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## Determining the Transcription Factor Genes Populating a Fruit Fly Pigmentation Gene Network and Their Regulatory Connections

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# **Determining the Transcription Factor Genes Populating a Fruit Fly Pigmentation Gene Network and Their Regulatory Connections**



Honors Thesis

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Advisor: Thomas Williams, Ph.D.

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## **Acknowledgements**

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## **ABSTRACT**

Morphological traits for organisms result from the concerted action of numerous genes that are interconnected into a gene network at the level of transcriptional regulation. In each network, transcription factors control the spatial, temporal, and even sex-specific patterns of gene transcription. To better understand how a gene network operates during development, I investigated the network controlling a male-specific pattern of *Drosophila melanogaster* abdominal pigmentation. Using RNA interference, I reduced the expression of 558 transcription factor genes to identify those needed for normal pigmentation by the occurrence of aberrant pigmentation patterns. From this, I identified 28 genes, which include several that are known to play major roles in establishing animal body plans and that regulate chromatin structure. With this new wealth of known network genes and the diversity of pigmentation patterns among fruit fly species, my thesis supports future studies into the gene network basis for trait development and evolution.

## **CHAPTER I: INTRODUCTION**

All animals possess a specific set of morphological traits that defines them, and the instructions to make these traits are encoded in its DNA sequence. Since many organisms share a considerable amount of the same genetic sequences, the question arises as to how diverse traits are formed. One explanation is that diversity results from variation in how genes are expressed, which has evolved over time to produce the wide variety of traits that exists in the animal kingdom. Thus, understanding gene expression regulation is essential to understand development and understanding how gene expression evolves is essential to understand evolution.

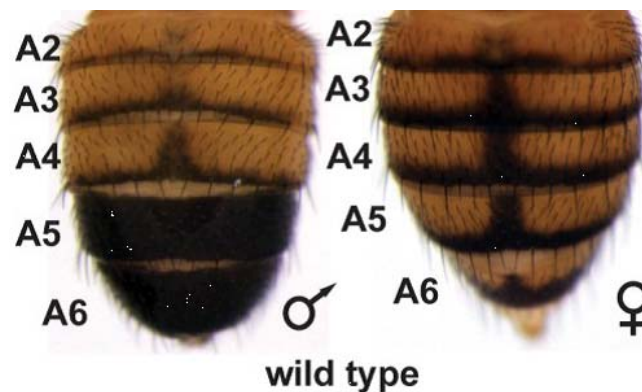
For any morphological trait, a **Gene Regulatory Network (GRNs)** is responsible for controlling the timing during development and the anatomical location where the relevant genes are expressed (Arnone & Davidson, 1997). At the heart of a GRN are transcription factor genes that encode proteins that bind to DNA in a sequence specific manner, and differentiation genes that encode proteins responsible for making a cell or tissue phenotype (Davidson, 2006). The expression patterns for network genes are controlled by a ***cis*-regulatory element (CRE)** or CREs. These DNA sequences are usually a non-coding section of DNA that typically possess binding sites for two or more transcription factor proteins (Arnone & Davidson, 1997).

Locating CREs is a complex task because they need not be near the genes that they regulate. While CREs can be found near these genes, they have also been found located far from the gene being regulated (Lettice et al., 2003) and in some cases on an entirely different chromosome (Spilianakis, Lalioti, Town, Lee, & Flavell, 2005). Transcription factors play prominent roles within a GRN as they regulate the expression

of numerous target genes, which can include additional transcription factors and differentiation genes (Weatherbee et al., 1999). In one study, over 500 target genes involved in mesoderm formation in *Drosophila (D.) melanogaster* were found to be regulated by a single transcription factor called Twist (Sandmann et al., 2007). While it remains unknown whether a similar number of target genes exist for most transcription factors, another study looked at 67 transcription factors in the *Drosophila* genome and estimated that each controls approximately 124 target genes (Stark et al., 2007). Considering the pleiotropy, which means to have multiple uses, of the transcription factors an organism possesses, the extensive variation in expression allowed is impressive.

Evolution of gene regulatory networks is a key component of variation in morphological traits across species. The main process through which these networks evolve has been reasoned to be through the evolution of CREs (Carroll, 2008). CREs can functionally evolve when there is a mutation in its sequence, leading to a gain or loss in binding sites for a transcription factor. Because the connectivity of a gene regulatory network is by linkages between a transcription factor and CRE binding sites, the mutations in a CRE can cause drastic changes in the network (Carroll, 2008). Although several gene regulatory networks have been thoroughly characterized, such as that for the formation of the *Drosophila melanogaster* mesoderm (Sandmann et al., 2007) and *Drosophila melanogaster* heart specification (Davidson & Erwin, 2006), the mechanisms of their evolution have not. In order to understand the linkages of a network and how those linkages evolve, an easily studied trait that has evolved over more recent time frames needs to be subjected to a GRN analysis.

The model I chose to study was the gene regulatory network that controls the formation of the dorsal abdominal pigmentation of *Drosophila melanogaster*. The abdomen of the fruit fly is made up of ten segments, referred to as A1 to A10, with A1 being the most anterior segment. The dorsal cuticle surface covering each of the A2 to A7 segments is referred to as a tergite. *Drosophila melanogaster* females have a line of dark pigmentation near the posterior edge of each tergite (Wittkopp, Carroll, & Kopp, 2003). *Drosophila melanogaster* displays sexually dimorphic pigmentation, meaning the pigmentation pattern differs between males and females (Figure 1). Specifically, males have fully pigmented **tergites** that cover the dorsal surface of the A5 and A6 abdominal segments, whereas the tergites covering the A2-A4 segments are pigmented similar to those covering the female abdominal segments (Williams et al., 2008). While this trait is a good model because it can be visualized easily, it also has undergone recent evolution in the *Drosophila* lineage (Salomone, Rogers, Rebeiz, & Williams, 2013), presenting opportunities to study the evolution of this gene regulatory network.



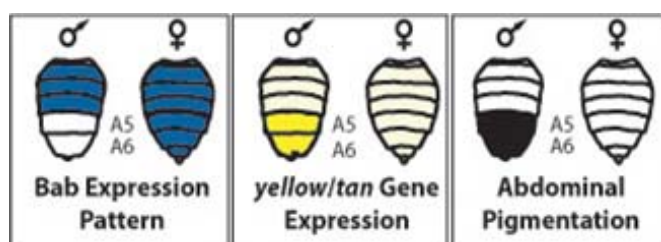
**Figure 1.** *Drosophila melanogaster* wild-type dorsal abdominal pigmentation.

For *Drosophila melanogaster*, the molecular mechanisms underlying the development and formation of abdominal pigmentation has been subjected to genetic



investigation, and many of the genes that encode enzymes involved in pigment metabolism have been identified (Wittkopp et al., 2003). The enzymes encoded by two genes have been identified as being necessary in forming black tergite pigmentation. The gene *yellow* is necessary to produce the black melanin pigment, most noticeably seen in the A5 and A6 segments of *Drosophila melanogaster* males. The Yellow protein is involved in melanin synthesis (Wittkopp, True, & Carroll, 2002). The *tan* gene also functions in producing black pigmentation because the enzyme it encodes is necessary for the production of dopamine, which in turn is involved in the production of black melanin (True et al., 2005). *ebony* is another important pigmentation gene, but the encoded Ebony enzyme functions in an opposite manner as *tan* and *yellow* to promote the yellow colored cuticle (Rogers et al., 2014). It does not function in the formation of black pigmentation, shown by studies with a mutated *ebony* gene that resulted in an increase in black coloring of fruit flies (Wittkopp et al., 2002).

In addition, a few transcription factors were known that play a role in regulating the expression of the pigmentation enzyme genes. The *bab* locus contains two genes, *bab1* and *bab2*, which both work to repress pigmentation (Couderc et al., 2002; Kopp, Duncan, & Carroll, 2000). *bab* shows sexual dimorphic expression, appearing in segments A2 to A6 in females but only weakly in the A2 to A4 in males (Salomone et al., 2013), which allows males' black pigmentation to develop in A5 and A6 (Figure 2).

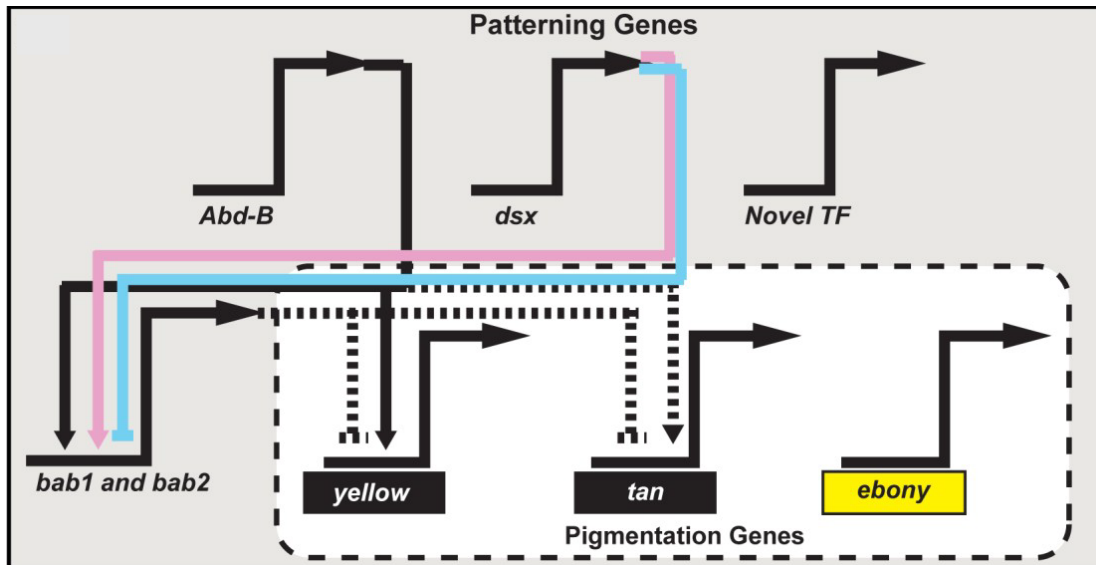


**Figure 2.** Gene expression patterns of pigmentation (*yellow* and *tan*) and patterning genes (*bab1* and *bab2* = *bab*), and the phenotypes those produce.

