dimethylformamide	For G418-TR conjugation	250 mL	$22.50
Whatman filter paper	5.5 cm, Grade 3	1 Pack of 100	$11.10
Fly food	To feed flies	~12 trays	$270.00
Dissection pinning needles	Black anodized minutien pins 0.15mm	1 Pack	$28.75
Embryo injection services	φC31 service	2 lines	$413.91
Primers	To amplify neo^R from genome (with extra sequence for restriction sites)	1 pair	$18
Primers	For sequencing	1 pair	$10
Miniprep kit	Qiaprep Miniprep SPIN 250X	1	$361.96
Competent cells	XL10-Blue SCC	5 x 2 mL	$201.38
		TOTAL	$1,866.60

Initial research plan explained

- **Develop a drug-response curve in D. melanogaster relating concentration of antibiotic to survivability, focusing on the highest dose conferring viability in wild-type flies.** While drugs are sometimes administered to fruit flies to deliver mutagens, response in terms of drug concentration is not commonly measured. Analysis of aminoglycoside drug response in fruit flies has never been measured, and no protocols for this process currently exist for determining a drug response curve. Therefore, in order to develop a model for toxicity and determine the LD_{50}, the dose that kills half of the flies tested, I needed to design a new process of reducing variability when dosing flies with aminoglycosides.

- **Determine which tissues accumulate the drug**. I planned to modify a previous protocol of conjugating gentimicin to Texas Red to instead conjugate G418 to the fluorophore. To make this change, I adjusted the volume of G418 to match the molar ratio previously used. I then fed the larvae this drug conjugate and fluorophore only control.

- **Analyze which tissues are affected by high doses of neomycin treatment**. I wanted to take advantage of the GAL4-UAS system to determine which tissues are most sensitive to G418's toxic effects. To understand this, I planned to insert a neomycin-resistance gene, neo^R, into the UAS sequence, and then drive its expression in several tissues using different GAL4 drivers. Results from dosing these crosses will tell me where this gene is necessary for survival. I planned to take a systematic approach to this problem, expressing the neo^R gene first in the entire fly, then separately in the endoderm, mesoderm, ectoderm, and so forth.

- **Alter the expression of genes to identify proteins that can increase tolerance to aminoglycosides and improve fruit fly survivability**. Once I characterized which tissues require the neo^R gene for survival, I planned to perform a genetic screen of genes highly expressed in these tissue of interest by using interference RNA (RNAi) to reduce gene expression. I will then assay for survivability to determine whether or not the gene is important in aminoglycoside toxicity, using the process characterized in Aim One.

Data

Result (Larval Dosing Method 1)

The survivorship of the first larval dosing method are shown in Figure 4. While there are several flaws with Method 1, it produced visible trends--flies that fail to produce offspring at the beginning of the dosing period do not improve, older flies do not produce as many offspring as younger flies, and there may be extraneous factors present on some days but not others, such as

lower quality food. These observations indicate that less variable results could be obtained by using younger parental flies and allowing them to lay eggs over a shorter time window. Data acquired using this method indicates that the maximal dose of 0.6 mg/mL G418 can kill Canton S flies, while neo^R flies survive the toxic effects. Also, G418 concentrations between 0.1 and 0.6 mg/mL detriment progeny, while doses less than 0.1 mg/mL do not have an effect on survivorship. Thus, the LD_{50} is within this range.

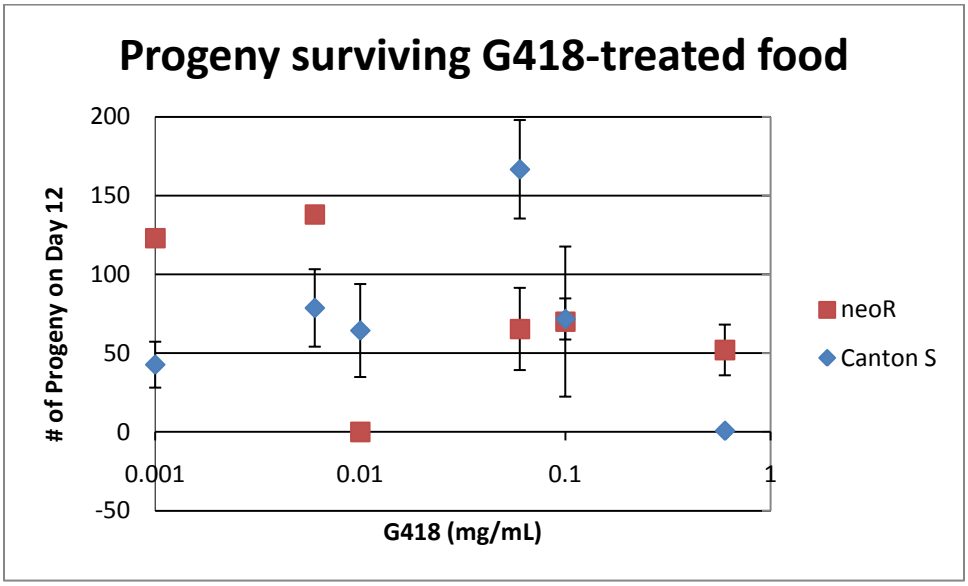


Figure 4 - Progeny surviving larval dosing method 1

Larval Dosing Methods 2 & 3

Survivorship counts using these methods are shown in Figure 5. The wild-type data shows the expected trend: the greater the dose, the less progeny survive to adulthood. Unfortunately, there appears to be a critical dose missing in method 2 because the survivorship drops sharply between 0.05 and 0.15 mg/mL G418. Furthermore, there is still high variability between each dose within the neo^R survivorship data. One potential source of this variability arises from high competition with the large number of larvae present in each vial. In method 3, I decreased the number of parental adults and the amount of time they laid eggs, which resulted in

less variable data and a survivorship curve that exponentially decayed in the wild-type data, as shown in Figure 5. Fitting a curve to the averages of the wild-type survivorship data at each dose resulted in an exponential decline of $y=149.7e^{-14.38x}$ with an $R^2 = 0.9614$ using Method 2. Using Method 3, this curve changed slightly to $y=224.02e^{-13.42x}$ with an $R^2 = 0.981$. A linear curve best fit the neo^R survivorship data. Using Method 2, a survivorship curve of $y = -61.944x + 113.38$ with an $R^2 = 0.6646$ resulted. Using Method 3, a survivorship curve of $y = -81.703x + 99.494$ with an $R^2 = 0.6372$ was produced. It is likely that detoxifying G418 is metabolically taxing to the flies, resulting in the negative survival trend of neo^R flies.

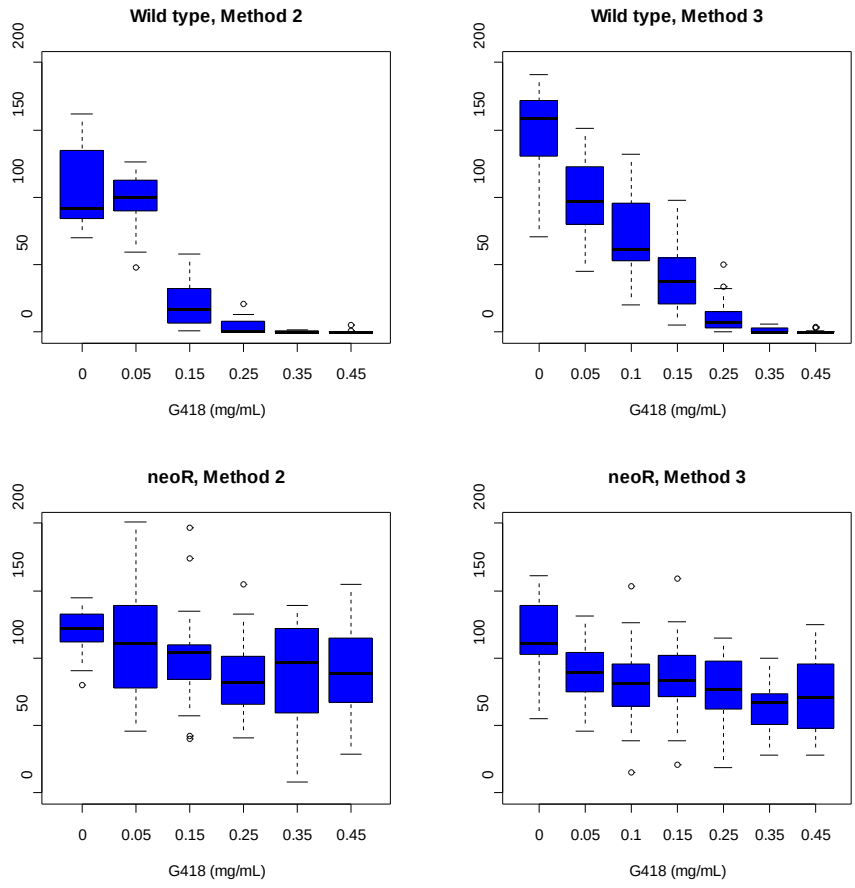


Figure 5 - Variability in survivorship of wild type and neo^R flies using larval dosing methods 2 & 3.

