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Developmental Expression Profile of Bric á Brac 2, Midline and H15 in the Developing Eye and Central Nervous System of *Drosophila melanogaster*

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Drosophila Melanogaster

by

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Abstract

Aberrant activity of a single gene can lead towards development of cancerous cells. *Drosophila melanogaster* is a useful model system to study cancer because there is high degree of evolutionary conservation in signaling pathways between humans and flies that play major roles in regulating cell proliferation and growth (Miles et al., 2011). At The University of Southern Mississippi (USM), Dr. Leal's lab has gathered evidence suggesting that *bab1* and *bab2* interact with the T-box gene *midline (mid)* and its paralog *H15*, while the early developmental function of *bab1* and *bab2* remains unknown. That is why elucidating the early interactions of *bab1*, *bab2, mid* and *H15* is an important first step towards determining whether they collaborate to regulate cell proliferation. Defects in their function may contribute to disruption of cell proliferation and subsequent development of cancerous tissue. In this study, we demonstrate the early developmental interactions of Bab2, Midline and H15 proteins in *Drosophila melanogster* by immunolabelling imaginal eye discs (third instar larval, P1 and P2 stages) and embryonic central nervous system (stages 14, 15, 17) with specific antibody probes to detect these proteins. We also present preliminary chromosomal deficiency mapping analyses to ensure that the *bab2* gene is the only gene of interest interacting with *mid* from a pool of other potential *mid*-interacting gene candidates. Our results demonstrate the early developmental interactions of Bab2, Midline and H15 proteins within the eye discs and the embryonic central nervous system (CNS). The model known as the "Nymc1-tbx2-tbx20" interaction model (Cai et al., 2005) is also presented as a reference for future research where I will determine the collaborative role of Bab1 in Bab2-Midline-H15 interactions in early development as a graduate student at USM (August, 2013).

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Introduction

The development of human cancer is a complex process. Aberrant activity of a single gene can lead toward errors in signaling, cell-cycle and cell-death pathways that respond to changes in the interactions between the tumor and the tumor microenvironment (Brumby and Richardson, 2005). In order to better understand the pathways that lead toward cancer development, the use of simple animal model systems is required to facilitate rapid discoveries in mechanisms regulating cell proliferation and growth.

Drosophila melanogaster is a useful model system to study cancer because there is high degree of evolutionary conservation in signaling pathways between humans and flies that play major roles in regulating cell proliferation and growth (Miles et al., 2011). Although *Drosophila* has a very small genome in comparison with humans, the lack of genetic redundancy of the genome in flies eases genetic analyses; typically in larger genomes, there are multiple isoforms of genes, but in *Drosophila,* there is one gene to analyze directly. In addition, understanding basic signaling pathways in *Drosophila* leads toward a better understanding of conserved pathways in humans (Miles et al., 2011).

Drosophila genetics research has identified various genes that when mutated or deregulated result in the development of specific cancers (Miles et al., 2011). The identification of several signaling pathways including the Hippo, Notch, Decapentaplegic (Dpp) and Janus kinase - Signal Transducer and Activator of Transcription (JAK-STAT) was largely accomplished utilizing the powerful genetics of *Drosophila*. These pathways are known to play key roles in regulating cell proliferation. As such, mutations in specific genes within these pathways result in a cancerous phenotype as shown by much published research using the *Drosophila* model system (Vidal and Cagan, 2006; Januschke and Gonzalez, 2008).

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Several cancer studies demonstrate the oncogenic activity of the BTB/POZ-ZF gene in humans (Nakayama et al., 2006; Courdec et al., 2002). BTB/POZ-ZF stands for *bric á brac/* poxvirus-zinc finger domain, and it is the human homolog of the *bric á brac (bab)* gene expressed in *Drosophila melanogaster.* BTB/POZ-ZF is composed of several proteins that participate in a variety of cellular functions including transcriptional regulation, cellular proliferation, apoptosis, cell morphology and degradation of ubiquition tagged proteins (Nakayama et al., 2006). Some of the proteins encoded by the BTB/POZ-ZF domain are involved in DNA repair systems, while others have shown pro-apoptotic and anti-apoptotic roles (Kelly and Daniel, 2012). Since the major signaling pathways between *Drosophila* and humans are highly conserved, researching the function of the *bric á brac* gene using the fruit fly as an animal model system can lead toward a better understanding of the BTB/POZ-ZF pathway and its oncogenic activity in humans.