The *bab* genes are regulated by the *Hox* gene *Abd-B*, which encodes a transcription factor that is the modulator that allows the activation of *bab* in A5 and A6 of females (Williams et al., 2008). *Hox* genes are involved in producing the basic morphology in an organism, which for *Drosophila* is their segmentation (Ronshaugen, McGinnis, & McGinnis, 2002). In females, Abd-B and the female isoform of the DSX (DSX<sup>F</sup>) transcription factor activate *bab* expression in the A5 and A6 segments which results in the repression of *yellow* and *tan* expression and thereby no melanic pigmentation (Jeong et al., 2008; Jeong, Rokas, & Carroll, 2006). The male isoform of DSX (DSX<sup>M</sup>) works in conjunction with Abd-B to repress *bab* expression in the male A5 to A6 segments (W. A. Rogers et al., 2013; Williams et al., 2008). Abd-B and DSX, male and female isoforms, bind to a CRE called the dimorphic element which is located in the non-coding sequence of the *bab1* gene. The connection between Abd-B and DSX and the dimorphic element is part of what allows the pigmentation genes *yellow*, *tan*, and *ebony* to be expressed and form the wild-type male and female dorsal abdominal pigmentation (Williams et al., 2008). All the aforementioned linkages were used to depict the gene regulatory network that currently represents our understanding of the formation of *Drosophila melanogaster* abdominal pigmentation (Figure 3).

The major goal of my thesis research was to determine the size of the regulatory tier of the gene regulatory network for *Drosophila melanogaster*'s sexually dimorphic abdominal pigmentation. As described above, the current knowledge suggested that the regulatory tier of this network is small, consisting of four transcription factors (Abd-B, DSX, Bab1, and Bab2) and the CREs regulating three genes (*yellow*, *tan*, *bab1*, and *bab2*) (Rogers et al., 2014). When compared to the aforementioned incredibly complex

networks that have been functionally and hierarchically described, the network underlying abdominal pigmentation seemed too simple.



**Figure 3.** Diagram of the current understanding of the GRN governing *D. melanogaster* abdominal pigmentation. The solid lines imply direct relationships where a transcription factor binds to a CRE controlling the regulated gene's expression. The dashed lines represent uncertainty of a direct or indirect relationship. The lines with arrow heads represent transcriptional activation and those with straight-lined heads represent repression. The Novel TF arrow represents other transcription factors that may be involved in the network.

I hypothesized that the tergite pigmentation gene regulatory network was more complex than current research suggested and that there would be more transcription factors involved in producing *Drosophila melanogaster* abdominal pigmentation. In order to investigate this hypothesis, I was part of a team that performed an RNA interference (RNAi) screen on 558 transcription factors to find genes of interest, which were ones that had F1 progeny that exhibited an abnormal tergite pigmentation phenotype. In order to further characterize the network, I was also interested in what type of pigment change

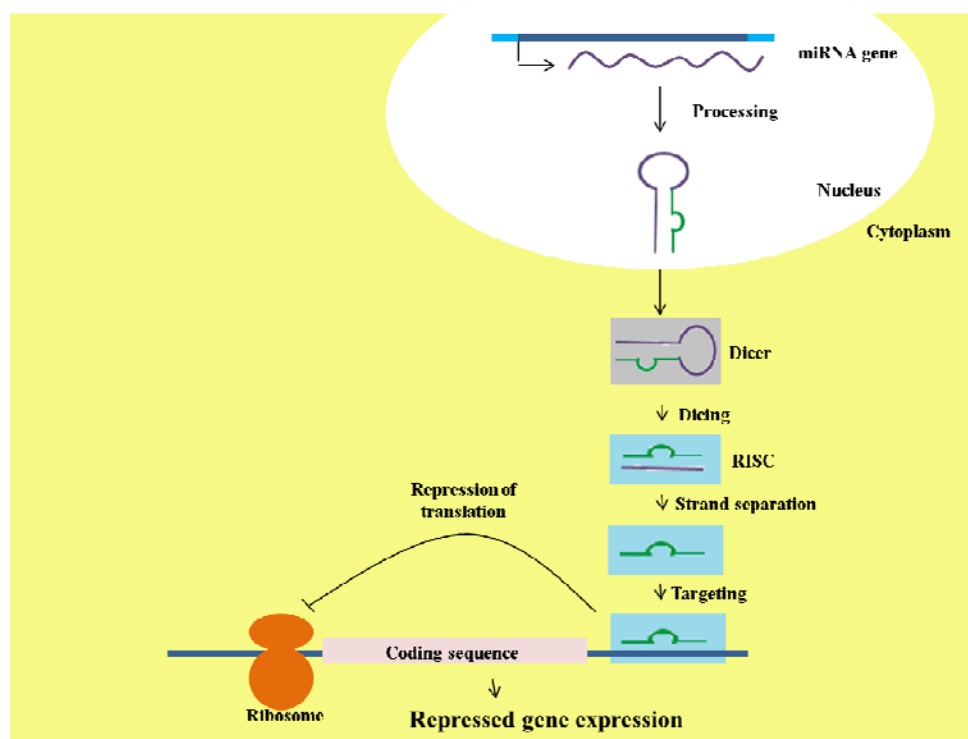
occurred. A class system was developed, where Class I was a loss of pigmentation, Class II was a gain in pigmentation, Class III was wild-type pigmentation, and Class IV was created when the RNAi screen caused other conspicuous phenotypes, possibly but not necessarily related to pigmentation (Rogers et al., 2014). Another important aspect of this research was the sexual dimorphism of the trait. We were interested in whether the transcription factors we found to be involved in the regulatory network affected either sexes or only one. These observations would give insight on the molecular mechanisms of the formation of sexually dimorphic traits. Through the RNAi screen, I was able to find 22 additional transcription factors that are a part of the regulatory network governing *Drosophila melanogaster* abdominal pigmentation, with 14 falling into Class I and 8 falling into Class II, therefore confirming my hypothesis that this gene regulatory network is more complex than previous researched showed.

## **CHAPTER II: MATERIALS AND METHODS**

### ***A. Methodological overview of an RNA interference screen***

The methodological goal of the RNAi screen was to reduce the expression of *Drosophila melanogaster* transcription factor genes individually along the dorsal midline of the abdomen. The reduction of gene expression occurs through the exploitation of the endogenous fruit fly RNA interference pathway. In general, RNAi reduces gene expression through inactivating specific mRNA molecules (Dietzl et al., 2007; Fire et al., 1998; Mummery-Widmer et al., 2009). The pathway begins with post-transcriptional modification of micro RNAs (miRNA), which are non-coding molecules that typically function in expression of genes during development. After processing, the miRNA becomes a smaller molecule in the structure of a stem-loop known as a pre-miRNA. The pre-miRNA has a double-stranded portion which is cleaved by the enzyme Dicer, forming the final miRNA product. The miRNA is separated into two single-stranded RNA molecules, which are incorporated into an RNA-induced silencing complex (RISC). The main component of an RISC is endonuclease proteins called argonaute proteins. The unwinding of the miRNA produces the guide strand, which pairs with a complementary sequence of the mRNA molecule, and the anti-guide strand, which is inactive in the RNAi pathway and degraded. The complex of the guide strand and the mRNA induce the argonaute proteins in the RISC to cleave the complex, resulting in silencing of the gene the mRNA was transcribed from and therefore no expression (Figure 4).

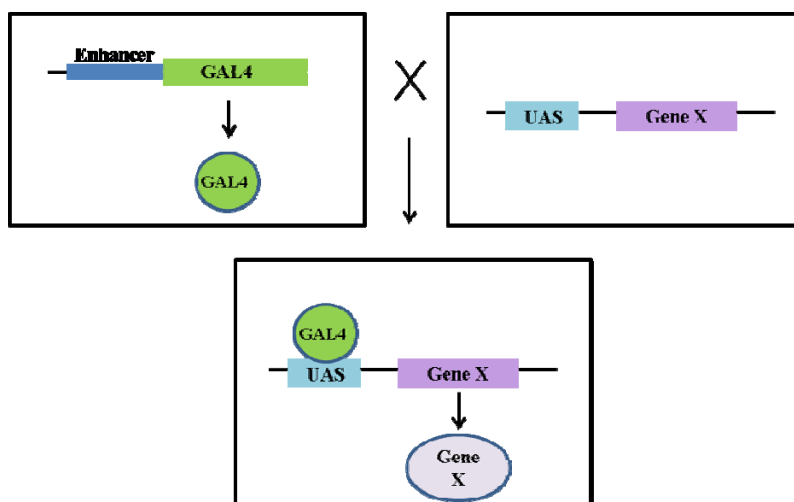
To control where the RNAi transgene is produced, the GAL4/UAS system was utilized (Brand & Perrimon, 1993). *GAL4* encodes a yeast transcription activator protein called Gal4, which binds to the Upstream Activation Sequence (UAS) to initiate gene



**Figure 4.** Cellular mechanism of RNA interference.

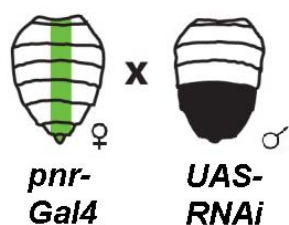
transcription. In order to make this system useful, UAS regions are inserted next to a gene of interest. Therefore, the gene of interest will be transcribed by cells producing Gal4 because the Gal4 will bind to the UAS. In my project, the genes of interest were genes encoding miRNAs that each would interact with a specific *Drosophila melanogaster* transcription factor gene's mRNA.