Adult Dosing

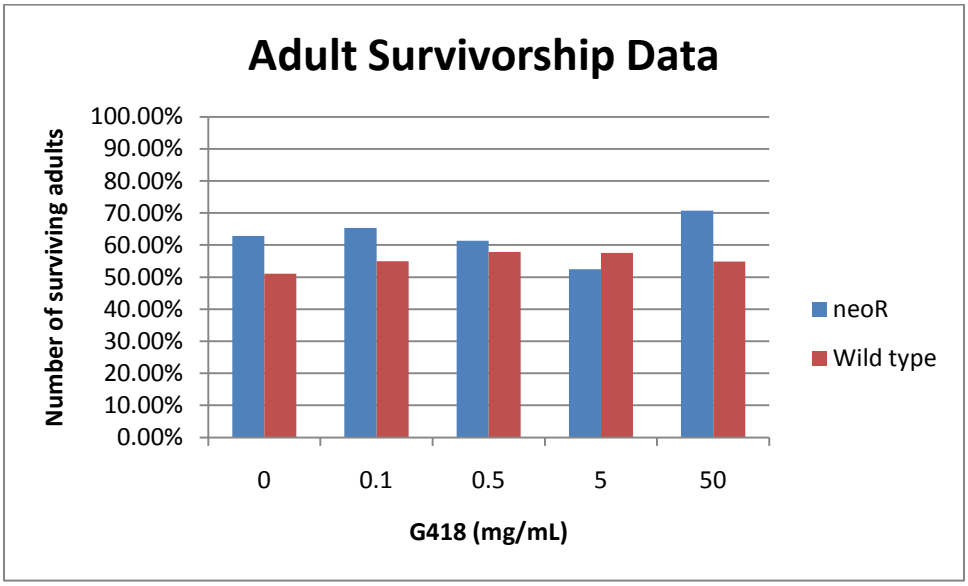


Figure 6 - Wild type and neo^R survivorship using adult dosing method. N = 100

After starving the flies for 8 hours, only 1 or 2 flies per bottle out of the original 100 flies were dead. After waiting 18 hours with the flies on the drug, the Whatman paper was still moist, indicating that flies still had adequate access to food (sucrose) and hydration. Nonetheless, 30-50% of both wild type and neo^R flies still died. The death of the flies was independent of drug concentration in both wild type and neo^R flies. One potential explanation is that the drug dose is not high enough to have an effect on the adult flies, even though 50 mg/mL is 100 times the lethal concentration for larvae. If a greater concentration than this is required to kill adults, drug cost and solubility at higher concentrations may become an issue. Furthermore, it will be difficult to see toxic effects when such a high proportion of the flies die regardless of the drug concentration. For these reasons, the larval dosing method appeared more feasible for the purposes of this project. However, a second round of dosing revealed that adult dosing is feasible, as shown in Figure 7.

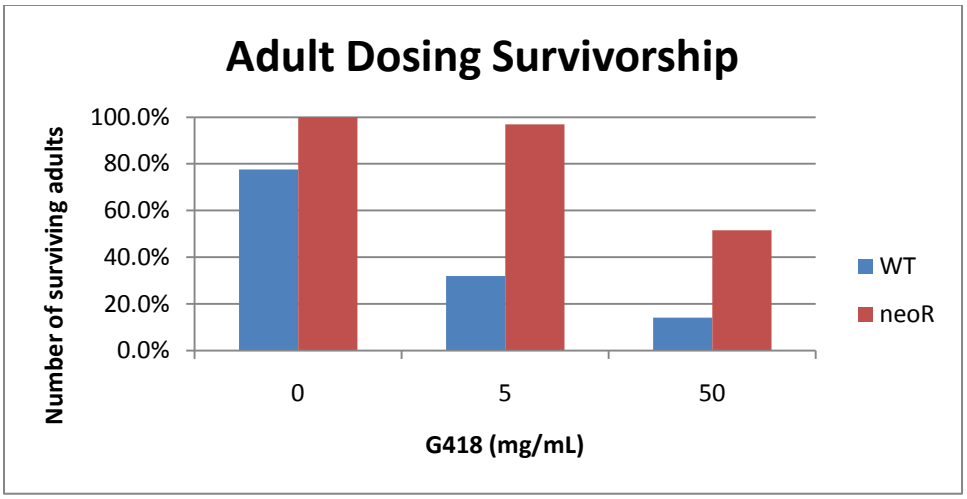


Figure 7 - Second round of adult dosing

The biggest change between the first and second round was adding new Whatman paper and a higher volume of drug, 1 mL rather than 0.75 mL, added to the bottle the second round. I reasoned that because the Whatman paper from the first round was still moist to touch, the combined water and drug may have been too much liquid and caused the flies to drown. I increased the volume of drug to 1 mL in order to moisten the entire piece of Whatman paper and to be consistent with the EMS protocol. Also, the new paper meant that the drug was effectively more concentrated, and thus had more of an effect on survivorship.

G418-TR conjugation

While dosing larvae at 0.45 mg/mL, I noticed that almost all wild-type flies died as first or second instars. Therefore, I imaged these larval stages with background fluorescence only (TR, no G418) and with the drug conjugate, G418-TR. I expected that the drug would accumulate in the gut and in the Malpighian tubule because when the larvae eat, food passes through these tissues. When the TR only is fed to the larvae, I see background levels. When the G418-TR is fed to the larvae, the fluorescent intensity above TR only intensity tracks the drug. Figure 8 shows the structure of the larval tissues where the drug accumulated.

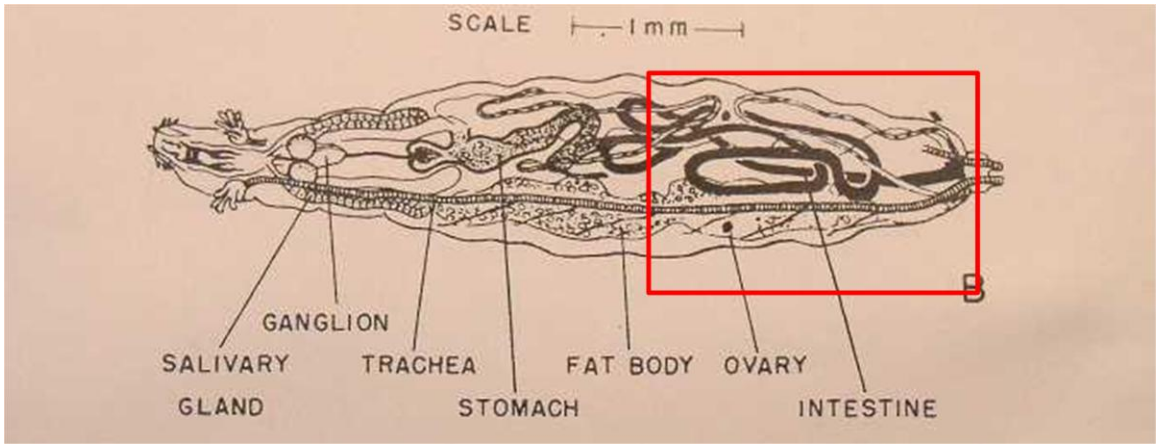
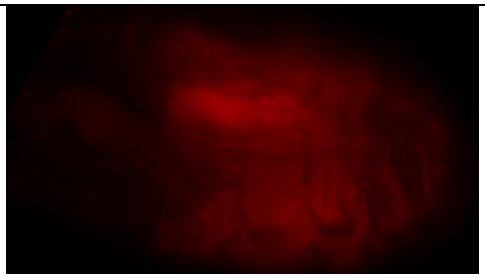
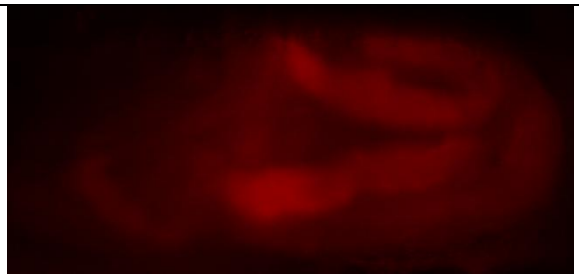
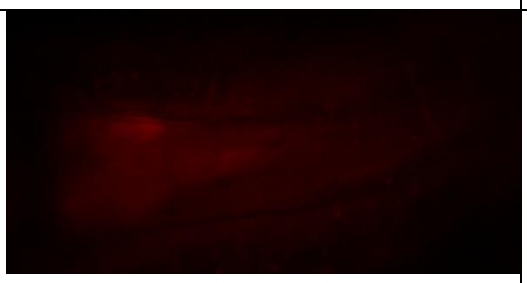
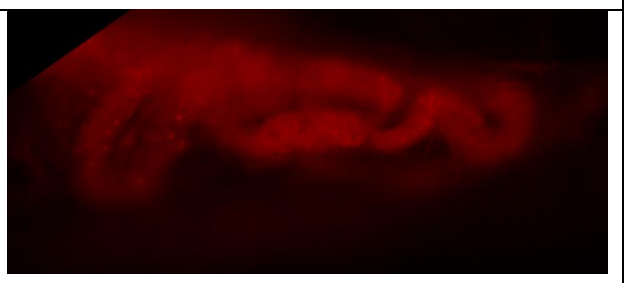


Figure 8 - Structures found in larvae. The red box indicates the region where the drug accumulated (intestine).

Table 1 shows the results of this drug-tracking assay.

Table 1 - G418-TR conjugation and unconjugated background accumulation in the gut of 1st and 2nd instar larvae. Images were all taken using the same exposure settings. All figures except the wild type G418-TR image are showing dorsal views of the intestine. The wild-type G418-TR image is a lateral view of the intestine.

	TR only (Background)	G418-TR
Neo^R		
Wild Type		

neo^R flies successfully metabolize the G418, indicated by the comparable fluorescence levels in the background and drug-conjugate comparisons. However, wild type flies cannot get

rid of the TR in the gut, indicated by the higher TR fluorescence intensities in the G418-TR dosed flies. Furthermore, the drug accumulates in tight puncta, which may be an attempt to package and excrete the drug.

Cloning

From the drug-conjugate experiment, I learned that G418 accumulates in the gut. This result suggests that this tissue may be most sensitive to aminoglycoside toxicity. In order to test this hypothesis and determine which tissues are most sensitive to G418, I wanted to take advantage of the GAL4-UAS system to drive expression of neo^R in different tissues. This goal requires a UAS-neo^R construct, however, which did not exist at the beginning of this project. Therefore, I decided to create one.

First, I obtained the pUASTattB vector from Basler et al, which already has a UAS sequence and takes advantage of the φC31 integrase-mediated transgenesis system to allow for site-specific integration (Bischof et al. 2007). My plan was to use this plasmid as a vector to make a UAS-neo^R construct, and then transform the construct into sites with high expression in most tissues. The construct map is shown in Figure 9.