In *Drosophila melanogaster,* the *bab* locus is composed of two paralogous genes, *bab1* and *bab2,* which are involved in patterning the adult abdomen, legs, and ovaries (Kopp et al., 2000). They are also expressed in the *Drosophila* eye and CNS tissues (modENCODE Temporal Expression Profile; Flybase.org). However, the functional role of *bab1* and *bab2* in patterning the eye and CNS of *Drosophila* during early developmental stages remains unknown.

At USM, Dr. Leal's lab has gathered evidence suggesting that *bab1* and *bab2* interact with the T-box gene *midline (mid) and* its paralog *H15*. This preliminary evidence is supported by the ModENCODE consortium that reported that *mid* and *bab2* are predicted to interact *in vivo* based upon *in vitro* studies. Completing a developmental expression profile of the Bric á brac, Midline and H15 proteins will lead towards better understanding of Bric á brac's developmental role in patterning the eye and CNS tissues of *Drosophila.* Elucidating the early interactions of

these three genes is an important first step towards determining whether they collaborate to regulate cell proliferation. Defects in their function may contribute to disruption of cell proliferation and the subsequent development of cancerous tissue. In this study, we will demonstrate the early developmental interactions of Bab2, Midline and H15 proteins in *Drosophila melanogster.*

Methods

FLY STOCKS

All *Drosophila melanogaster* strains are maintained at 25°C on standard cornmeal-yeast-agar media with a 12 hours light/dark cycle. We used Oregon-R flies as wild type controls for the experiments.

IMMUNOFLOURESCENCE STUDIES

Larval Eye Disc Fixation

Developmentally staged third-instar larval eye discs were dissected from wild-type *Drosophila melanogaster* in cold phosphate buffered saline (PBS: 10 mM NaH_2PO_4, 10 mM Na_2HPO_4, pH 7.0) and the tissues were fixed in 3.7% paraformaldehyde in 0.1 M 3-Morpholinopropanesulfonic acid (MOPS) buffer (pH 7.2) for 15 mins at 25°C (Panin et al., 1997) followed by two washes of PBS containing 0.1% Triton-X 100 (PTX) and two washes of PTX supplemented with 1% bovine serum albumin (PBT). Eye discs were incubated in PBT containing 1% goat serum for at least one hour at 25°C and then incubated with primary antibodies for 4 hrs at 25°C or overnight at 4°C.

Pupal Eye Disc Fixation

We developmentally staged pupal eye discs after puparium formation (APF) and dissected P0 (prepupae), P1 (0-1hr APF), P2 (1-3hrs APF), P3 (3-6hrs APF) in PEMF buffer containing 0.1 M PIPES (pH 7.0), 1mM $MgSO_4$, 2mM EGTA and 4% formaldehyde (Skeath and Caroll, 1994). The eye discs are fixed in PEMF buffer with 4% formaldehyde for 30 minutes on

ice with continuous shaking, followed by three washes with PAXD (PBS containing 1% BSA, 0.3% Triton X-100, and 0.3% sodium deoxycholate), and then incubated with the primary antibodies for 4 hrs at 25°C or overnight at 4°C (Frankfort et al., 2004).

Primary Antibodies

We used the following primary antibodies at the indicated dilutions for this study: guinea pig anti-H15 (1:2000), rabbit anti-Mid (1:300) (Leal et al., 2009) and rabbit anti-Bab2 (1:4000) (Courdec et al., 2002). We used Alexafluor 488 (H15), 594 (Mid) and 633 (Bab2) secondary antibodies with appropriate species specificity for immunofluorescent labeling (Molecular Probes).