For this project, flies were crossed that possessed the *GAL4* gene under the regulation of the *pannier* (*pnr*) gene regulatory sequences (Calleja et al., 2000) to flies possessing an RNAi transgene with UAS binding sites as *cis*-regulatory element sequences (Figure 5). This cross resulted in F1 progeny that possessed the *UAS-RNAi* transgene and the *pnr-GAL4* gene, and progeny that had the *UAS-RNAi* transgene and the



**Figure 5.** Schematic of the GAL4/UAS system.

*TM3* chromosome that lacked the *pnr-GAL4* gene (control individuals). The presence of the *pnr-GAL4/UAS-RNAi* chromosome in flies could be identified by the serration of their wings, which would indicate the fly did not possess those chromosomes.



**Figure 6.** Cross between *pnr-GAL4* females and RNAi transgene males to obtain F1 progeny of interest. The phenotype will be shown in the area of the green line.

This *pnr-GAL4* bearing chromosome drives expression of the *GAL4* gene exclusively in the dorsal midline of fruit flies throughout development (Figure 6). The anticipated outcomes for cross progeny were described through a class system. Class I represents a loss of pigmentation, Class II represents a gain of pigmentation, and Class III represents wild-type pigmentation (Figure 7).



**Figure 7.** System used to classify the phenotypes of the F1 progeny.

### ***B. RNA interference (RNAi) Screen***

All fly stocks were maintained at 21°C using a sugar food recipe that was previously described (Salomone et al., 2013). Out of the 749 transcription factors found in the *Drosophila melanogaster* genome (Pfreundt et al., 2010), we were able to obtain RNAi lines for 558 of these transcription factors genes (Appendix 1). RNAi lines were investigated in groups of about 30. The majority of these lines came from the Transgenic RNAi Project at Harvard Medical School (<http://www.flyrnai.org/TRiP-HOME.html>), where each UAS-RNAi transgene was introduced into the attP2 site on the third chromosome (Groth & Calos, 2004; Groth, Fish, Nusse, & Calos, 2004). The only RNAi transgenes integrated into another genome location were for those targeting the *bab1* and *bab2* genes. These transgene lines came from the Vienna Drosophila RNAi Center (Dietzl et al., 2007). The Transgenic RNAi Project lines were derived by use of the VALIUM1 and VALIUM10 vectors which include long dsRNA hairpin (Ni et.al., 2008, 2009), while lines produced from the VALIUM20, VALIUM21, and VALIUM22 vectors made a short hairpin RNA (Haley et.al., 2010, 2008; Ni et.al., 2001). The effectiveness of long and short RNAi hairpins to target a specific gene was shown previously for several developmental genes (Ni et al., 2008). Males from UAS-RNAi lines (Appendix 1) were crossed with virgin females with the genotype *pnr-GAL4/TM3-Ser1* (Bloomington



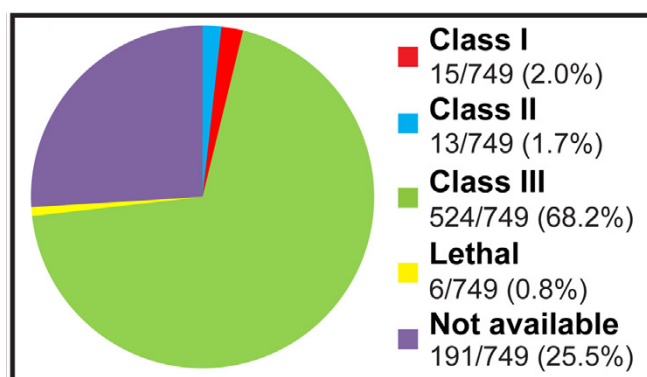
Drosophila Stock Center Stock #3039). Phenotypes were observed for the F1 progeny, making sure that at least 7 of each sex were evaluated in order to determine whether or not any phenotypes had variable penetrance and/or expressivity.

### ***C. Microscopic Imaging of Phenotypes***

Bright field images of the abdomens for progeny were taken using a zoom stereomicroscope (Olympus SZX-16) that is outfitted with a digital camera (Olympus DP72). For each RNAi transgene evaluated, images were taken of the dorsal surface for 3 adult flies. Based on phenotypic observation, the flies were organized into phenotypic classes based on the presence of a wild-type or mutant abdominal pigmentation. Images were saved in the Tagged Image File Format (TIFF). Images were cropped and edit using Adobe Photoshop CS3 and Figures were constructed using Adobe Illustrator CS3.

### CHAPTER III: RESULTS

Out of the 558 transcription factors genes I screened (Appendix 1), 28 genes resulted in an abnormal dorsal pigmentation along the abdominal midline. As described previously, the results were categorized using a class system (Figure 8).

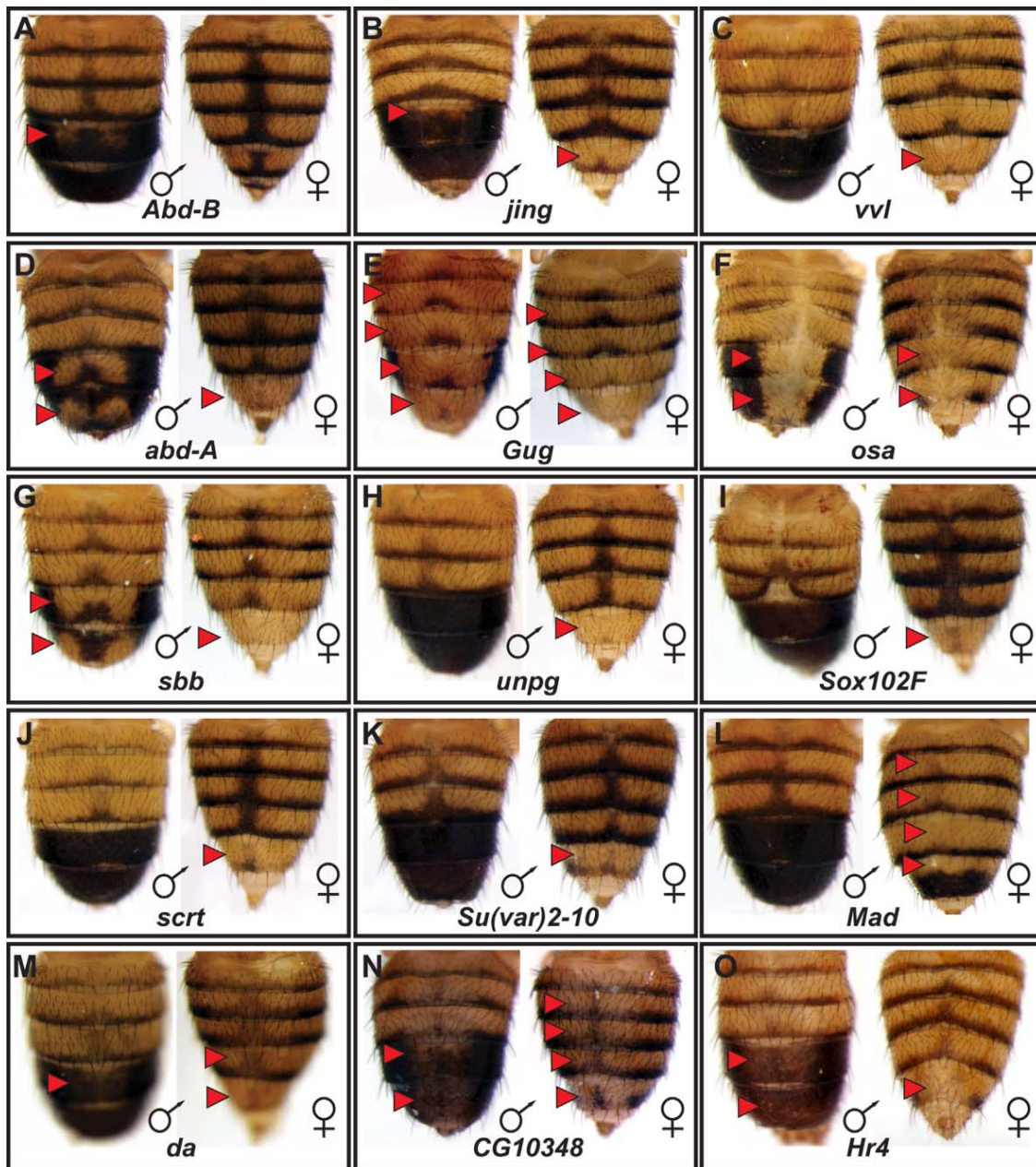


**Figure 8.** Breakdown of results based on class system. Class I represents a loss of pigmentation, Class II a gain of pigmentation, and Class III no change in pigmentation.