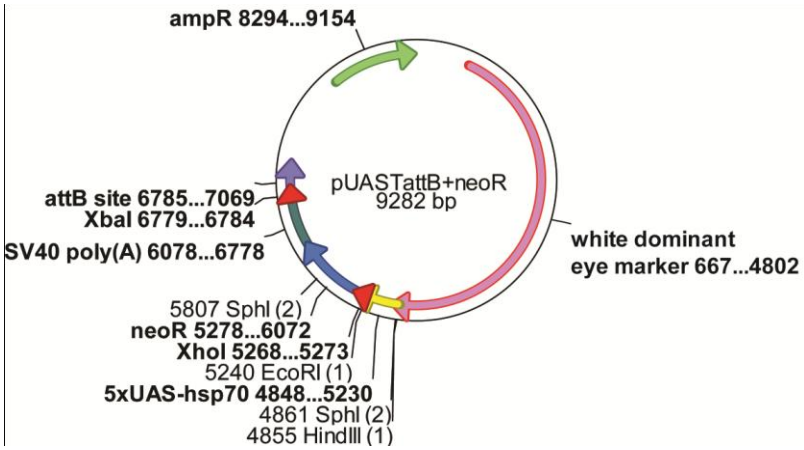
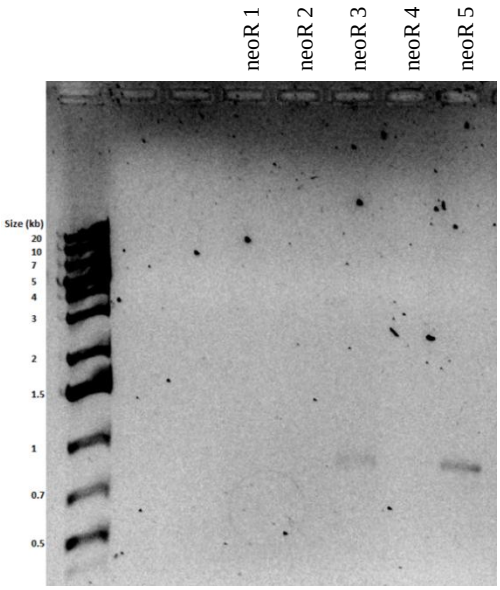


Figure 9 - Vector map of pUASTattB vector with neo^R insert. Restriction sites shown were used to verify the size of plasmid fragments.

I chose XhoI and XbaI within the multiple cloning site because they work most optimally in the same buffer (NEB2 + BSA), which allowed me to perform a double digest. I chose primers at the ends of the coding region of neo^R gene and added restriction sites. While the gene is relatively small (795 bp), the ends have low GC content. When the Kozak sequence, restriction sites, and overhanging ends were added, the primers were long (35 bp and 32 bp) and not optimal. In order to remove unwanted product, I performed a gel extraction. Figure 10 shows the initial amplification. As anticipated, the amplification of the neo^R gene was very poor because of the primer constraints. In order to get more of the neo^R gene, I pooled all five products, PCR amplified the neo^R gene again in a larger volume, and gel extracted the resulting product again. Figure 10 and 11 show amplification of the neo^R gene from genomic and amplified template, respectively.



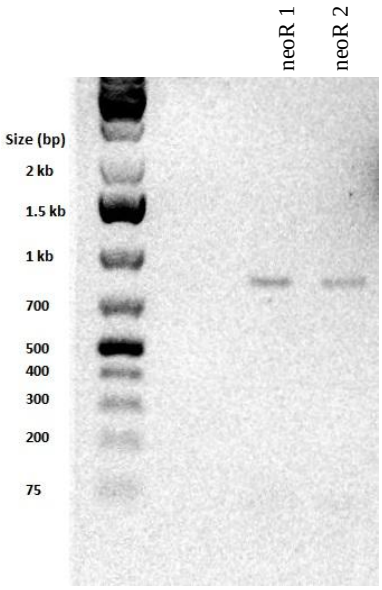


Figure 10 - Genomic amplification neo^R gene. **Figure 11 - Re-amplification of neo^R gene.**

After gel extracting the neo^R gene from the gel shown in Figure 10, I obtained 30 µL of DNA at a concentration of only 1.8 ng/µL. I digested 25 µL of my product and the pUASTattB vector overnight with XhoI and XbaI. I ligated my insert and vector at a 3:1 ratio in order to

kinetically drive the insert into the vector. I transformed DH5α cells with my ligation reaction. With an efficiency of $1 * 10^6$ cfu/µg supercoiled DNA * 0.1 µg ligated product * 1/10 quality supercoiled/ligated product, I should have gotten $1 * 10^4$ colonies. Unfortunately, when I plated my cells, I had a higher background than potential product. While the supercoiled control was as expected with a lawn forming when plating ~1 mL of my transformation, I only had 9 colonies total for my entire pUASTattB + neo^R transformation. Furthermore, my pUASTattB only reaction should have given me no colonies, but instead gave me a background of 198 colonies. Nonetheless, I miniprepped all 9 colonies that potentially contained the vector + insert. Then, I digested each miniprep with SphI (sites shown in Figure 9), which should cut once in the vector and once in the insert and produce a 1 kb and 8.3 kb fragment. The enzyme clearly worked because the cut vector ran faster on the gel than the uncut vector. However, none of the colonies had two fragments in the expected ranges.

I re-amplified the neo^R gene from the remaining 5 µL of previously amplified, gel-extracted, and digested DNA, meaning I should have no contaminating product. As a result, I should not need to gel extract my product and will lose minimal DNA from the PCR purification. The results of this PCR purification are shown in Figure 12. Most of the product leaked out because the gel lanes tore when removing the comb, but a faint band between 700 and 1000 bp indicates that the neoR gene is still present.

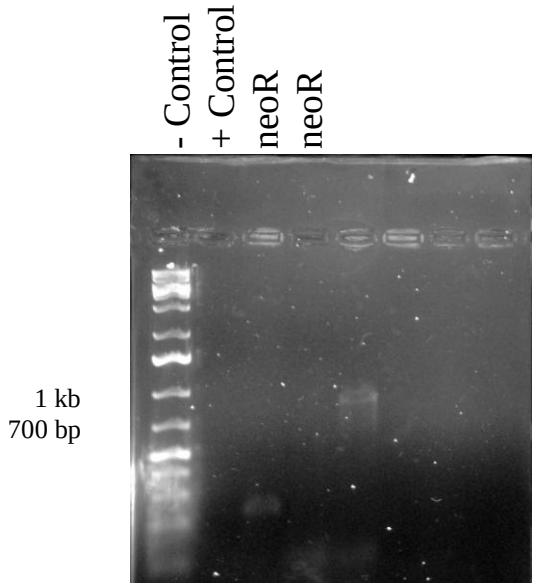


Figure 12 - PCR amplification of neoR gene.

I digested the insert and vector again overnight with XhoI and XbaI. In order to increase my yield of colonies, I used XL10-Gold® Ultracompetent cells during the second transformation, which guarantees 5 x 10^3 more cfu/µg pUC18 DNA than DH5α cells. The result of the second transformation was much more successful: my background had ~200 colonies, while my pUASTattB + neoR ligation had nearly a lawn when I plated 1 mL of my transformation. I chose 18 colonies to miniprep because our centrifuge rotor holds this many tubes. I then digested DNA from each miniprep with SphI, as previously. The resulting gel is shown in Figure 13, with white arrows indicating potential pUASTattB-neo^R colonies.

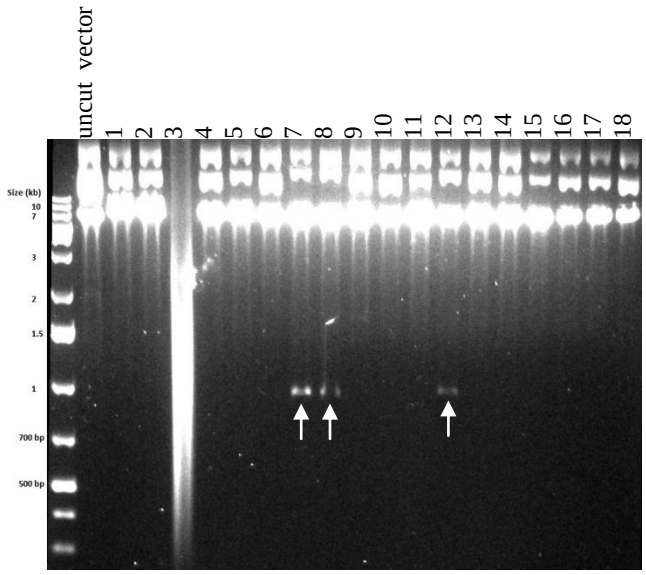


Figure 13 - SphI digest of potential UAS-neo^{R} colonies. White arrows indicate colonies that were cut twice and have a small band indicating the neo^{R} gene was inserted successfully.

As shown above, colonies 7, 8, and 12 all appear to have the neo^{R} insert. I designed two sets of primers for sequencing across the 5' and 3' junctions of the neo^{R} gene, 5'neoRF1, 5'neoRR1, 3'neoRF1, and 3'neoRR1. Unfortunately, these primers were too dilute, indicating that I did not spin them down properly or the company that synthesized them did not have as much product as they thought. As a result, these primers did not give a good sequence and were also unsuccessful at amplifying regions of vector + insert. Another researcher (AA) in the lab was using an attB vector, which has a very similar sequence to the pUASTattB vector but was missing the UAS region. She designed sequencing primers, pattB-AAF1 and pattB-AAR1. While her primers were further from the insertion ends than mine, she got excellent sequencing results using hers. I used her primers in a sequencing reaction with DNA that I miniprepped from colonies 8 and 12 and obtained good results. While I didn't get sequencing over the entire coding region using the pattB-aa primers, I found that the ends of the neo^{R} gene were intact with the vector and that all of the coding sequence looked good so far. I redesigned primers (5'neoRF2 and 3'neoRR2) closer to the end of the neo^{R} gene than the pattB-AA primers. A map of the

locations of all of my sequencing results is shown in Figure 14. A contig showing the full sequence from all primers shown in Figure 14 is shown in the appendix.

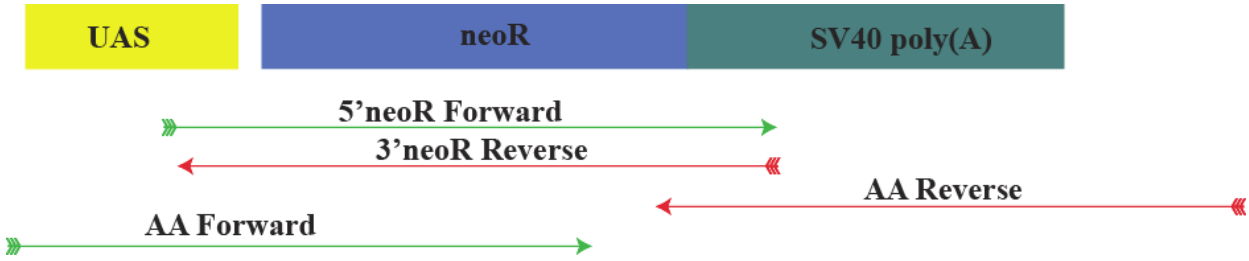


Figure 14 - Sequence results map. AA Forward and Reverse indicate reads from the pattB-AA primers.