CONFOCAL SCANNING MICROSCOPY

We used Zeiss LSM10 META confocal microscope and Zeiss LSM Image browser software (version5) to scan and analyze the immunofluorescently labeled tissues. Immunofluorescent probes are excited at 488 nm (to detect H15) and 594 nm (to detect Mid) and 633nm (to detect Bab2) of the absorption spectrum.

EMBRYO COLLECTION

Drosophila melanogaster eggs were collected and their chorion was removed with 50% bleach in distilled water by incubating for 3 minutes. The embryos were rinsed extensively with distilled water for about 3 minutes. We proceeded fixation the tissues by placing the embryos in a 10 ml test tube containing 2 ml heptane and 2 ml 37% formaldehyde followed by gentle rocking for 2 minutes. The embryos gathered at the interface of the solvents. We removed the formaldehyde and added 6-10 ml of methanol. The tube was shaken vigorously by hand for 1-2

minutes. The devitellinized embryos sank to the bottom of the tube after shedding their fatty, buoyant vitelline membrane. The embryos were transferred with a glass pasteur pipette to a 1.5 ml eppendorf tube and rinsed 4-5 times with 1 ml methanol.

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The methanol was removed and 1 ml of PTX (Triton-X and PBS) was added. The mixture of embryos was rocked gently on the rotator for 10 minutes followed by a second rinse of PTX for a 10 minute incubation. One ml of PBT (Bovine serum albumen + Triton-X; recipe on page 3) was added and the embryos were rocked on the rotator for 10 minutes. This step was repeated two more times, but for 5 minutes per rinse, respectively. The PBT solution was removed, and 300 microliters of the primary antibody was added. The tube was laid down on its side so the embryos were well exposed to the antibody. The embryos were incubated in the primary antibody for 2 hours at room temperature (typically 25 degrees C) or at 4 hours at 4 degrees Celsius. After the incubation with the primary antibody was complete, the primary antibody was removed and recycled by returning it to its original tube. The embryos were rinsed 3 times with 1 ml of PBT for 10 minutes each rinse. After the last rinse, secondary antibody was added. (Muller, 2008)

CHROMOSOMAL DEFICIENCY MAPPING

The following chromosomal deficiency lines were crossed to the *mid-RNAi* transgenic fly line (*UAS-mid-RNAi/CyO;GMR-Gal4/TM3*): *Df(3L)Exel6086, P{w[+mC]=XP-U}Exel6086/TM6B, Tb[1], w[1118]; Df(3L)BSC178/TM6B, Tb[+], w[1118];*

Df(3L)BSC289/TM6C, Sb[1] cu[1], w[1118]; *Df(3L)BSC311/TM6C, Sb[1] cu[1], w[1118]*; *Df(3L)BSC363/TM6C, Sb[1] cu[1], w[1118]*; *Df(3L)BSC431/TM6C, Sb[1] cu[1], and w[1118]*; *Df(3L)BSC632/TM6C, cu[1] Sb[1]*. One-day old female F1 progeny with straight wings and normal bristles were collected (example UAS-mid-RNAi/ w[1118] ;Df(3L)BSC632/TM3). The *Drosophila's* eyes were transfixed to the glass slide with clear nail polish with one eye facing up.

Drosophila eyes were observed under a Leica M165 stereomicroscope with a DFC 295 digital camera. The microscope is connected to a computer that allows us to combine several multifocal images into one image to create the optimal picture or montaged image. Eye bristles were counted using Image-Pro Analyzer software (Media Cybernetics, Houston, TX). The bristle counts from mutant F1 progeny were compared to wild-type, Oregon-R flies to determine the *mid-RNAi* mutant phenotype was modified (suppressed or enhanced).