The first possibility was that the silencing of the transcription factor gene resulted in a loss of pigmentation, which I referred to as a Class I phenotypic outcome. This suggests the transcription factor being investigated is involved in some manner in producing pigmentation. 54% of the interesting transcription factors (15 of 28) fell into this category. One of these genes, *Abd-B*, is a known activator of the pigmentation genes *yellow*, *bab1*, and *bab2* (Jeong et al., 2006; Williams et al., 2008). Therefore, the loss of pigmentation when *Abd-B* is suppressed is to be expected. 14 more transcription factors were found to also cause a decrease in abdominal pigmentation (Figure 9). The loss of pigmentation ranges from severe loss, such as with the genes *Abd-A*, *Gug*, *osa*, and *sbb* (Figure 9D-G) to minimal loss (Figure 9A-B, H-N). Pigmentation was lost in a variety of

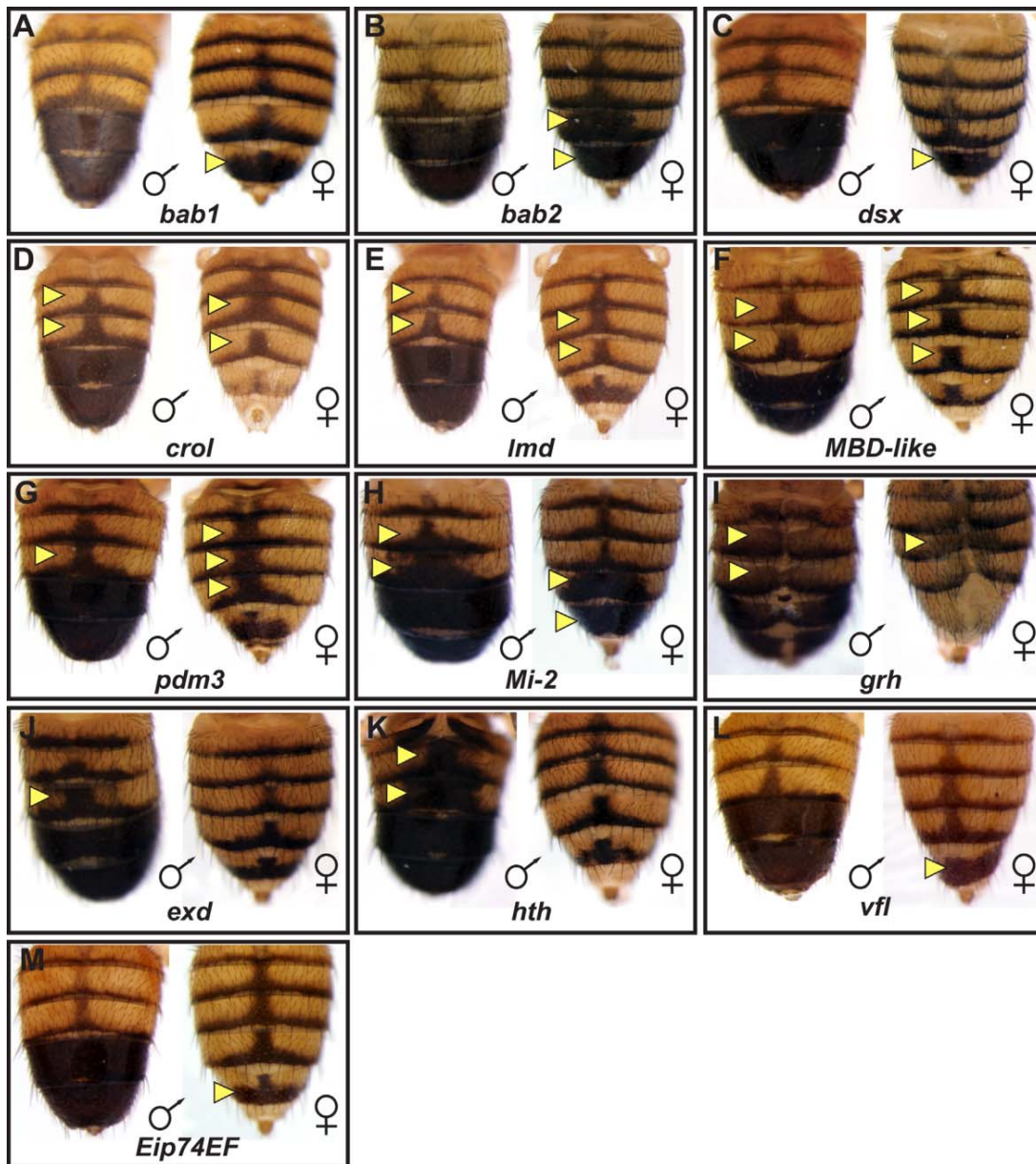
patterns. One common one was the loss of midline pigmentation in the A5 segment of females, which was shown in every gene but *Abd-B* (Figure 9B-N). Several transcription factors seem to contribute to pigmentation in only one sex, shown by the silencing of genes *Abd-B*, *vvl*, *unpg*, *Sox102F*, *scrt*, *Su(var)2-10*, and *Mad*. *Abd-B* was the only gene that resulted in male-specific loss of pigmentation (Figure 9A,C,H-L). The remaining genes that presented abnormal pigmentation in only one sex were seen in the females (Figure 9C, H-L).

The next class, Class II, is characterized by a gain in pigmentation resulting from the loss of expression of a single transcription factor. Therefore, the transcription factors in this class can be assumed to be involved in repressing pigmentation in the dorsal abdomen of *Drosophila melanogaster*. The remaining 13 of 28 transcription factor genes of interest are part of this class. Out of the 13 genes, 5 of them (*bab1*, *bab2*, *dsx*, *exd*, *hth*) have been studied before and are known to repress pigmentation (Couderc et al., 2002; Ryoo, Marty, Casares, Affolter, & Mann, 1999; Williams et al., 2008). Therefore, reduction of expression of these genes would result in a gain in pigmentation, which is what occurred. 8 more novel transcription factors were found that seem to also be repressors of pigmentation. As with Class I, there was a variation between the severities of the resulting phenotypes. *Bab2*, *Mi-2*, and *grh* showed more extreme gains of pigmentation than the remaining five genes (Figure 10). Females commonly had ectopic pigmentation throughout the whole midline or increased pigmentation in the A5 and A6 sections, some so severe that they looked extremely similar to the male wild-type phenotype (Figure 10A-I, L-M). Males also experienced an overall gain in pigmentation down the midline but some also showed ectopic pigmentation in the A3 and



**Figure 9.** Images of flies that displayed a loss of pigmentation in response to the silencing of a single transcription factor. Red arrows point to areas of interest where pigmentation was lost.

A4 tergites (Figure 10D-K). Several genes in this class also only affected one sex, with only *exd* and *hth* being male-specific transcription factor genes and *bab1*, *bab2*, *dsx*, *vfl*, and *Eip74EF* being female-specific (Figure 10A-C, J-M).



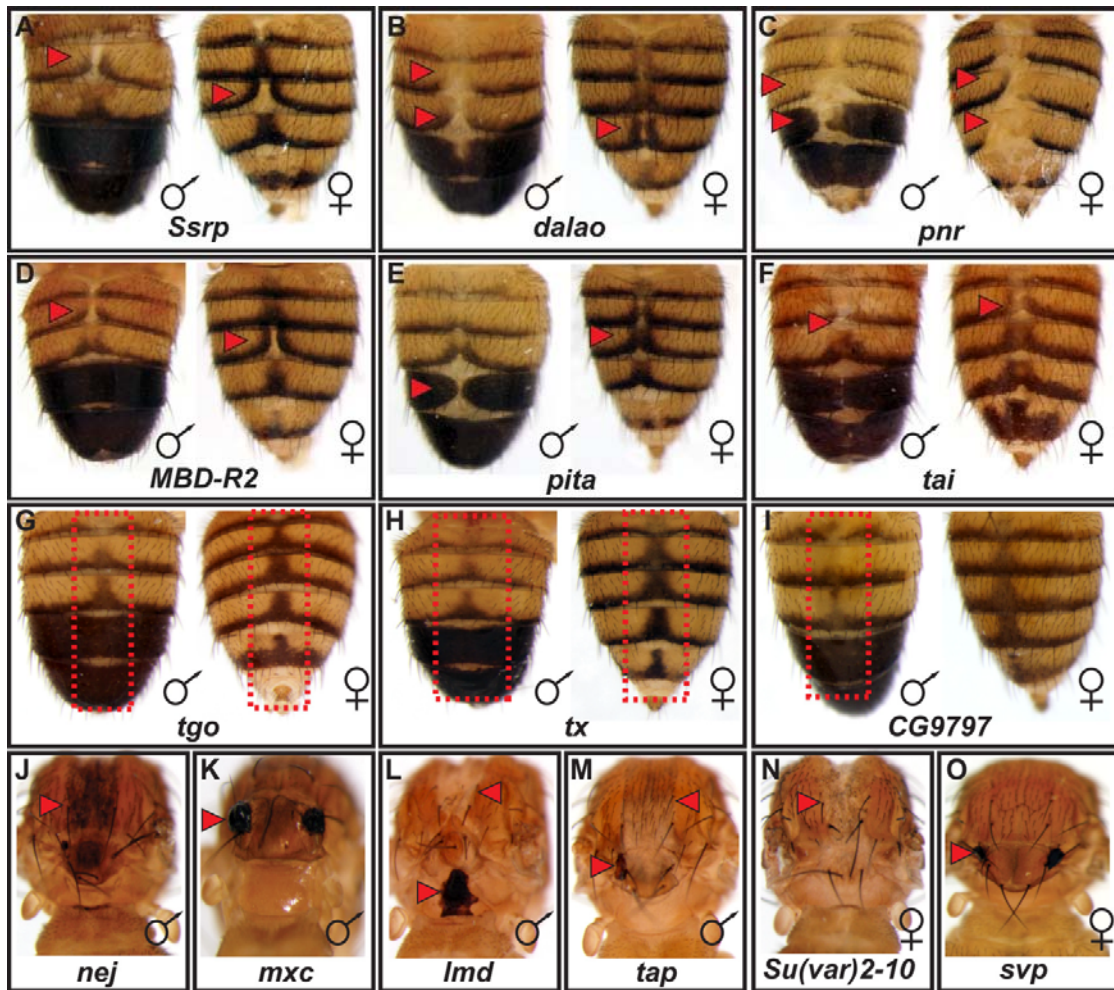
**Figure 10.** Images of flies that displayed a gain in pigmentation following the silencing of a single transcription factor gene. Yellow arrows point to areas of ectopic pigmentation.