The aligned sequences are shown in the appendix. An example of the sequencing chromatogram I obtained using the pattB-AA primers is shown in Figure 15.

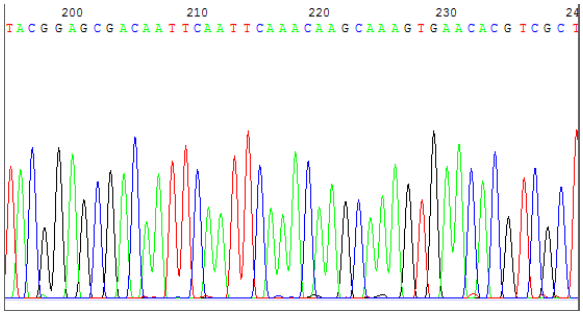


Figure 15 - Sample 5' sequencing read of colony 8 using pattB-aa primer.

Figure 15 shows that there is almost no background signal from the sequence, which means that there is no ambiguity in the base identity for those bases shown. The sequence using these primers gives positive results of base identity from the end of the loxP region to the attB site with a gap of approximately 100 bp near the 3' end of the neo^R gene (see vector map in Figure 9). The sequence I obtained using the 5'neoRF2 and 3'neoRR2 primers yielded sequence across the entire neo^R coding region, which proved that I had what I wanted. A sample of this sequencing read is shown in Figure 16.

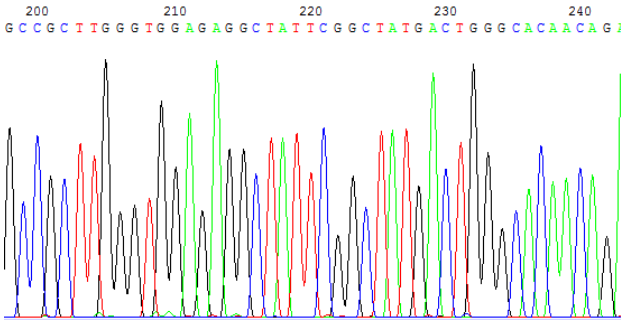


Figure 16 - Sample 5' sequencing read of colony 8 using 5'neoRF2 primer.

After seeing these convincing results, I prepared a maxiprep of colony 8 DNA, digested it overnight to confirm that the plasmid DNA was the correct size, and ran it on a gel. I linearized the vector using EcoRI, which cuts once in neo^R, and HinDIII, which cuts in the vector, as shown in Figure 9. The result of this gel is shown in Figure 17. I previously saturated the EcoRI and HinDIII enzymes, and therefore used a 1:20 dilution of DNA in the digests, which is why the bands are so much fainter than the uncut vector.

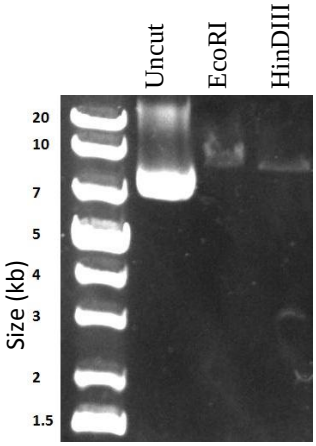


Figure 17 - Maxiprep vector gel verification of colony 8.

The linearized vector should be approximately 9.3 kb with the insert or 8.5 kb without. Figure 17 shows a band closer to the 10 kb band than the 7 kb band, indicating that the vector has an insert. After running this verification gel, I sent 100 µg of plasmid DNA containing the

neo^{R} insert to Genetics Services, Inc. for injections into white-eyed flies containing the attP40 and attP2 landing sites, which are located on the 2nd and 3rd chromosomes, respectively.

UAS-neo^{R} Overexpression Screen

Because my plasmid contains the white gene, producing a functional red eye when present, I knew my plasmid had integrated into the genome when the flies had red eyes. After receiving larvae, I selected red eyed progeny and homozygosed flies containing UAS-neo^{R} insertion. I used the crossing strategy shown in to generate homozygous flies.

$$Flies\ from\ Genetics\ Services \times w; \frac{Sp}{CyO}; \frac{Ly}{TM3,Sb}$$

$$♂ \frac{w}{¬}; \frac{+}{Sp\ or\ CyO}; \frac{P\{UAS-neoR\}}{TM3,Sb} \times ♀ \frac{w}{w}; \frac{+}{Sp\ or\ CyO}; \frac{P\{UAS-neoR\}}{TM3,Sb}$$

$$♂ \frac{w}{¬}; \frac{+}{+}; \frac{P\{UAS-neoR\}}{P\{UAS-neoR\}} \times ♀ \frac{w}{w}; \frac{+}{+}; \frac{P\{UAS-neoR\}}{P\{UAS-neoR\}}$$

Figure 18 - Crossing strategy employed to generate homozygous UAS-neoR strain for attP2 site. P{UAS-neoR} is marked with the white gene resulting in a red-eyed phenotype. Sp = Sternopleural bristles, CyO = Curly wings, TM3, Sb = Stubble bristles, Ly = Lyra wings.

After generating homozygous UAS-neoR flies, I crossed flies with the UAS-neo^{R} insertion in the attP2 site to 16 different GAL4 lines with varying expression patterns. I used the attP2 site because these took fewer generations to homozygose and were therefore available sooner. An example of the genotypes of a cross with a homozygous and heterozygous GAL4 driver is shown in Figure 19.

Homozygous CY2 GAL4 cross:

$$♀\ \frac{w}{w};\frac{+}{+};\frac{P\{UAS-neoR\}}{P\{UAS-neoR\}} \times ♂\ \frac{w}{¬};\frac{P\{w[+mW.hs]GAL4=GawB\}CY2}{P\{w[+mW.hs]GAL4=GawB\}CY2}$$

Heterozygous cb16 GAL4 cross:

$$♀\ \frac{w}{w};\frac{+}{+};\frac{P\{UAS-neoR\}}{P\{UAS-neoR\}} \times ♂\ \frac{w}{¬};\frac{Pin^{1}}{Cyo,P\{w[+mW.hs]GAL4=GawB\}cb16}$$

Figure 19 - Example crosses of homozygous and heterozygous GAL4 drivers to UAS-neoR. Heterozygous GAL4 drivers produce 50% GAL4 and UAS, and 50% UAS only. Pin = pin bristles.

The GAL4 drivers that I crossed to UAS-neo^{R} and their expression patterns are shown in Table 2.

Table 2 - Expression data for GAL4 drivers crossed to UAS-neo^{R}. Expression data was obtained from FlyAtlas, FlyBase, and a previous grad student in the lab[41].

GAL4 drivers	Expression data during larval development
act5c	ubiquitous
c42	principal cells of main segment (but with some cross-talk in bar-shaped cells) of the Malpighian tubule
cb13	somatic musculature (weak), CNS, PNS
cb16	EP, somatic musculature, CNS, PNS
cb21	Midgut, PNS
cb24	EP, CNS (weak), lymph gland
cb32	subset most tissues
cb37	foregut, PNS, clypeolabrum
CY2	Not established in larval stages. During oogenesis, high expression in all follicle cells over the oocyte.
elav	nervous system, ectoderm
fkh	salivary gland
twi (II) 25707	in a ventral stripe 12-14 cells wide at cellular blastoderm, TRiP, UAS-Dicer 2
twi (II) 2517	in a ventral stripe 12-14 cells wide at cellular blastoderm
twi (X) 914	embryonic mesoderm
Uro (II)	principal cells of main segment ONLY in third instar and adult Malpighian tubule
Uro (X)	principal cells of main segment ONLY in third instar and adult Malpighian tubule

The total survivorship on Day 17 for each line crossed to UAS-neo^{R} in the attP2 site and dosed at 0.45 mg/mL G418 is shown in Figure 20.

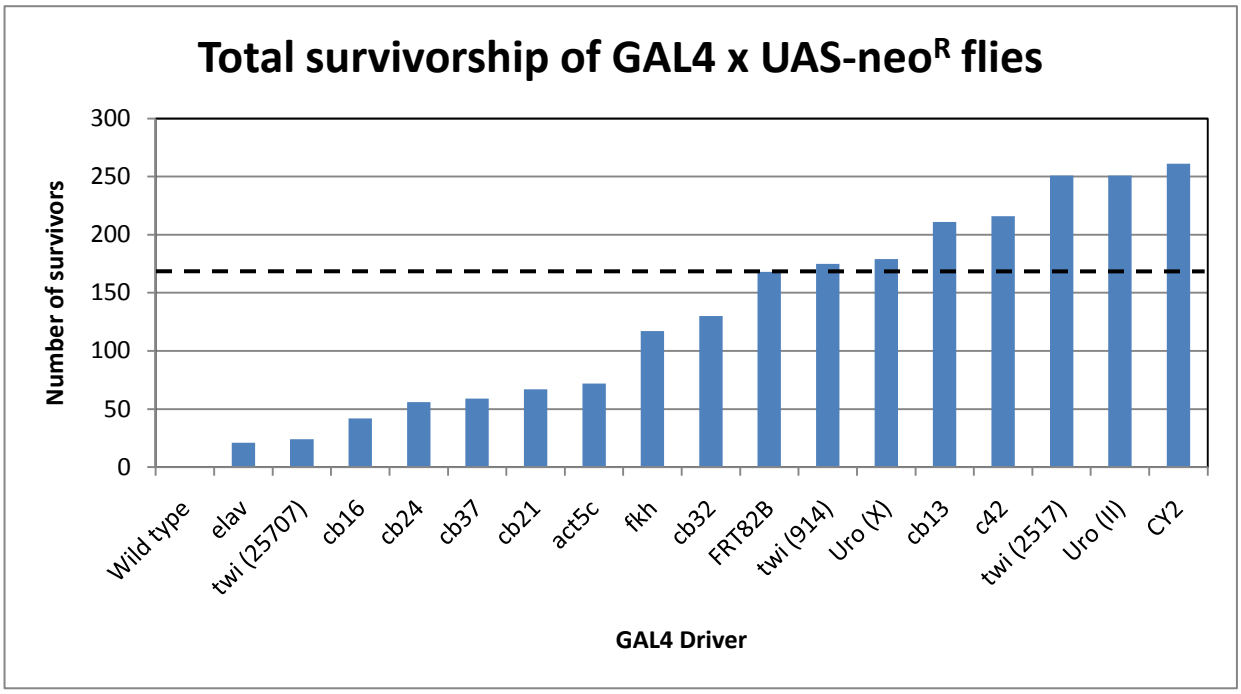


Figure 20 - Total survivorship of GAL4 x UAS-neo^R flies in 0.45 mg/mL G418. Note: the black dotted line indicates the survivorship of the flies I isolated the neo^R gene from, which express the neo^R gene ubiquitously.