Results

We dissected imaginal eye discs of pupal stages (P1, P2) in cold PEMF and third-instar larvae in PBS. After fixation (*Pupal Eye Disc Fixation* – Methods), imaginal eye discs were co-immunolabeled with guinea pig anti-H15 (1:2000), rabbit anti-Mid (1:1000) (Leal et al., 2009) and rabbit anti-Bab2 (1:4000) antibodies (Courdec et al., 2002). We used Alexafluor 488 (H15), 594 (Mid) and 633 (Bab2) secondary antibodies with appropriate species specificity for immunofluorescent labeling (Molecular Probes) following the immunoflorescent studies reported by Das et al. (2013) . Using DABCO (1,4-Diazabicyclo[2.2.2]octane) solution to prevent fading of the immunofluors, imaginal eye discs were placed on a slide with a cover slip and scanning confocal images were taken (Fig. 1). Figure 1 depicts the immunolabeling of P1, P2 and third-instar larvae imaginal eye discs where the Bab2, Mid and H15 expression patterns overlap.

We collected embryos using the protocol (Material and Methods). The embryos were labeled by guinea pig anti-H15 (1:2000), rabbit anti-Mid (1:1000) (Leal et al., 2009) and rabbit anti-Bab2 (1:4000) (Courdec et al., 2002) We used Alexafluor 488 (H15), 594 (Mid) and 633 (Bab2) secondary antibodies with appropriate species specificity for immunofluorescent labeling (Molecular Probes) following immunoflorescent studies procedure.

Using DABCO solution, we dissected ventral nerve chords (CNS) out staged (14, 15, 17) embryos. CNS chords were fixed on the slide and pictures were taken by the confocal microscope (Figure 2.)