The third and final class that displayed abnormal phenotypes resulting from the reduction in expression of specific transcription factor genes is Class IV, which represents interesting phenotypes such as abnormal tergite and thorax formation. The

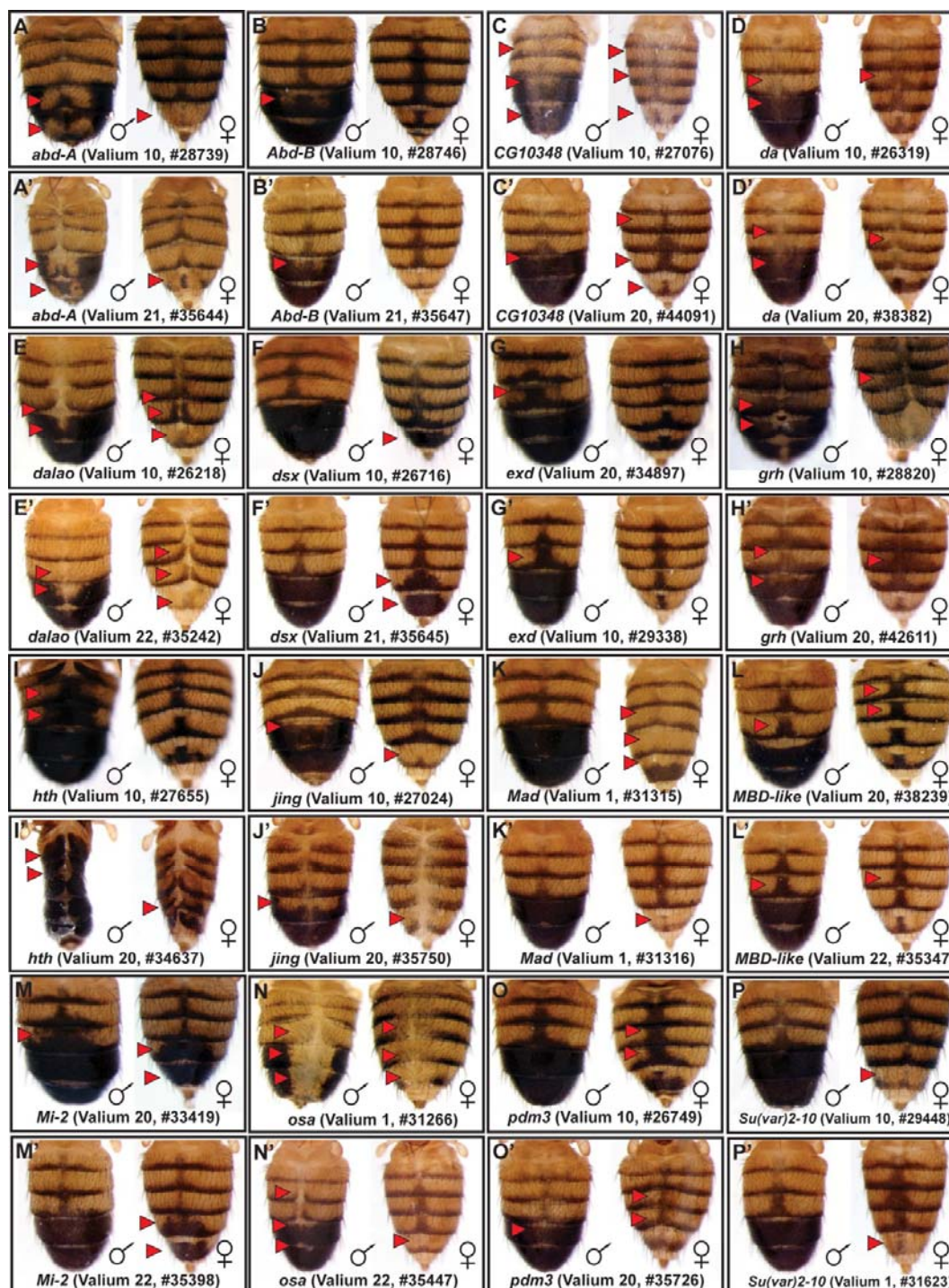
knockdown of genes *Ssrp*, *dalao*, *pnr*, *MBD-R2*, *pita*, and *tai* resulted in a mutant pigmentation phenotype but also in unusual tergite formation, causing an inability to state the abnormal pigmentation was from the RNAi and not a secondary characteristic of unusual tergite formation (Figure 11A-F). Small bristles on the dorsum of *Drosophila melanogaster* known as microchaetae did not form after the silencing of the *tgo*, *tx*, and *CG9797* genes (Figure 11G-I). Another developmental problem that occurred was abnormal thorax phenotypes, including ectopic pigmentation, lack of bristles, and a mutant shape (W. a Rogers et al., 2014). Different combinations of these phenotypes were seen when the expression of genes *nej*, *mxc*, *lmd*, *tap*, *Su(var)2-10*, and *svp* was reduced (Figure 11J-O). It can be concluded that while the genes included in this class do not necessarily directly play a role in producing dorsal abdominal pigmentation, they are involved in the development of *Drosophila melanogaster*.

For several RNAi transgenic lines that showed an abnormal pigmentation pattern, I obtained secondary transgenic lines that acted on a different part of the gene to confirm that the knockdown of the gene did indeed result in a mutant phenotype. Secondary lines were obtained for 16 genes of interest and 11 of those (*abd-A*, *abd-B*, *da*, *dalao*, *dsx*, *exd*, *grh*, *hth*, *jing*, *Mi-2*, and *Su(var)2-10*), produced a similar conspicuous phenotype that occurred when the primary lines was tested (Figure 12A'-B',D'-J',M',P'). Four of the secondary lines (*CG10348*, *Mad*, *MBD-like*, and *osa*) showed a less extreme phenotype than the primary line (Figure 12C',K'-L',N'). The final secondary line aimed to silence *pdm3* and interestingly produced a reciprocal pattern as that shown in the progeny of the primary line (Figure 12O').





**Figure 11.** Images of flies with abnormal developmental issues not directly related to abdominal pigmentation. **A-F.** RNAi targeting showed mutant pigmentation patterns but also incorrect tergite formation. **G-I.** Flies displayed a reduced amount of bristles down the midline. **J-O.** Silencing of genes resulted in abnormal thorax formation and pigmentation.



**Figure 12.** Secondary lines of genes that showed interesting phenotypes after screening the primary lines. **A-P.** Phenotypes of the primary line. **A'-P'.** Phenotypes of the secondary lines.



## **CHAPTER IV: DISCUSSION**

My thesis research was a collaborative effort with Sumant Grover and William Rogers of the University of Dayton and Jennifer Parks of West Carrollton High School to better define the network of transcription factors that regulate the production of *Drosophila melanogaster* abdominal tergite pigmentation. We have shown that a greater cohort of transcription factor genes populate the network of genes that generate the pattern of tergite pigmentation for *Drosophila melanogaster*. This cohort includes major body plan transcription factor genes, such as ABD-A, EXD, and HTH, and genes that encoded components of chromatin remodeling complexes. HTH and EXD were found to have effects limited to the more anterior tergites of males, providing evidence that this pigmentation network has a male-specific circuit that accompanies the female-specific circuit governed by the Bab transcription factors. Collectively, these results bear upon the regulatory complexity of a gene network for a secondary sex trait, and reveal more candidate loci for investigations into the evolutionary diversification of fruit fly pigmentation patterns.

### ***Comparing gene networks for embryonic and secondary sex-trait development***

Over the past several decades, a genetic understanding of embryonic development has emerged from studies in various model organisms and model traits. A common theme from these differing models is that development requires a large number of genes interconnected at the level of gene expression regulation and that development proceeds by temporal changes in cellular gene expression states (E H Davidson, 2006; M. Levine

& Davidson, 2005). Extensive studies of the *Drosophila* segmentation, dorsal-ventral patterning, and mesoderm development have revealed regulatory networks that include some 30 to over 50 genes (Bonn & Furlong, 2008). Most of these genes encode transcription factors that interact with an even far greater number of CREs. This complexity in transcription factor content and regulatory wiring seems logical, as embryonic development involves coordination of cell proliferation, death, determination, and differentiation events. Regarding a late developing secondary sex-trait, such as abdominal tergite pigmentation, the question could be asked whether a simpler network would be utilized.

The most prominent feature of *D. melanogaster* tergite pigmentation is the completely melanic A5 and A6 tergites of males that is comparatively lacking in females. Thus, it was expected that upstream regulatory inputs would include those that are expressed in these posterior abdominal segments and inputs whose expression differs between males and females. A key spatial regulator is the Hox gene *Abd-B*, whose expression is limited to the posterior most abdominal segments, including A5 and A6, which regulates the expression of the pigmentation gene *yellow* in these segments (Jeong et al., 2006). Key sex-specific regulators are the Bab transcription factors which function as dominant repressors of pigmentation and which are broadly expressed in the female abdominal segments but poorly expressed in the male abdomen, most prominently in the A5 and A6 segments. Sexual dimorphism for Bab results from its direct regulation by the sex specific isoforms of the DSX transcription factor (Williams et al., 2008), a terminal transcriptional effector of the somatic sex-determination pathway (Williams & Carroll, 2009). Together, the patterns of expression and molecular activities of these transcription

factors seemed sufficient to account for this species abdominal pigmentation pattern. In this study, a survey of over 75% of the genome encoded transcription factor genes revealed that *Abd-B*, *bab1*, *bab2*, and *dsx* represent an important but small subset of network transcription factor genes that likely includes at least 24 additional genes. Many of these genes, such as *abd-A*, *Gug*, *osa*, *sbb*, and *Mi-2*, resulted in mutant phenotypes of equal or greater magnitude (Figure 9; Figure 10).