Expressing neo^R in the nervous system decreases fly survivorship, as indicated by the low survival of elav and cb16 GAL4 drivers. Because the ubiquitous drivers act5c and cb32 GAL4 drivers also express in the nervous system, their decreased survivorship may be explained by a threshold effect. The hsp70 promoter that drives the expression of neo^R in the FRT82B strain expresses at low levels in all cells. The act5c and cb32 GAL4 drivers may express more GAL4 and thus neo^R when crossed to UAS-neo^R in the nervous system, which detriments fly survivorship. To test this hypothesis, I could quantify neo^R levels in nervous system tissue in FRT82B, GAL4-act5c x UAS-neo^R, and GAL4-cb 32 x UAS-neo^R flies.

cb13 was originally characterized to express only in the nervous system as well, which seems to contradict the idea that expressing neo^R in the nervous system detriments fly survivorship. However, closer analysis of the expression pattern by crossing cb13-GAL4 to a nuclear localizing UAS-GFP revealed that this GAL4 driver also strongly expresses in the

salivary gland, brain, and anterior spiracles. It also weakly expresses in the gastric caeca and just underneath the cuticle throughout the larvae.

One of the twi lines (25707) survived very poorly, while the other twi lines survived better than even FRT82B flies. twi (25707) also overexpresses Dicer 2, a protein required for RNAi. While this shouldn't have an effect on survivorship, expressing it is clearly detrimental to survivorship in some way. It is possible that overexpressing Dicer 2 interferes with the native RNAi machinery because increasing reactants (Dicer 2 + long pieces of dsRNA) drives reaction kinetics toward products (short pieces of dsDNA), enabling more RNAi to be present in the cell. If the RNAi downregulates genes important for survival such as actin, reduced survival would be expected whether or not neo^R is present. Therefore, to test this hypothesis, I could assay for survivorship with and without UAS-neo^R. I would expect to find that twi (25707) do not survive as well as the other two twi lines in both cases.

Interestingly, seven GAL4 lines crossed with UAS-neo^R performed better than the original neo^R flies, the FRT82B strain: twi (2 lines), Uro (2 lines), cb13, c42, and CY2. Uro and c42 expression is specific to the Malpighian tubule, and twi expression is specific to the mesoderm. CY2-GAL4 expresses strongly in the follicle cells during oogenesis, though its expression pattern during other developmental time points has not yet been characterized. It is interesting to note that Uro-GAL4 is only expressed in third instar larvae because they show delayed time to eclosion. This trend is shown in Figure 21.

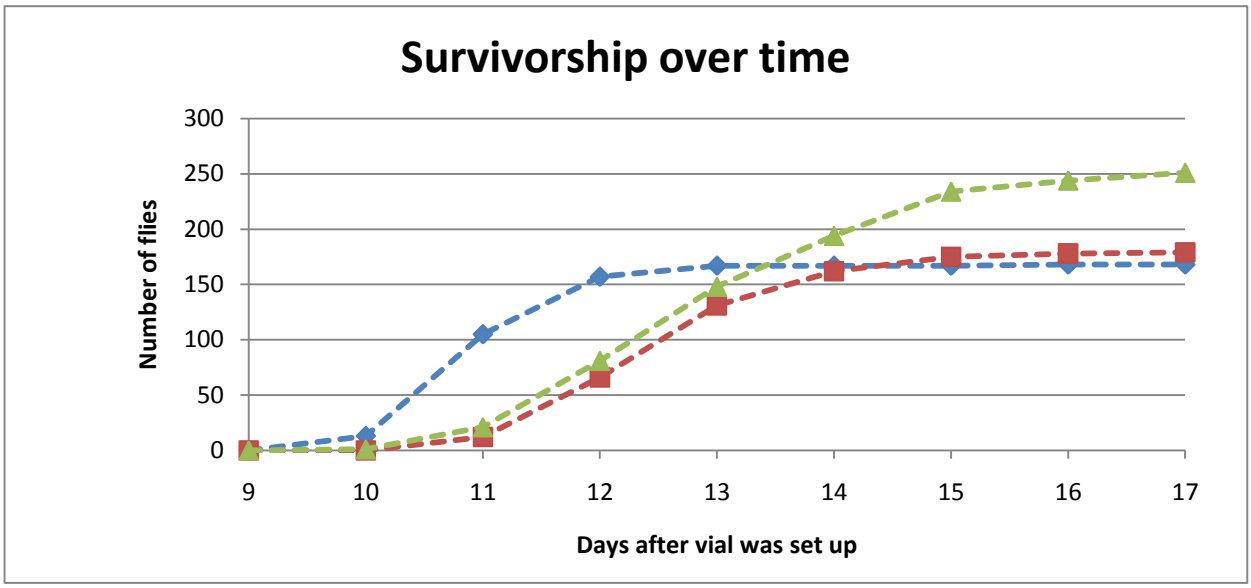


Figure 21 - Uro survivorship counts over time. **FRT82B flies,** **Uro (X) flies,** **Uro (II) flies.**

With respect to FRT82B flies, both Uro lines appeared to be delayed slightly more than one day during development. This delay likely means that the neo^{R} gene is useful during the second instar larval stage or earlier.

After observing that all UAS-neoR flies crossed with a GAL4 line had more survivorship than wild type, I wanted to ascertain whether the attP2 and attP40 UAS-neoR lines alone increased survivorship. Based on my previous data, I chose several lines that survived well and even better than FRT82B flies. The total survivorship on Day 17 for each of the GAL4 line I crossed to both UAS-neo^{R} lines and dosed at 0.45 mg/mL G418 is shown in Figure 22.

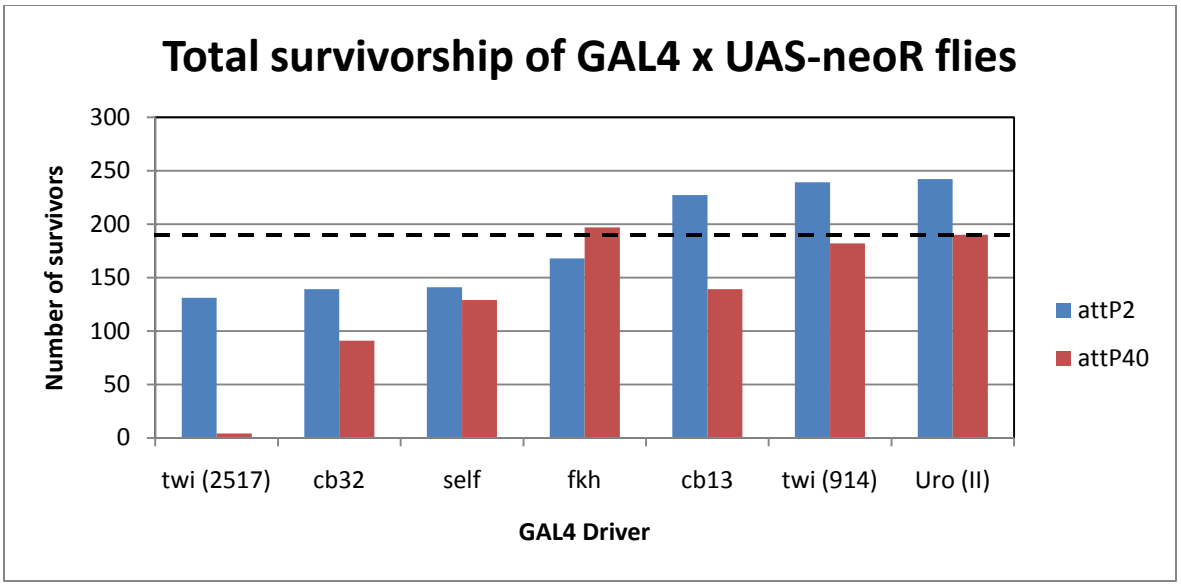


Figure 22 - Total survivorship of GAL4 x UAS-neoR flies in 0.45 mg/mL G418. Note: the black dotted line indicates the survivorship of FRT82B flies. No wild type flies survived at this drug concentration. The "self" category indicates UAS-neoR only, though its expression was homozygous.

Interestingly, the number of survivors are not in the same order as they were in the previous crosses. This result suggests that future studies should include several vials of each cross to determine statistical averages of survivorship.

RNAi Gene Knockdown Screen

My goal in screening was to see if there were any *Drosophila* genes that I could knock down to increase survivorship. Having narrowed down tissue sensitivity to the mesoderm and drug concentration to the gut, I decided to reduce the expression of genes that are normally highly expressed in these tissues in an RNAi screen. FlyAtlas is a database that researchers set up after mapping the expression of 18770 transcripts comprising the vast majority of the genome using 44 Affymetrix Dro2 expression arrays (Chintapalli et al. 2007). Essentially, FlyAtlas allows researchers to search for genes and determine how highly they are expressed in 14 different tissues. The enrichment score, which gives an indicator of expression in each tissue, tells how much higher the gene is expressed in a specific tissue than in the whole fly. Another useful resource FlyAtlas provides is a list of the 50 most highly expressed genes in each tissue. I

used this list to identify genes that could have a role in aminoglycoside sensitivity expressed highly in the Malpighian tubule, hindgut, midgut, and foregut. I also searched FlyBase and FlyAtlas for genes that previously showed some role in transporting molecules across a membrane. FlyBase is a database describing all fly gene's characterized molecular functions, biological process in which they may play a role, and subcellular localization, in addition to other genomics information. The genes that I chose to knock down are shown in Table 3.

Table 3 - Genes to knock down to see if survivorship increases. Upregulation was determined from a high enrichment score on FlyAtlas.