Figure 2 shows the results of immunolabeling the CNS where the interactions of Bab2, Mid and H15 are visible as a merge of three colors in D, H, and L. Localization of Bab2, Mid and H15 in CNS is also shown.
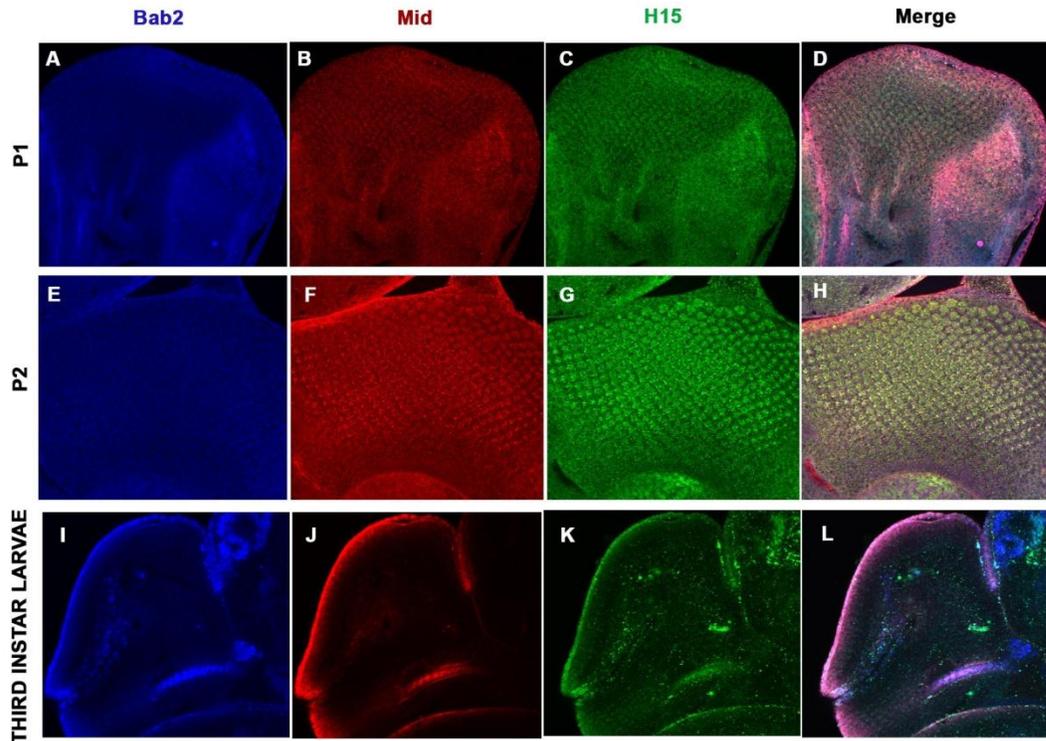
Figure 1: Immunolabeling of P1, P2 and third instar larvae imaginal eye discs. A:Bab2 in P1 imaginal eye disc. B:Mid in P1 imaginal eye disc. C:H15 in P1 imaginal eye disc. D:Merge of Bab2, Mid and H15 in P1 imaginal eye disc. E:Bab2 in P2 imaginal disc. F:Mid in P2 imaginal eye disc. G:H15 in P2 imaginal eye disc. H:Merge of Bab2, Mid and H15 in P2 imaginal eye disc. I:Bab2 in third instar larvae imaginal eye disc. J:Mid in third instar larvae imaginal eye disc. K:H15 in third instar larvae imaginal eye disc. L:Merge of Bab2, Mid and H15 in third instar larvae imaginal eye disc
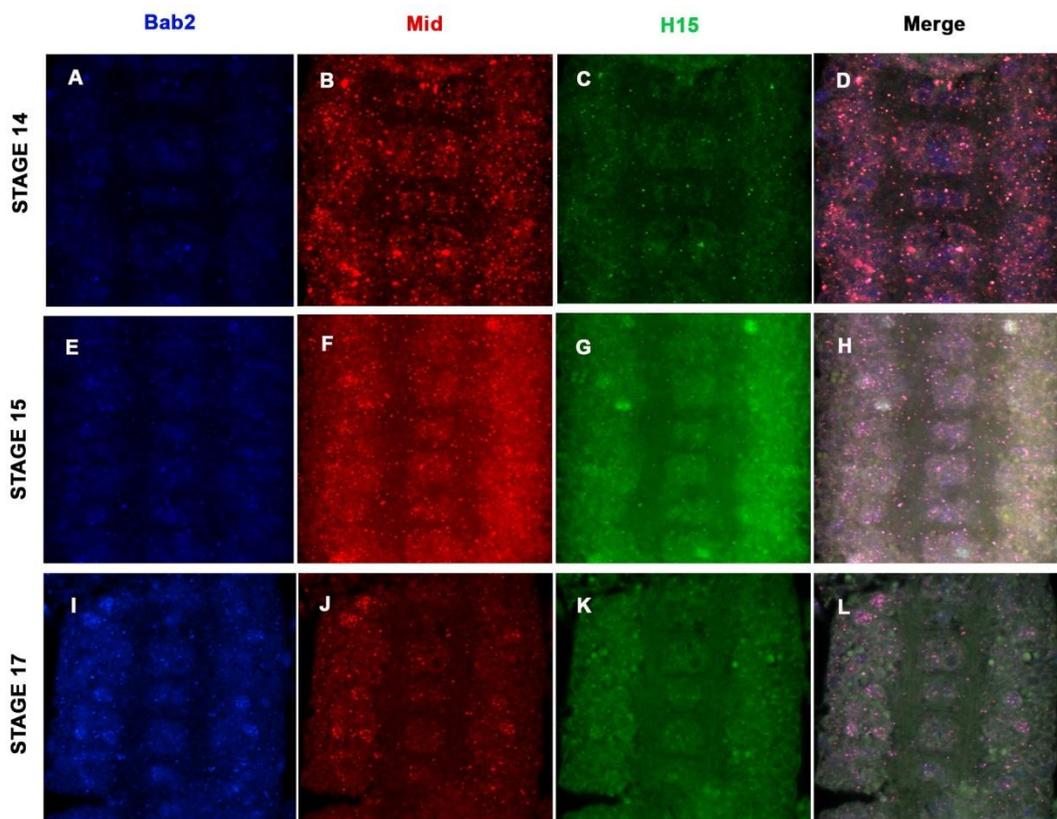
Figure 2. Immunostaining of CNS. A:Bab2 in CNS stage 14. B:Mid in CNS stage 14. C:H15 in CNS stage 14. D:Merge of Bab2, Mid, and H15 in CNS stage 14. E:Bab2 in CNS stage 15. F:Mid in CNS stage 15. G:H15 in CNS stage 15. H:Merge of Bab2, Mid, and H15 in CNS stage 15. I:Bab2 in CNS stage 17. J:Mid in CNS stage 17. K:H15 in CNS stage 17. L:Merge of Bab2, Mid, and H15 in CNS stage 17.