### ***A network with male and female specific circuits***

The outcomes for loss- and gain-of-function studies supported a model where *Abd-B* promoted pigmentation whereas *bab* suppressed pigmentation in regions where co-expressed with *Abd-B*, and in anterior segments of both sexes where *Abd-B* is not expressed (Couderc et al., 2002; Kopp et al., 2000). Hence, the production of pigmentation in males occurs on the A5 and A6 tergites where *Abd-B* is and *bab* is not expressed. However, it was later found that *Abd-B* also acts an activator of *bab*, revealing that *Abd-B* is both for and against the formation of pigmentation and indicating that this network was complex in connectivity between transcription factors and CREs. More recently, *Bab* expression was found to be nearly absent in male abdomens during late pupal development when pigmentation patterning is occurring (Salomone et al., 2013). This indicates that *bab*'s pigmentation function is essentially limited to females, a function wired into this network as a female-specific circuit. Here we found that reduced expression of *exd* and *hth* resulted in ectopic pigmentation on the male A3 and A4

tergites. These findings indicate that the anterior limitation of pigmentation is wired into the network as a male-specific circuit that includes these two genes.

***Abdominal pigmentation: a composite of pattern elements and regulatory genes***

The pattern of tergite pigmentation does not only consist of the dimorphic A5 and A6 tergites, but rather is composite of pattern elements that include posterior stripes, dorsomedial pigmentation spots, and the yellow coloration of the non-melanic tergite regions. Thus, the regulatory structure of this network must include transcription factors that pattern these sub-elements. Consistent with this expectation, pigmentation stripes were reduced by RNAi for *sbb*, *Gug*, and *osa*, and widened by antagonizing *pdm3*. Several genes were implicated in the patterning of midline spots. *da* and *Mad* were required for spot development, whereas *crol*, *lmd*, *MBD-like*, *pdm3*, and *hth* were needed to limit the spot size and number. RNAi targeting of transcription factor genes, including *Ssrp*, *dala*, *MBD-R2*, *pita*, and *pnr* disrupted pigmentation along the dorsal midline, however these affects seem to be a consequence of disrupted tergite formation. Lastly, *da*, *CG10348*, and *grh* were implicated in the overall coloration of tergites, as their suppression resulted in alterations along the entire dorsomedial domain of RNAi transgene expression.

### ***A role for chromatin remodeling in pigmentation development***

In addition to spatial- and sex-specific transcription factor inputs, gene expression is going to depend upon the chromatin state at the promoters and CREs for network genes. Consistent with the important role for chromatin in gene regulation, many of the novel transcription factor genes identified in this screen encode components of chromatin-modifying complexes or transcription factors that have been previously found to interact with these complexes.

*Ssrp* (Structure specific recognition protein) encodes a protein with an HMG box DNA-binding domain and that functions in the FACT complex that interacts with nucleosomes where it can remove H2A-H2B histone dimers (Winkler & Luger, 2011). This complex has been found on *Hox* gene regulatory sequences and shown to interact with another transcription factor to regulate *Hox* gene expression (Shimojima et al., 2003). Strong genetic differentiation between tropical and temperate populations of *D. melanogaster* was found at *Ssrp*, suggesting this loci might have been a target for environmental adaptation (M. T. Levine & Begun, 2008).

SWI/SNF complexes function to remodel nucleosomes which can favor DNA-binding by transcription factors (Cote et al. 1994). Two complexes exist in *Drosophila*, BAP and PBAP that have common (such as Brahma) and unique protein components (Mohrmann et al., 2004). We found that RNAi suppression of the *osa* and *dalao* genes resulted in tergite defects that included pigmentation. These genes encode the Osa and Dalao (BAP111) proteins, the former which is specific to the BAP complex and the latter which occurs in both complexes. Though these two proteins bind to DNA, binding was

non-specific *in vitro* (Collins, Furukawa, Tanese, & Treisman, 1999; Papoulas et al., 2001). In contrast, *in vivo* studies showed that *osa* (Brumby et al., 2002; Terriente-Félix & de Celis, 2009; Treisman, Luk, Rubin, & Heberlein, 1997; Vázquez, Moore, & Kennison, 1999) and more generally the SWI/SNF complex has specific targets of regulation (Holstege et al., 1998). It remains uncertain to what extent factors like Osa and Dalao contribute to target gene discrimination, perhaps a question that can be addressed within the increasingly well understood pigmentation network.

RNAi suppression of Grunge/Atrophin (Gug/Atro) resulted in a dramatic loss of tergite pigmentation. Gug has a SANT domain that resembles a DNA-binding domain; however this capability has not been demonstrated (Wang & Tsai, 2008). Gug acts as a necessary repressor of many developmental genes through physical interactions with transcription factors that include Eve, Hkb, and Tll (Wang, Rajan, Pitman, McKeown, & Tsai, 2006; Wehn & Campbell, 2006; Zhang, Xu, & Lee, 2002), where Gug recruits HDAC1 and HDAC2 through its ELM2 and SANT domains (Wang, Charroux, Kerridge, & Tsai, 2008; Wang et al., 2006). While a prominent role for Gug is to function as a transcriptional co-repressor, it was classified as a trithorax gene as it is required for some *Hox* gene functions (Kankel, Duncan, & Duncan, 2004). In our study, Gug suppression had a dramatic function on pigmentation, but tergite development otherwise appeared to be normal. Therefore, Gug function appears to operate within the pigmentation network during pupal development. It remains to be worked out how this specificity is achieved, perhaps through spatial-limited gene expression, interactions with other network transcription factors, or through selective binding to target gene cis-regulatory elements.

The connection between chromatin remodeling complexes and tergite pigmentation include the *Drosophila* nucleosome remodeling and deacetylase (dNuRD) complex. In our study, suppression of *dMi-2* led to ectopic pigmentation on the male A4 tergite and the female A5 and A6 tergites. dMi-2 functions as the ATPase subunit which has been found to regulate gene expression through its inclusion of both histone deacetylases and histone binding proteins (Bouazoune & Brehm, 2006). The dMi-2 protein has several noteworthy motifs in addition to the ATPase domain, including a putative DNA-binding domain (Kehle et al., 1998). However, the significance of this latter domain and more specifically whether it functions to bind DNA in a sequence-specific manner has not been shown. dMi-2 can repress gene expression through several mechanisms, including interactions with various transcription factor proteins (Kehle et al., 1998; Murawsky et al., 2001) and through the disruption of higher order chromatin structures by destabilizing interactions between Cohesin and chromosomes (Fasulo et al., 2012). dMi-2 is also part of an abundant binary complex (called dMec) with dMEP-1, and this complex contributes to the repression of proneural genes through a SUMOylation-mediated mechanism (Kunert et al., 2009). dMi-2 function is not universally repressive, as it has been found to be associated with genes that are transcriptionally active (Murawska et al., 2008; Murawska, Hassler, Renkawitz-Pohl, Ladurner, & Brehm, 2011). Interestingly, both dMEP-1 and dMi-2 were found expressed higher in females than males (Kunert et al., 2009; Murawska et al., 2008), a result that corresponds with the prominent female pigmentation phenotype and suggests that dMi-2 might act in parallel or in conjunction with Bab to generate a female-specific expression of pigmentation network genes.

### *Tracing the network structure through target gene CRE interactions*

Within the *D. melanogaster* pigmentation network, few direct interactions between transcription factors and CREs are known that structure this sexually dimorphic trait's production. The few that are known include direct CRE interactions between ABD-B and the *yellow* body element CRE (Jeong et al., 2006) and both ABD-B and DSX with the *bab* loci dimorphic element CRE (Williams et al., 2008) (Figure 3). Repression of *yellow* (Jeong et al., 2006) and presumably the *tan* gene expression is mediated by the Bab proteins, but whether regulation is direct or indirect remains unknown. Moreover, no direct regulators of *ebony* are known.

In order to elucidate the relative network position and regulatory association for this new wealth of network transcription factor genes, William Rogers evaluated on a small scale the effects that the reduced expression had on the regulatory activity of *tan*, *ebony*, and *bab* CREs (W. a Rogers et al., 2014). This revealed that all tested transcription factors act upstream of *tan* and *ebony* CREs, whereas a smaller subset were positioned upstream in the hierarchy of the *bab* CRE. One interesting outcome was the finding that *abd-A* acts as a double agent, by promoting (activator and repressor of *tan* and *ebony* respectively) and suppressing (activating *bab* expression) the formation of pigmentation. He also observed a regulatory outcome indicating that *hth*, but not *exd*, functioned as an activator of female-specific *bab* expression. This exclusive utilization of *hth* differed from the regulation of *tan* and *ebony* where both factors played similar activating (*tan*) or repressing (*ebony*) functions. Although reduced *sbb* and *Mi-2* expression respectively resulted in a loss and gain of female A6 pigmentation, he observed no corresponding alteration in the female specific activity of the *bab* CRE.



These outcomes suggest that *sbb* and *Mi-2* are either downstream of *bab* or act in an independent regulatory circuit. A similar approach could be used to better understand the network role for the remaining transcription factors identified in this RNAi screen. Ultimately, what needs to be accomplished is the mapping of the actual direct binding events between these transcription factors and their network CREs targets.