Gene	CG#	Description
	CG6901	Transporter activity. Upregulated in the larval and adult midgut
	CG30272	Transmembrane transporter. Upregulated in the larval and adult midgut.
TrpA1	CG5751	Calcium channel activity, thermotaxis. Upregulated in adult midgut. (Sun et al. 2009)
	CG31751	Aminoglycoside Phosphotransferase. Highly upregulated in most tissues.
	CG42611	Low-density lipoprotein receptor activity (Megalin, family containing GP330) (Moestrup et al. 1995)
ABCB7	CG14408	ATPase, heme, transmembrane transport. Up in brain, larval CNS, and salivary gland.
tko	CG7925	Mitochondrial ribosomal protein S12. Up in ovary, down in most tissues. Mechanosensory behavior. (Royden et al. 1987)
	CG18432	sentinel. Up in male accessory gland. (Owens et al. 2009)
	CG18631	sentinel. Cilium assembly. Up in hindgut, testis, larval midgut. Down in most tissues. (Owens et al. 2009)
	CG11659	Fatty acid transporter, metabolic activity. Highly up in adult tubule and down in larval tubule.
	CG9270	Transmembrane ATPase transporter. Up in growing S2 cells.
	CG33281	Monosaccharide transmembrane transporter. Up in larval and adult tubule.
	CG33282	Monosaccharide transmembrane transporter. Up in larval and adult tubule.
	CG15088	Neurotransmitter transport, potassium, amino acid symport. Up in tubule, hindgut, brain.
	CG18095	Protein binding. Up in adult and larval tubule.
	CG13905	Function unknown. Up in larval and adult tubule.
	CG3212	Scavenger receptor activity. Defense response. Up in salivary gland.
	CG15408	Transmembrane transporter. Up in larval and adult tubule.
	CG8837	Transmembrane transporter. Up in brain, CNS, tubule, testis.
	CG31106	Transmembrane transporter. Up in adult and larval tubule.
	CG14606	Transmembrane transporter, phagocytosis, engulfment. Up in hindgut and adult and larval tubule.

scarlet	CG4314	ATPase, transmembrane movement, ommochrome biosynthesis. Up in head, eye, and tubule.
Mucin 68E	CG33265	Chitin binding protein. Up in larval and adult midgut

Some of these genes have already been shown to have some interaction with aminoglycosides. For example, Glycoprotein 330 (GP330) is an endocytic receptor found in epithelia within the renal proximal tubule and inner ear cells, and binding studies show that gentamicin efficiently binds to this receptor (Moestrup et al. 1995). A study in zebrafish searched for genetic modifiers of hair cell sensitivity by randomly inducing mutations using ethylnitrosourea. Next, they treated mutants with neomycin and staining with a vital dye that is differentially taken up by neuromast hair cells called DASPEI. A high level of DASPEI staining correlates with normal hair cell function, while a low level of staining indicates hair cell damage. From this study, these researchers found that mutations in 5 genes (persephone, sentinel, trainman, bane, and merovingian) conferred resistance to neomycin (Owens et al. 2008). One of these genes, sentinel, has been cloned and sequenced and has a *Drosophila* homologue. In principal, this study is similar to mine--it is looking for genes that can be knocked out to confer some beneficial resistance to neomycin toxicity. It differs, however, in that I am searching for genes that overall help the fly cope with aminoglycosides, not a tissue-specific benefit.

A transient receptor potential (TRP) gene, TrpA1, is a component of mechanosensitive transduction channels in vertebrate hair cells. Its mRNA in hair epithelia appears at the same time as the onset of mechanosensitivity. Inhibiting its expression in mouse and zebrafish inner ears inhibits receptor function (Corey et al. 2004). In *Drosophila*, TRP genes are expressed in Johnston's organ where hearing is mediated, and several members of this gene family are required for hearing (Eberl & Boekhoff-Falk 2007).

Experimental/design decisions made by the student during the course of the project

I made changes to the larval dosing protocol between each round of dosing to decrease the variability in survivorship data. The decision for these changes came about by a discussion with Celeste. I decided that a drug tracking technique would be useful, which is where the drug-fluorophore experiment came into the project. I perused the literature to find a method previously used with a similar drug and together adapted this method to G418 with my mentor. I also decided which stages of larvae to image after the pulse-chase experiment. I had to come up with a method of adding the drug-fluorophore conjugate to the food, which proved challenging due to its poor quality. During the cloning portion of my project, my mentor and I came up with an overall strategy, and I designed primers and chose restriction sites to add and use to put the neo^R gene into the plasmid. Together, my mentor and I came up with a list of candidate genes to knock down and assay for increased tolerance to G418.

Analysis and conclusions

From the larval dosing data, it is clear that the most effective method for reducing variability was my final method: method 3. The decline of wild type occurred as expected with increasing G418 concentration. The neo^R survivorship was more variable. It slightly declined, which could be explained by the metabolic energy requirement for expressing this gene. Furthermore, it is possible that expressing the neo^R gene in some tissues, such as the nervous system, may detriment the fly more than help it. This hypothesis is evidenced by the low survivorship of the elav and cb16 GAL4 flies when crossed with UAS-neo^R flies and dosed at 0.45 mg/mL G418.

During the first dosing round of adult flies, the EMS method of drug delivery was insufficient for dosing flies. However, a second attempt revealed a key issue in the first adult

dosing method. When I added the drug and sucrose directly to the old Whatman paper used to hydrate the flies, approximately 1/3 of wild type and neo^{R} flies died, regardless of drug concentration. This result suggests that new Whatman paper should be used for the drug, which was changed in the second dosing round. It is likely that too much liquid was present in the bottle, causing some flies to drown when eating. Furthermore, adding the drug to previously hydrated Whatman paper dilutes the drug, reducing the effective concentration of drug exposure. These two reasons could explain the failure of the first adult dosing method. In the second adult dosing method, it became clear that adults were indeed affected by the drug concentration, not just starvation. Furthermore, the high dose of 50 mg/mL G418, 100 times the larval lethal dose, was enough to kill both wild-type and neo^{R} flies, suggesting that the neo^{R} enzyme was saturated. At 5 mg/mL, however, neo^{R} fly survivorship was unaffected by the drug.

Conjugating G418 to Texas Red turned out to be a successful method of tracing the drug. While other studies indicate that concentration does not necessarily correlate with toxicity, it is still interesting to note where the drug is present in high and low quantities. The fact that the drug and fluorophore were not present in the nervous system, for example, suggests that while this tissue could be sensitive to aminoglycosides, the drug does not traffic there. This point may be true in humans as well since few drugs effectively cross the blood-brain barrier.

I chose to use the attP2 and attP40 injection sites for the UAS-neo^{R} construct because other researchers found that these sites had low basal levels of expression and some of the highest levels of inducible expression in the nervous system, fat body, and muscle (Markstein et al. 2008). However, one issue that I came across was that the basal level was not necessarily low enough to act like wild-type. At 0.45 mg/mL G418, all Canton S flies died, while attP2 and attP40 UAS-neo^{R} flies alone with no GAL4 driver survived at low levels, as shown in Figure 22.

Nonetheless, I was able to rescue more flies than in the starting strain, FRT82B, with some GAL4 drivers, indicating that a higher level of inducible expression than expected was achieved. Interestingly, some of the GAL4-UAS-neo^{R} crosses resulted in lower survivorship than the UAS-neoR alone, suggesting that neoR expressed in these tissues is detrimental.

Suggestions for future work

While the RNAi screen is useful for screening a small number of candidate genes, a high-throughput method of screening the genome for genes that are differentially expressed when G418 is present would also be useful. One potential method of screening would be to perform a microarray (Schena et al. 1995) to find the most upregulated or downregulated genes when dosed with high concentrations of G418. Others have used microarray analysis to identify genes that are differentially expressed in an altered environment, then analyzed the role of these genes through mutational analysis. For example, one group performed a microarray analysis of flies on a high sugar diet and during starvation. Upon sugar ingestion, they found that *sugarbabe* is the highest and earliest activated gene. Further mutational analysis revealed that this zinc finger protein is involved in dietary fat breakdown and absorption (Zinke et al. 2002). Based on their success in using this method, it is plausible that performing a microarray of DNA from flies that have ingested G418 would reveal genes that are crucial for getting rid of the drug. Many of the genes identified in a genome-wide screen are likely to have unknown functions in *Drosophila*, so it will be useful to refer to studies performed on homologous genes in other organisms to determine whether or not they are interesting in the context of aminoglycoside tolerance.

A method of sorting out which genes are interesting would need to be carefully analyzed. For example, in humans, cytochrome p450 interacts with up to 1/5 of drugs. A mutant homolog of this gene that increases tolerance would be interesting, though not necessarily specific to

aminoglycosides. Therefore, an alternative method of screening the genome to find genes of interest would be to dose flies that are mutant in a large number of genes with a dose lethal to wild-type flies to see if survivorship is increased. While this method would take considerably longer and be more costly, the only genes identified would increase survivorship of aminoglycoside toxicity.

It would also be interesting to make a construct with UAS-neoR tagged with a reporter. By doing this, it would be possible to track the neoR gene and see if neoR and G418 co-localize in drug conjugation studies.

The drug dosing methods that I used in the project indicated that both larval and adult wild type flies are indeed affected by a range of G418 concentrations. Furthermore, using the GAL4-UAS tissue-specific expression system indicated that some tissues are differentially sensitive to aminoglycosides. These results suggest that the fruit fly can indeed be used as a realistic model to understand the molecular basis of toxicity, as well as why some tissues are more sensitive than others. In addition, the fact that *Drosophila* has well developed genetic tools indicate that it can be used to identify key players in clearing aminoglycosides from the system. A genome-wide analysis would likely add many more genes to the short list of those already identified as important in aminoglycoside clearance. Furthermore, additional characterization of gene function identified using high-throughput methods would be far simpler in *Drosophila* than in humans, primates, zebrafish, and other organisms used previously to identify genetic modifiers of aminoglycoside toxicity.