We also crossed *UAS-mid-RNAi* males with deficiency lines 7565, 9609, 23674, 24337, 24387, 24935, and 25723, genotypes *w[1118]; Df(3L)Exel6086, P{w[+mC]=XP-U}Exel6086/TM6B, Tb[1], w[1118]; Df(3L)BSC178/TM6B, Tb[+], w[1118]; Df(3L)BSC289/TM6C, Sb[1] cu[1], w[1118]; Df(3L)BSC311/TM6C, Sb[1] cu[1], w[1118]; Df(3L)BSC363/TM6C, Sb[1] cu[1], w[1118]; Df(3L)BSC431/TM6C, Sb[1] cu[1], and w[1118];*

Df(3L)BSC632/TM6C, cu[1] Sb[1] respectively. Figure 3 shows the rescue effect of the *mid-RNAi*/deficiency lines in female progeny.
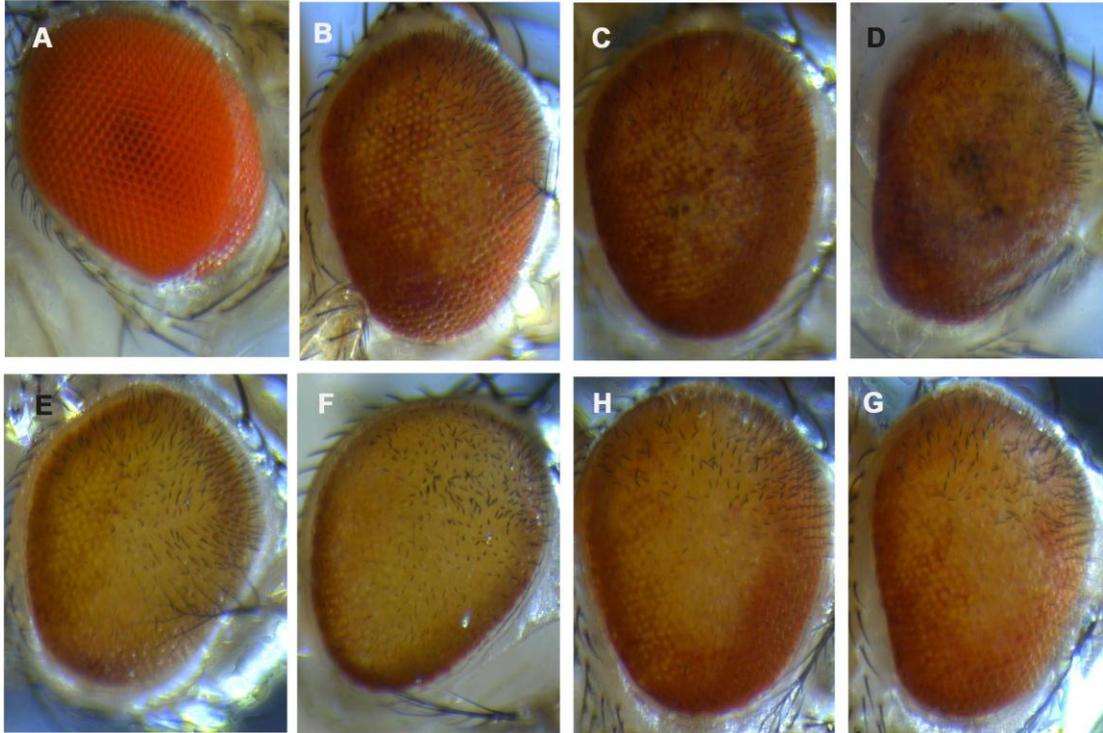
Figure 3. Eye pictures showing the rescue effects in crosses with deficiency lines in females. A: wild type. B: UAS-mid-RNAi/ w[1118] ; Df(3L)Exel6086, P{w[+mC]=XP-U}Exel6086/TM3. C: UAS-mid-RNAi/ w[1118] ; Df(3L)BSC178 /TM3. D: UAS-mid-RNAi/ w[1118] ; Df(3L)BSC289 /TM3. E: UAS-mid-RNAi/ w[1118] ; Df(3L)BSC311 /TM3. F: UAS-mid-RNAi/ w[1118] ; Df(3L)BSC363/TM3. H: UAS-mid-RNAi/ w[1118] ; Df(3L)BSC431/TM3. G: UAS-mid-RNAi/ w[1118] ;Df(3L)BSC632/TM3