### ***Understanding morphological diversity through gene network evolution***

Identifying the regulatory genes and mapping their target gene CRE interactions for the *Drosophila melanogaster* abdominal pigmentation network will provide insights about the production of secondary-sex traits. However, the evolutionary insights to emerge from this pigmentation model may transcend these developmental insights. The fruit fly phylogeny is robust in certain clades, (Jeong et al., 2006; van der Linde, Houle, Spicer, & Steppan, 2010) aiding inferences about the direction of pigmentation pattern evolution. Moreover, abdominal pigmentation patterns vary at the population (Kopp, Graze, Xu, Carroll, & Nuzhdin, 2003; Parkash, Sharma, & Kalra, 2008; Pool & Aquadro, 2007) and species levels (Jeong et al., 2006), and male-specific tergite pigmentation is thought to have evolved convergently (Gompel & Carroll, 2003). Thus, a well-resolved gene network for *D. melanogaster* will provide a necessary reference point from which to tease about the mechanistic processes whereby a homologous network has been modified in gene composition or connectivity to encode convergent and diverse phenotypic outcomes. I believe that this expansion of the *D. melanogaster* pigmentation gene network makes more feasible answering these evolutionary questions.

## **CHAPTER V: APPENDICES**

**1: RNAi Transgene Lines.** The table below presents the identity of the RNAi line stocks that were used in my thesis project. The fly stocks can be obtained from the Bloomington Drosophila Stock Center: <http://flybase.org/reports/FBst0026741.html>

<b>Targeted Gene</b>	<b>Vector</b>	<b>RNAi Stock Number</b>
A3-3	Valium 10	26741
ab	Valium 20	32378
abd-A	Valium 10	28739
Abd-B	Valium 10	26746
ac	Valium 10	29586
Acf1	Valium 10	31340
achi	Valium 20	34652
acj6	Valium 10	29335
Ada2b	Valium 1	31347
Adf1	Valium 10	28680
Aef1	Valium 10	31942
al	Valium 10	26747
Alh	Valium 20	39057
Antp	Valium 10	27675
aop	Valium 20	34909
ap	Valium 10	26748
AP-2	Valium 10	36132
apt	Valium 10	26236
ara	Valium 10	27060
ase	Valium 10	31895
Atac1	Valium 1	31086
ATbp	Valium 10	26763
Atf-2	Valium 10	26210
Atf6	Valium 10	26211
ato	Valium 20	35017
Awh	Valium 10	31772
az2	Valium 10	26230
bab1	Valium 21	35707
bab2	Valium 21	35720
bap	Valium 10	27061
Bap170	Valium 10	26308
Bap60	Valium 20	32503
bbx	Valium 10	26215
bcd	Valium 10	28586
BEAF-32	Valium 10	29734

B-H1	Valium 1	31711
B-H2	Valium 20	33647
bi (omb)	Valium 10	28341
bimax	Valium 10	29325
bin	Valium 20	34718
Blimp-1	Valium 22	36634
bowl	Valium 10	27074
br	Valium 20	33641
brk	Valium 20	37493
brp	Valium 10	25891
bs	Valium 10	26755
bsh	Valium 10	29336
BtbVII	Valium 10	28912
btd	Valium 10	29453
Bteb2	Valium 10	27075
bun	Valium 10	28322
C15	Valium 20	35018
cad	Valium 22	34702
Camta	Valium 10	27062
cas	Valium 20	34701
cato	Valium 20	26317
cbt	Valium 20	38276
cg	Valium 20	34668
CG10147	Valium 10	31943
CG10267	Valium 10	29360
CG10274	Valium 10	26239
CG10321	Valium 10	26764
CG10348	Valium 10	27076
CG10366	Valium 10	26765
CG10462	Valium 10	31945
CG10543	Valium 10	31964
CG10631	Valium 10	28001
CG10654	Valium 10	27998
CG10669	Valium 10	31946
CG10949	Valium 10	26231
CG10959	Valium 10	29361
CG10979	Valium 10	29362
CG11071	Valium 10	26766
CG11247	Valium 10	31947
CG11294	Valium 10	28641
CG11317	Valium 10	28065
CG11398	Valium 20	36109
CG11456	Valium 10	26240
CG11696	Valium 10	27999
CG11723	Valium 10	29349

CG11902	Valium 10	29363
CG11906	Valium 10	26767
CG12071	Valium 10	26768
CG12219	Valium 10	28000
CG12236	Valium 10	31949
CG12299	Valium 20	33957
CG1233	Valium 10	31950
CG12391	Valium 10	31951
CG12605	Valium 10	29364
CG12769	Valium 10	26769
CG12942	Valium 10	27077
CG13123	Valium 10	29545
CG13204	Valium 10	31919
CG13624	Valium 10	25983
CG13897	Valium 10	29613
CG14655	Valium 10	26770
CG14667	Valium 10	29365
CG14710	Valium 10	26771
CG14711	Valium 1	31726
CG14767	Valium 20	34679
CG14962	Valium 10	31953
CG15011	Valium 1	31589
CG15073	Valium 10	29366
CG15365	Valium 20	36856
CG15514	Valium 10	31940
CG15710	Valium 10	26773
CG1602	Valium 10	31920
CG1603	Valium 10	27063
CG1663	Valium 10	27078
CG16778	Valium 10	31915
CG17181	Valium 10	26775
CG17186	Valium 10	27079
CG17328	Valium 10	28898
CG17359	Valium 10	26776
CG17385	Valium 10	31954
CG17568	Valium 10	31955
CG17803	Valium 10	29367
CG17806	Valium 10	31956
CG17912	Valium 10	27996
CG1792	Valium 10	26709
CG18262	Valium 20	33348
CG18265	Valium 10	28560
CG1832	Valium 10	27080
CG18446	Valium 20	33735
CG1845	Valium 10	27081

CG18476	Valium 10	26710
CG18619	Valium 10	29542
CG2116	Valium 10	26712
CG2199	Valium 10	27082
CG2202	Valium 10	26241
CG2678	Valium 10	28630
CG30020	Valium 10	29368
CG31224	Valium 20	33969
CG31670	Valium 10	26778
CG32050	Valium 1	31560
CG32105	Valium 10	31905
CG32343		
CG32532	Valium 10	26750
CG32772	Valium 22	35803
CG32778	Valium 10	26715
CG32830	Valium 10	29407
CG33213	Valium 10	29458
CG33695 (cana)	Valium 22	35475
CG34422	Valium 1	31754
CG3711	Valium 20	33422
CG3838	Valium 10	31922
CG3919	Valium 20	33355
CG3995	Valium 10	27997
CG4133	Valium 10	28370
CG42724		33737
CG4282	Valium 10	26313
CG4328	Valium 10	27987
CG4404	Valium 10	31923
CG4575		31975
CG4617	Valium 10	25993
CG5343	Valium 22	36640
CG5591	Valium 10	25994
CG6272	Valium 20	33652
CG6276	Valium 10	27064
CG6701	Valium 1	31213
CG6791	Valium 20	32395
CG6808	Valium 1	31727
CG6813	Valium 10	29369
CG6905 (Roc1b)	Valium 1	31067
CG7045	Valium 10	28656
CG7046	Valium 10	28657
CG7556	Valium 20	32511
CG7839	Valium 10	25992
CG8108	Valium 10	27562

CG8119	Valium 22	38893
CG8216	Valium 10	27069
CG8301	Valium 22	41643
CG8359	Valium 10	27066
CG8478	Valium 20	39033
CG8765	Valium 10	29447
CG8909	Valium 1	31105
CG8924	Valium 10	29348
CG8944	Valium 10	31925
CG9418	Valium 10	26216
CG9437	Valium 10	26754
CG9609	Valium 20	34030
CG9650	Valium 10	26713
CG9705	Valium 10	31901
CG9727	Valium 10	26762
CG9797	Valium 20	32858
CHES-1-like	Valium 20	34928
chinmo	valium 20	33638
chm	Valium 20	32484
chn	Valium 10	26779
ci	Valium 10	28984
cic	Valium 10	25995
Clk	Valium 10	31660
cnc	Valium 10	25984
coop	Valium 10	29350
CoRest	Valium 10	31743
Cp190	Valium 20	33903
crc	Valium 10	25985
CrebA	Valium 10	31900
CrebB-17A	Valium 10	29332
crm	Valium 1	31338
croc	Valium 20	34647
crol	Valium 20	41669
crp	Valium 10	31896
ct	Valium 20	33967
CTCF	Valium 22	35345
CTPsyn	Valium 10	31924
cwo	Valium 10	27736
cyc	Valium 10	31897
D	Valium 20	34672
D1	Valium 20	33655
D19A	Valium 20	33371
d4	Valium 10	28623
da	Valium 20	29326
dac	Valium 10	26758

Dad	Valium 20	33759
dalao	Valium 10	26218
danr		28378
dar1	Valium 10	31987
Dbx	Valium 10	31904
Deaf1	Valium 20	32512
dei	Valium 10	25973
Dfd	Valium 10	26751
Dh44-R2	Valium 10	29610
Dif	Valium 10	30513
dimm	Valium 10	26976
Dip3	Valium 10	27067
disco	Valium 10	28659
disco-r	Valium 20	41683
dl	Valium 20	34938
Dll	Valium 10	29337
dm	Valium 10	25784
dmrt11E	Valium 21	35657
dmrt93B	Valium 10	27657
dmrt99B	Valium 10	31982
Doc1	Valium 10	31931
Doc3	Valium 10	31932
Dp	Valium 20	33372
dpn	Valium 10	26320
Dr	Valium 10	26224
Dref	Valium 10	31941
dsf	Valium 10	29373
Dsp1	Valium 10	31960
dsx	Valium 10	26716
dve	Valium 10	26225
dwg	Valium 21	35666
dys	Valium 10	26321
E(bx)	Valium 20	33658
E(spl)	Valium 10	26322
E(var)3-9	Valium 10	31948
e(y)3	Valium 20	32346
E(z)	Valium 10	27993
E2f	Valium 10	27564
E2f2	Valium 10	27995
ecd	Valium 20	41676
EcR	Valium 10	29374
eg	Valium 10	29629
egg	Valium 1	31352
Eip74EF	Valium 10	29353
Eip75B	Valium 10	26717