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Appendix

UAS-neoR sequence from the UAS to SV40 region (over the neoR insertion). Contig assembled using Sequencher:

```
UAS_SV40 ----------------------------GGATCCAAGCTTGCATGCC
5'neoRF2 ----------------------------------------------
3'neoRR2 ----------------------------------------------
AAR1     ----------------------------------------------
AAF1     GNCTCNAAGTTGCTTCGAAGTTTGCTAGCGGATCCA-GCTTGCATGCC

UAS_SV40 TGCAGGTCGGAGTACTGTCCTCCGAGCGGAGTACTGTCCTCCGAGCGG
5'neoRF2 ----------------------------------------------
3'neoRR2 ----------------------------------------------
AAR1     ----------------------------------------------
AAF1     TGCAGGTCGGAGTNCTGTCGTCCGAGCGGAGTACTGTCCTCCGAGCGG

UAS_SV40 AGTACTGTCCTCCGAGCGGAGTACTGTCCTCCGAGCGGAGTACTGTCC
5'neoRF2 ----------------------------------------------
3'neoRR2 ----------------------------------------------
AAR1     ----------------------------------------------
AAF1     AGTACTGTCCTCCGAGCGGAGTACTGTCCTCCGAGCGGAGTACTGTCC

UAS_SV40 TCCGAGCGGAGACTCTAGCGAGCGCCGGAGTATAAATAGAGGCGCTTC
5'neoRF2 ----------------------------------------------
3'neoRR2 ----------------------------------------------
AAR1     ----------------------------------------------
AAF1     TCCGAGCGGAGACTCTAGCGAGCGCCGGAGTATAAATAGAGGCGCTTC

UAS_SV40 GTCTACGGAGCGACAATTCAATTCAAACAAGCAAAGTGAACACGTCGC
5'neoRF2 ----------------------------------------------
3'neoRR2 ----------------------------------------------
AAR1     ----------------------------------------------
AAF1     GTCTACGGAGCGACAATTCAATTCAAACAAGCAAAGTGAACACGTCGC

UAS_SV40 TAAGCGAAAGCTAAGCAAATAAACAAGCGCAGCTGAACAAGCTAAACA
5'neoRF2 ----------------------------------------------
3'neoRR2 ----------------------------------------------
AAR1     ----------------------------------------------
AAF1     TAAGCGAAAGCTAAGCAAATAAACAAGCGCAGCTGAACAAGCTAAACA

UAS_SV40 ATCTGCAGTAAAGT-GCAAGTTAAAGTGAATCAATT-AAAAGTAACC-
5'neoRF2 ---------NTCGTAGGCAGTTAA-GTGAATCAATT-AAAAGTAACC-
3'neoRR2 ----------------------------NGAATCAATTNAAAAGTAACCC
AAR1     ----------------------------------------------
AAF1     ATCTGCAGTAAAGT-GCAAGTTAAAGTGAATCAATT-AAAAGTAACC-

UAS_SV40 AGCAACC-AAGTAAA-T-CAACTGCAACT-ACTGAAATCTG-CCAAGA
5'neoRF2 AGCAACC-AAGTAAA-T-CAACTGCAACT-ACTGCNATCTG-CCAAGA
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3'neoRR2 AGCAACCCAAGTAAAATTCAACTGCNACTTACTGAAATNTGNCCAAGA
AAR1     ----------------------------------------------
AAF1     AGCAACC-AAGTAAA-T-CAACTGCAACT-ACTGAAATCTG-CCAAGA

UAS_SV40 A-GTAATTATTGAATACAAGAA-GAGAACTCTGAATAGGG-AATTGGG
5'neoRF2 A-GTAATTATTGAATACAAGAA-GAGAACTCTGAATAGGG-AATTGGG
3'neoRR2 ANGTAATTATTGAATACAAGAAAGAGAACTCTGAATAGGGGAATTGGG
AAR1     ----------------------------------------------
AAF1     A-GTAATTATTGAATACAAGAA-GAGAACTCTGAATAGGG-AATTGGG

UAS_SV40 AATTCGTTAACAGATCTGCGGCCGCGG-CTCGAGCCACATGATTGAAC
5'neoRF2 AATTCGTTAACAGATCTGCGGCCGCGG-CTCGAGCCACATGATTGAAC
3'neoRR2 AATTCGTTAACAGATCTGCGGCCGCGGNCTCGAGCCACATGATTGAAC
AAR1     ----------------------------------------------
AAF1     AATTCGTTAACAGATCTGCGGCCGCGG-CTCGAGCCACATGATTGAAC

UAS_SV40 AAGATGGATTGCACGCAGGTTCTCCGGCCGCTTGGGTGGAGAGGCTAT
5'neoRF2 AAGATGGATTGCACGCAGGTTCTCCGGCCGCTTGGGTGGAGAGGCTAT
3'neoRR2 AAGATGGATTGCACGCAGGTTCTCCGGCCGCTTGGGTGGAGAGGCTAT
AAR1     ----------------------------------------------
AAF1     AAGATGGATTGCACGCAGGTTCTCCGGCCGCTTGGGTGGAGAGGCTAT

UAS_SV40 TCGGCTATGACTGGGCACAACAGACAATCGGCTGCTCTGATGCCGCCG
5'neoRF2 TCGGCTATGACTGGGCACAACAGACAATCGGCTGCTCTGATGCCGCCG
3'neoRR2 TCGGCTATGACTGGGCACAACAGACAATCGGCTGCTCTGATGCCGCCG
AAR1     ----------------------------------------------
AAF1     TCGGCTATGACTGGGCACAACAGACAATCGGCTGCTCTGATGCCGCCG

UAS_SV40 TGTTCCGGCTGTCAGCGCAGGGGCGCCCGGTTCTTTTTGTCAAGACCG
5'neoRF2 TGTTCCGGCTGTCAGCGCAGGGGCGCCCGGTTCTTTTTGTCAAGACCG
3'neoRR2 TGTTCCGGCTGTCAGCGCAGGGGCGCCCGGTTCTTTTTGTCAAGACCG
AAR1     ----------------------------------------------
AAF1     TGTTCCGGCTGTCAGCGCAGGGGCGCCCGGTTCTTTTTGTCAAGACCG

UAS_SV40 ACCTGTCCGGTGCCCTGAATGAACTGCAGGACGAGGCAGCGCGGCTAT
5'neoRF2 ACCTGTCCGGTGCCCTGAATGAACTGCAGGACGAGGCAGCGCGGCTAT
3'neoRR2 ACCTGTCCGGTGCCCTGAATGAACTGCAGGACGAGGCAGCGCGGCTAT
AAR1     ----------------------------------------------
AAF1     ACCTGTCCGGTGCCCTGAATGAACTGCAGGACGAGGCAGCGCGGCTAT

UAS_SV40 CGTGGCTGGCCACGACGGGCGTTCCTTGCGCAGCTGTGCTCGACGTTG
5'neoRF2 CGTGGCTGGCCACGACGGGCGTTCCTTGCGCAGCTGTGCTCGACGTTG
3'neoRR2 CGTGGCTGGCCACGACGGGCGTTCCTTGCGCAGCTGTGCTCGACGTTG
AAR1     ----------------------------------------------
AAF1     CGTGGCTGGCCACGACGGGCGTTCCTTGCGCAGCTGTGCTCGACGTTG

UAS_SV40 TCACTGAAGCGGGAAGGGACTGGCTGCTATTGGGCGAAGTGCCGGGGC
5'neoRF2 TCACTGAAGCGGGAAGGGACTGGCTGCTATTGGGCGAAGTGCCGGGGC
3'neoRR2 TCACTGAAGCGGGAAGGGACTGGCTGCTATTGGGCGAAGTGCCGGGGC
AAR1     ----------------------------------------------
AAF1     TCACTGAAGCGGGAAGGGACTGGCTGCTATTGGGCGAAGTGCCGGGGC

UAS_SV40 AGGATCTCCTGTCATCTCACCTTGCTCCTGCCGAGAAAGTATCCATCA
5'neoRF2 AGGATCTCCTGTCATCTCACCTTGCTCCTGCCGAGAAAGTATCCATCA
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3'neoRR2 AGGATCTCCTGTCATCTCACCTTGCTCCTGCCGAGAAAGTATCCATCA
AAR1     ----------------------------------------------
AAF1     AGGATCTCCTGTCATCTCACCTTGCTCCTGCCGAGAAAGTATCCATCA

UAS_SV40 TGGCTGATGCAATGCGGCGGCTGCATACGCTTGATCCGGCTACCTGCC
5'neoRF2 TGGCTGATGCAATGCGGCGGCTGCATACGCTTGATCCGGCTACCTGCC
3'neoRR2 TGGCTGATGCAATGCGGCGGCTGCATACGCTTGATCCGGCTACCTGCC
AAR1     ----------------------------------------------
AAF1     TGGCTGATGCAATGCGGCGGCTGCATACGCTTGATCCGGCTACCTGCC

UAS_SV40 CATTCGACCACCAAGCGAAACATCGCATCGAGCGAGCACGTACTCGGA
5'neoRF2 CATTCGACCACCAAGCGAAACATCGCATCGAGCGAGCACGTACTCGGA
3'neoRR2 CATTCGACCACCAAGCGAAACATCGCATCGAGCGAGCACGTACTCGGA
AAR1     ----------------------------------------------
AAF1     CATTCGACCACCAAGCGAAACATCGCATCGAGCGAGCACGTACTCGGA

UAS_SV40 TGGAAGCCGGTCTTGTCGATCAGGATGATCTGGACGAAGAGCATCAGG
5'neoRF2 TGGAAGCCGGTCTTGTCGATCAGGATGATCTGGACGAAGAGCATCAGG
3'neoRR2 TGGAAGCCGGTCTTGTCGATCAGGATGATCTGGACGAAGAGCATCAGG
AAR1     ----------------------------------------------
AAF1     TGGAAGCCGGTCTTGTCGATCAGGATGATCTGGACGAAGAGCATCAGG

UAS_SV40 GGCTCGCGCCAGCCGAACTGTTCGCCAGGCTCAAGGCGCGCATGCCC-
5'neoRF2 GGCTCGCGCCAGCCGAACTGTTCGCCAGGCTCAAGGCGCGCATGCCC-
3'neoRR2 GGCTCGCGCCAGCCGAACTGTTCGCCAGGCTCAAGGCGCGCATGCCC-
AAR1     ----------------------------------------------
AAF1     GGCTCGCGCCAGCCGAACTGTTCGCCAGGCTCAAGGCGCGCATGCCCC

UAS_SV40 GACGGCGAGGATCTCGTCGTGACCCATGGCGATGCCTGCTTGCCGAAT
5'neoRF2 GACGGCGAGGATCTCGTCGTGACCCATGGCGATGCCTGCTTGCCGAAT
3'neoRR2 GACGGCGAGGATCTCGTCGTGACCCATGGCGATGCCTGCTTGCCGAAT
AAR1     ----------------------------------------------
AAF1     GACGGCGAGGATCTCGTCGTGACCCATGGCGATGCCTGCTTGCCGAAT

UAS_SV40 ATCATGGTGGAAAATGGCCGCTTTTCTGGATTCATCGACTGTGGCCGG
5'neoRF2 ATCATGGTGGAAAATGGCCGCTTTTCTGGATTCATCGACTGTGGCCGG
3'neoRR2 ATCATGGTGGAAAATGGCCGCTTTTCTGGATTCATCGACTGTGGCCGG
AAR1     ----------------------------------------------
AAF1     ATCATGGTGGAAA-------------------------------------

UAS_SV40 CTGGGTGTGGCGGACCGCTATCAGGACATAGCGTTGGCTACCCGTGAT
5'neoRF2 CTGGGTGTGGCGGACCGCTATCAGGACATAGCGTTGGCTACCCGTGAT
3'neoRR2 CTGGGTGTGGCGGACCGCTATCAGGACATAGCGTTGGCTACCCGTGAT
AAR1     ----------------------------------------------
AAF1     ----------------------------------------------