Table 1 presents the rescue effects of 7565, 9609, 23674, 24337, 24387, 24935, and 25723 in females in comparison with the wild-type (WT). The *mid-RNAi*/23674 mutant eye shows the greatest reduction in bristles (41.34%) while the *mid-RNAi*/24337 mutant eye shows the smallest reduction in bristles (11.2%).

	dorsal	ventral	overall	rescue percentage (number of bristles/number of bristles of WT)
wild type (WT)			403.55	100
UAS-mid-RNAi/ w[1118] ; Df(3L)Exel6086, P{w[+mC]=XP-U}Exel6086/TM3	246.25	86	332.25	82.33
UAS-mid-RNAi/ w[1118] ; Df(3L)BSC178 /TM3	216.33	79	295.33	73.18
UAS-mid-RNAi/ w[1118] ; Df(3L)BSC289 /TM3	164.25	45.25	209.5	58.66
UAS-mid-RNAi/ w[1118] ; Df(3L)BSC311 /TM3	244.33	114.33	358.66	88.88
UAS-mid-RNAi/ w[1118] ; Df(3L)BSC363/TM3	241	94	335	83.01
UAS-mid-RNAi/ w[1118] ; Df(3L)BSC431/TM3	206	105.5	311.5	77.19
UAS-mid-RNAi/ w[1118] ;Df(3L)BSC632/TM3	225.5	82	307.5	76.20

Table 1: Bristle count of the wild type and progeny of UAS-mid-RNAi crosses with deficiency lines 7565 (UAS-mid-RNAi/ w[1118] ; Df(3L)Exel6086, P{w[+mC]=XP-U}Exel6086/TM3)**, 9609** (UAS-mid-RNAi/ w[1118] ; Df(3L)BSC178 /TM3) **, 23674** (UAS-mid-RNAi/ w[1118] ; Df(3L)BSC289 /TM3)**, 24337** (UAS-mid-RNAi/ w[1118] ; Df(3L)BSC311 /TM3)**, 24387** (UAS-mid-RNAi/ w[1118] ; Df(3L)BSC363/TM3)**, 24935** (UAS-mid-RNAi/ w[1118] ; Df(3L)BSC431/TM3)**, and 25723** (UAS-mid-RNAi/ w[1118] ;Df(3L)BSC632/TM3) **females.**

Discussion

The *mid* and *H15* genes are both important for the regulation of cell fate specification. The *mid* gene is also involved in the regulation of cardiovascular system development, neuronal differentiation, cellular component organization, neuroblast differentiation and regionalization. As a *mid* homolog in vertebrates, *tbx20* misexpression leads to the development of several child tumors (Cai et al., 2005). Considering that expression of Tbx20 and its homologues in cardiac structures has been conserved from *Drosophila* to mammals (Plageman and Yutzey, 2005), interactions of *mid* with the DNA-dependent transcriptional factor *bab2* present a possible mechanism that can lead towards the aberrant proliferation of cells.

So far, according to several studies, homologues of *mid* and *bab2* are involved in the etiology of several cancers and play important roles in the development of cancerous cells. A specifically identified conserved Bab domain has proven to be essential for mediating protein-protein interactions. In humans, BTB/POZ-ZF interacts with a candidate tumor suppressor gene that is hypermethylated in cancer – HIC-1 (Guerardel et al., 1999). The authors of this study state that many BTB/POZ and zinc finger proteins are transcriptional repressors such as *Drosophila tramtrack* and vertebrate repressors including ZF5, QF1-binding protein isoform B (QFBP-B), PLZF and BCL6.