Eip78C	Valium 10	26718
emc	Valium 10	26738
ems	Valium 10	28726
en	Valium 20	33715
ERR	Valium 10	27085
esg	Valium 10	28514
Ets21C	Valium 20	39069
Ets65A	Valium 20	41682
Ets96B	Valium 10	31935
Ets97D	Valium 10	25795
Ets98B	Valium 10	28700
eve	Valium 10	28734
ewg	Valium 1	31225
exd	Valium 20	34897
ey	Valium 10	29339
eyg	Valium 10	26226
fd59A	Valium 10	31937
fd96Cb	Valium 10	26761
Fer1	Valium 10	27737
Fer2	Valium 10	28697
Fer3	Valium 10	25974
fkx	Valium 10	27072
foxo	Valium 10	25997
foxP	Valium 10	26774
fru	Valium 1	31593
ftz	Valium 20	33761
ftz-fl	Valium 10	27659
fu2	Valium 10	28554
GATAd	Valium 20	33747
GATAe	Valium 20	34641
gce	Valium 10	26323
gcm	Valium 10	28913
gcm2	Valium 10	28904
gem	Valium 10	26214
gl	Valium 10	26780
grh	Valium 10	28820
grn	Valium 10	27658
grp	Valium 10	27277
gsb	Valium 10	29600
gsb-n	Valium 10	28078
gt	Valium 10	26742
Gug	Valium 20	32961
h	Valium 10	27738
ham	Valium 10	26728
Hand	Valium 10	28977



hang	Valium 21	35674
hb	Valium 10	29630
hbn	Valium 10	31906
Hcf	Valium 20	32453
Her	Valium 10	27654
Hey	Valium 10	31898
HGTX	Valium 10	29382
hkb	Valium 10	31976
HLH106	Valium 10	25975
HLH3B	Valium 10	26324
HLH4C	Valium 10	25976
HLH54F	Valium 10	28698
HLHm3	Valium 10	25977
HLHm5	Valium 10	26201
HLHm7	Valium 10	29327
HLHmbeta	Valium 10	26202
HLHmdelta	Valium 10	26203
HLHmgamma	Valium 10	25978
HmgD	Valium 1	31344
HmgZ	Valium 10	26219
Hnf4	Valium 10	29375
Hr38	Valium 10	29376
Hr39	Valium 10	27086
Hr4	Valium 10	31868
Hr46	Valium 10	27254
Hr51	Valium 20	39032
Hr78	Valium 10	31990
Hr96	Valium 10	27992
Hsf	Valium	27070
hth	Valium 10	27655
ind	Valium 10	28662
inv	Valium 20	41675
Iswi	Valium 1	31111
Jarid2	Valium 10	26184
jim	Valium 22	35609
jing	Valium 10	27024
Jra	Valium 1	31595
jumu	Valium 22	35438
kay	Valium 10	27722
Kdm2	Valium 1	31360
ken	Valium 20	34739
key	Valium 22	35572
klu	Valium 10	28731
kn	Valium 10	31916
kni	Valium 10	27259

knrl	Valium 20	36664
Kr	Valium 10	27666
l(1)sc	Valium 10	27058
l(3)mbt	Valium 10	28076
lab	Valium 10	26753
Lag1	Valium 10	29340
lbe	Valium 10	28374
lbl	Valium 21	35698
Lhr	Valium 20	38240
lid	Valium 10	28944
lilli	Valium 10	26314
Lim1	Valium 10	29341
Lim3	Valium 10	26227
lin-28	Valium 10	29564
Lin29	Valium 10	26711
lola	Valium 10	26714
luna	Valium 10	27084
lz	Valium 10	27985
Mad	Valium 1	31315
maf-S	Valium 10	25986
mamo	Valium 21	37486
Max	Valium 10	29328
MBD-like	Valium 20	38239
MBD-R2	Valium 10	30481
mbf1	Valium 10	28550
Med	Valium 10	31928
Mef2	Valium 10	28699
MEP-1	Valium 20	3367
MESR4	Valium 22	35618
Met	Valium 10	26205
Mi-2	Valium 20	33419
mib1	Valium 10	27320
Mio	Valium 10	27059
mip120	Valium 20	32461
mirr	Valium 10	31907
Mitf	Valium 20	34835
mld	Valium 10	31952
Mnf	Valium 10	27994
Mnt	Valium 10	29329
mod	Valium 10	28314
mor	Valium 20	34919
Mrtf	Valium 10	31930
MTA1-like	Valium 20	33745
MTF-1	Valium 20	33381
mus201		31052

mx	Valium 10	25970
Myb	Valium 10	26237
N	Valium 10	28981
nau	Valium 10	31899
nej	Valium 10	27724
nerfin-1	Valium 10	28324
nerfin-2	Valium 10	28551
net	Valium 10	26204
Nfl	Valium 10	25845
Nf-YA	Valium 10	25991
NK7.1	Valium 10	31908
noc	Valium 10	29370
Not1	Valium 10	28681
nub	Valium 10	28338
Oaz	Valium 10	25923
oc	Valium 10	29342
Octbeta2R	Valium 20	34673
odd	Valium 10	28295
OdsH	Valium 10	31909
Oli	Valium 10	25979
onecut	Valium 10	29343
opa	Valium 20	34706
Opbp	Valium 20	36120
Optix	Valium 10	31910
osa	Valium 1	31266
ovo	Valium 22	35413
p53	Valium 10	29351
pad	Valium 10	31944
pan	Valium 10	26743
pb	Valium 10	29595
pdm2	Valium 10	29543
pdm3	Valium 10	26749
Pdp1	Valium 10	26212
peb	Valium 10	28735
Pep	Valium 20	32944
pfk	Valium 10	31914
pho	Valium 1	31609
Pif1A	Valium 20	36706
Pif1B	Valium 20	36706
pita	Valium 21	35724
pnr	Valium 10	28935
pnt	Valium 10	31936
polybromo	Valium 20	32840
Poxm	Valium 10	26757
Poxn	Valium 10	26238

Pph13	Valium 10	31911
ppl	Valium 10	27700
prd	Valium 20	33965
pros	Valium 10	26745
psq	Valium 10	28693
Pur-alpha	Valium 22	36849
pzg	Valium 22	35448
Rabex-5	Valium 10	29357
Ravus	Valium 1	31345
Rel	Valium 10	28943
repo	Valium 10	28339
retn	Valium 10	26309
Rfx	Valium 10	29355
rgr	Valium 10	31926
ro	Valium 10	28671
row	Valium 10	25971
run	Valium 10	28673
RunxA	Valium 20	33353
Rx	Valium 10	28674
sage	Valium 10	25980
salm	Valium 20	33714
salr	Valium 10	29549
sbb	Valium 10	27049
sc	Valium 10	26206
Scamp	Valium 20	38277
Scr	Valium 10	28676
scro	Valium 10	29387
s crt	Valium 10	27025
sd	Valium 10	29352
sens	Valium 10	27287
sens-2	Valium 10	27285
Set2	Valium 1	31355
shn	Valium 20	34689
sim	Valium 10	26739
sima	Valium 10	26207
sisA	Valium 10	29330
Six4	Valium 10	30510
slbo	Valium 10	27043
slou	Valium 10	29344
slp1	Valium 10	29354
slp2	Valium 20	34634
Smox	Valium 10	26756
Smr	Valium 10	27068
sna	Valium 10	28679
so	Valium 10	31912

sob	Valium 20	34648
Sox100B	Valium 21	35656
Sox102F	Valium 10	26220
Sox14	Valium 10	26221
Sox21a	Valium 10	31902
SoxN	Valium 10	25996
Sp1	Valium 20	35777
sr	Valium 10	27701
srp	Valium 10	28606
ss	Valium 10	26208
Ssb-c31a	Valium 22	35437
Ssrp	Valium 10	26222
Stat92E	Valium 1	31318
stwl	Valium 22	35415
Su(H)	Valium 10	28900
su(Hw)	Valium 1	31600
Su(Tpl)		33399
Su(var)205	Valium 20	33400
Su(var)2-10	Valium 10	29448
Su(var)3-7	Valium 22	35368
Su(z)12	Valium	31191
sug	Valium 10	27026
sv	Valium 10	27269
svp	Valium 10	28689
tai	Valium 10	28971
tap	Valium 10	26209
tara	Valium 1	31634
tef	Valium 1	31046
TFAM	Valium 10	26744
tgo	Valium 10	26740
tin	Valium 10	28539
tio	Valium 20	35812
tj	Valium 10	25987
tll	Valium 10	27242
tna	Valium 10	29372
toe	Valium 10	29345
tou	Valium 1	31637
toy	Valium 10	29346
trem	Valium 20	40881
trh	Valium 10	27986
Trl	Valium 20	40940
tsh	Valium 10	28022
ttk	Valium 10	26315
twi	Valium 10	25981
Ubx	Valium 10	31913

unc-4	Valium 20	36713
unpg	Valium 10	29393
Usf	Valium 10	25982
ush	Valium 10	29516
usp	Valium 10	27258
vfl	Valium 22	35468
vis	Valium 20	35738
vnd	Valium 10	27733
vri	Valium 10	25989
Vsx2	Valium 10	26223
vvl	Valium 10	26228
wdn	Valium 21	35654
wek	Valium 21	35680
Xbp1	Valium 10	25990
Xrp1	Valium 20	34521
yemalpha	Valium 22	35425
z	Valium 10	29446
zen	Valium 10	26229
zen2	Valium 20	34649
zf30C	Valium 21	35678
zfh1	Valium 10	29347

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