UAS_SV40 ATTGCTGAAGAGCTTGGCGGCGAATGGGCTGACCGCTTCCTCGTGCTT
5'neoRF2 ATTGCTGAAGAGCTTGGCGGCGAATGGGCTGACCGCTTCCTCGTGCTT
3'neoRR2 ATTGCTGAAGAGCTTGGCGGCGAATGGGCTGACCGCTTCCTCGTGCTT
AAR1     ----------------------------------------------
AAF1     ----------------------------------------------

UAS_SV40 TACGGTATCGCCGCTCCCGATTCGCAGCGCATCGCC-TTCTATCGCC-
5'neoRF2 TACGGTATCGCCGCTCCCGATTCGCAGCGCATCGCCNTTCTATCGCCN
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3'neoRR2 TACGGTATCGCCGCTCCCGATTCGCAGCGCATCGCC-TTCTATCGCC-
AAR1     --------------TCCCGATTCGCAGCGCATCGCC-TTCTATCGCN-
AAF1     ----------------------------------------------

UAS_SV40 TTCTTGACGAGTTCTTCTGATCTAGAGGATCTTTGTGAAGGAACCTT-
5'neoRF2 TTCTTGACGAGTTCTTCTGATCTANAGGATCTTTGTGAAGGAACCTTT
3'neoRR2 TTCTTGACGAGTTCTTCTGATCTAGAGGATCTTTGTGAAGGAACCTT-
AAR1     TTCTTGACGAGTTNTTCTGATCTAGAGGATCTTTGTGAAGGAACCTT-
AAF1     ----------------------------------------------

UAS_SV40 ACTTCTGTGGTGTGACATAATTGGACAAACTACCTACAGAGATTTAAA
5'neoRF2 ACTTCTGTGGTGTGACATAATTGGACAAACTACCTACAGAAATTTAAA
3'neoRR2 ACTTCTGTGGTGTGACATAATTGGACAAACTACCTACAGAGATTTAAA
AAR1     ACTTCTGTGGTGTGACATAATTGGACAAACTACCTACAGAGATTTAAA
AAF1     ----------------------------------------------

UAS_SV40 GCTCTAAGGTAAA-TATAAAATTTTT-AAGTGTATAATGTGTT-AAAC
5'neoRF2 GCTCTAAGGTAAAATATAAAATTTTN-AAGTGTATAATGGGTTNAAAC
3'neoRR2 GCTCTAAGGTAAA-TATAAAATTTTT-AAGTGTATAATGTGT--AAAC
AAR1     GCTTTAAGGTAAA-TATAAAATTTTTTAAGTGTATAATGTGTT-AAAC
AAF1     ----------------------------------------------

UAS_SV40 TACTGATTCTAATTGTTTG-TGTATTTTAGATTCCAACCTATGGAACT
5'neoRF2 TACTGATTCTAATTGTTTG-NG-------------------------
3'neoRR2 TACTGATTCTAAT-GTTGGAT-------------------------
AAR1     TACTGATTCTAATTGTTTG-TGTATTTTAGATTCCAACCTATGGAACT
AAF1     ----------------------------------------------

UAS_SV40 GATGAATGGGAGCAGTGGTGGAATGCCTTTAATGAGGAAAACCTGTTT
5'neoRF2 ----------------------------------------------
3'neoRR2 ----------------------------------------------
AAR1     GATGAATGGGAGCAGTGGTGGAATGCCTTTAATGAGGAAAACCTGTTT
AAF1     ----------------------------------------------

UAS_SV40 TGCTCAGAAGAAATGCCATCTAGTGATGATGAGGCTACTGCTGACTCT
5'neoRF2 ----------------------------------------------
3'neoRR2 ----------------------------------------------
AAR1     TGCTCAGAAGAAATGCCATCTAGTGATGATGAGGCTACTGCTGACTCT
AAF1     ----------------------------------------------

UAS_SV40 CAACATTCTACTCCTCCAAAAAAGAAGAGAAAGGTAGAAGACCCCAAG
5'neoRF2 ----------------------------------------------
3'neoRR2 ----------------------------------------------
AAR1     CAACATTCTACTCCTCCAAAAAAGAAGAGAAAGGTAGAAGACCCCAAG
AAF1     ----------------------------------------------

UAS_SV40 GACTTTCCTTCAGAATTGCTAAGTTTTTTGAGTCATGCTGTGTTTAGT
5'neoRF2 ----------------------------------------------
3'neoRR2 ----------------------------------------------
AAR1     GACTTTCCTTCAGAATTGCTAAGTTTTTTGAGTCATGCTGTGTTTAGT
AAF1     ----------------------------------------------

UAS_SV40 AATAGAACTCTTGCTTGCTTTGCTATTTACACCACAAAGGAAAAAGCT
5'neoRF2 ----------------------------------------------
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3'neoRR2 --------------------------------------------------
AAR1     AATAGAACTCTTGCTTGCTTTGCTATTTACACCACAAAGGAAAAAGCT
AAF1     --------------------------------------------------

UAS_SV40 GCACTGCTATACAAGAAAATTATGGAAAAATATTTGATGTATAGTGCC
5'neoRF2 ---------------------------------------------------
3'neoRR2 ---------------------------------------------------
AAR1     GCACTGCTATACAAGAAAATTATGGAAAAATATTTGATGTATAGTGCC
AAF1     ---------------------------------------------------

UAS_SV40 TTGACTAGAGATCATAATCAGCCATACCACATTTGTAGAGGTTTTACT
5'neoRF2 ----------------------------------------------------
3'neoRR2 ----------------------------------------------------
AAR1     TTGACTAGAGATCATAATCAGCCATACCACATTTGTAGAGGTTTTACT
AAF1     ----------------------------------------------------

UAS_SV40 TGCTTTAAAAAACCTCCCACACCTCCCCCTGAACCTGAAACATAAAAT
5'neoRF2 -----------------------------------------------------
3'neoRR2 -----------------------------------------------------
AAR1     TGCTTTAAAAAACCTCCCACACCTCCCCCTGAACCTGAAACATAAAAT
AAF1     -----------------------------------------------------

UAS_SV40 GAATGCAATTGTTGTTGTTAACTTGTTTATTGCAGCTTATAATGGTTA
5'neoRF2 -----------------------------------------------------
3'neoRR2 -----------------------------------------------------
AAR1     GAATGCAATTGTTGTTGTTAACTTGTTTATTGCAGCTTATAATGGTTA
AAF1     -----------------------------------------------------

UAS_SV40 CAAATAAAGCAATAGCATCACAAATTTCACAAATAAAGCATTTTTTTC
5'neoRF2 -----------------------------------------------------
3'neoRR2 -----------------------------------------------------
AAR1     CAAATAAAGCAATAGCATCACAAATTTCACAAATAAAGCATTTTTTTC
AAF1     -----------------------------------------------------

UAS_SV40 ACTGCATTCTAGTTGTGGTTTGTCCAAACTCATCAATGTATCTTATCA
5'neoRF2 -----------------------------------------------------
3'neoRR2 -----------------------------------------------------
AAR1     ACTGCATTCTAGTTGTGGTTTGTCCAAACTCATCAATGTATCTTATCA
AAF1     -----------------------------------------------------

UAS_SV40 TGTCTG-----------------------------------------------
5'neoRF2 -----------------------------------------------------
3'neoRR2 -----------------------------------------------------
AAR1     TGTCTGGATCCACTAGTGTCGACGATGTAGGTCACGGTCTCGAAGCCG
AAF1     -----------------------------------------------------

UAS_SV40 -----------------------------------------------------
5'neoRF2 -----------------------------------------------------
3'neoRR2 -----------------------------------------------------
AAR1     CGGTGCGGGTGCCAGGGCGTGCCCTTGGGCTCCCCGGGCGCGTACTCC
AAF1     -----------------------------------------------------

UAS_SV40 -----------------------------------------------------
5'neoRF2 -----------------------------------------------------
```

```
3'neoRR2 ------------------------------------------------
AAR1     ACCTCACCCATCTGGTCCATCATGATGAACGGGTCGAGGTGGCGGTAG
AAF1     ------------------------------------------------

UAS_SV40 ------------------------------------------------
5'neoRF2 ------------------------------------------------
3'neoRR2 ------------------------------------------------
AAR1     TTGATCCCGGCGAACGCGCGGCGCACCGGGAAGCCCTCGCCCTCGAAA
AAF1     ------------------------------------------------

UAS_SV40 ------------------------------------------------
5'neoRF2 ------------------------------------------------
3'neoRR2 ------------------------------------------------
AAR1     CCGCTGGGCGCGGTGGTCACGGTGAGCACGGGACGTGCGACGGCGTCG
AAF1     ------------------------------------------------

UAS_SV40 ------------------------------------------------
5'neoRF2 ------------------------------------------------
3'neoRR2 ------------------------------------------------
AAR1     GCGGGTGCGGATACGCGGGGCAGCGTCAGCGGGTTCTCGACGGTCACG
AAF1     ------------------------------------------------

UAS_SV40 ---------------------------------------
5'neoRF2 ---------------------------------------
3'neoRR2 ---------------------------------------
AAR1     GCGGGCATGTCGACACTAGTTCAGCCAGCTTTCCNTTAN
AAF1     ---------------------------------------
```