Previous study (Cai et al., 2005) has already shown that interaction of two t-box genes, *tbx20* (homolog of midline) and *tbx2* can result in overexpression of Nymc1 and higher proliferation of cell. In *Drosophila melanogaster*, *mid* is reported to interact with *bab1* (Mod Encode consortium). However the interaction of *mid* and *bab2* has not been discovered *in vivo* yet.

In this study, immunolabeling of Mid, Bab2, and H15 in *Drosophila* eye imaginal discs and the CNS has shown that these proteins are co-expressed in imaginal eye disc tissues and the CNS. Figure 1 and figure 2 show the overlapping expression pattern of Mid, Bab2, and H15 in imaginal eye discs and the CNS, respectively. The co-expression patterns of Mid, Bab2, and H15 suggest these proteins interact throughout the development of *Drosophila melanogaster.*

From the preliminary data, the probability that Bab2 and Mid interact is high given the co-expression patterns detected during eye and CNS development. However, for future studies, the interaction of Mid and Bab2 has to be identified by using techniques like high-throughput chromatin immunoprecipitation with microarray technology (*"chip"*) (ChIP-chip) experiments in *Drosophila* as it was done with Bab1 and Mid interaction.

The *bab1* and *bab2* genes have the same orientation and show several structural similarities, suggesting that they are the result of a chromosomal duplication. (FlyBase.org) Both genes have four introns, three of which are at homologous positions in the coding region. Sequence analysis of the *bab1* and *bab2* transcript predicts proteins of 967 and 1067 amino acids, respectively. (FlyBase.org) Bab1 and Bab2 have two evolutionarily conserved domains in common. Outside of these domains Bab1 and Bab2 show only low sequence similarity to each other and no significant similarity to other proteins. (FlyBase.org) *bab* and *BTB-II* have been shown to constitute a gene complex. The domain architecture of Bab1 and Bab2, together with their nuclear localization, suggests that these proteins function as transcriptional regulators (Courdec et al., 2002).

Based upon our results, the possible explanation for the co-expression patterns of Bab2, Mid and H15 could be that these proteins interact. At present, the unavailability of anti-Bab1 antibody precludes the detection of Bab1 proteins within eye imaginal discs and the CNS of

Drosophila using immunolabelling techniques. For future studies, if funds are available, we are planning to make our own Bab1 antibody, in addition to generating a *bab1* transcript probe that would be marked with fluorescence, to merge in parallel with an antibody probe for Mid.

Tbx20 has been shown to serve an important role by inducing the overexpression of Nymc1 which leads to a higher rate of cell proliferation (Cai et al., 2005). For future studies, based on this and previous research, we propose a model where *mid* as a T-box gene plays a crucial role as a repressor and co-regulatory member with the transcription factors Bab1/Bab2. We conjecture that this association leads toward the aberrant proliferation of the cell effecting the expression of a gene similar to that of Nymc1 according to the "tbx20-tbx2-Nymc1" model. To support this model, it is necessary to point out the similarity between *tbx2* (*omb* in *Drosophila*) and the *bab* locus as transcriptional repressors.

According to World Health Organization, cancer is a leading cause of death worldwide and the total number of cases on the global level is increasing. Understanding the cause of cancer can help the early detection, prevention, and/or development of gene therapy for specific types of cancer. As mentioned before, several cancer studies demonstrate the oncogenic activity of the BTB/POZ-ZF gene in humans (Nakayama et al., 2006; Courdec et al., 2002). A better understanding of cell proliferation regulated by the Bab2-Mid-H15 interaction can represent a step forward toward finding a solution to block the oncogenic activity of the BTB/POZ-ZF gene in humans.

In conclusion, we identified a potential early interaction between Bab2, Mid, and H15. The future step in the continuation of this study is to determine whether Mid and H15 interact with Bab2 only, or the Bab1-Bab2 complex, in order to gain a clearer picture of the early roles of

bab1 and *bab2* in the development of *Drosophila melanogaster*. The next step will be testing the discussed model for the Bab2-Mid-H15 interaction.

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