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REGULATORS OF *repo*: A SEARCH FOR *gcm/repo* TRANSCRIPTION FACTORS

A Dissertation

presented in partial fulfillment of requirements

for the degree of Doctor of Philosophy

in the Department of Biology

The University of Mississippi

by

KATHY A. NIPPER

May 2014

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## ABSTRACT

The role of the *Drosophila melanogaster* gene *glial cells missing* (*gcm*) is that of a binary switch in both the central nervous system (CNS) and the peripheral nervous system (PNS) in specification of the glial fate for multipotent precursor cells. However, Gcm is also essential for maturation of hematocytes and tendon cells (Alfonso and Jones, 2002; Jones, et al., 1995; Hosoya et al., 1995; Soustell et al., 2004). The varied outcomes of *gcm* expression imply the interaction of co-factors capable of giving the Gcm protein different “meanings” in different developmental contexts. The Gcm target *repo* is expressed exclusively in glial cells and its protein is essential for differentiation and migration of glia (Yausa et al., 2003; Lee and Jones, 2005). Defining the transcriptional role of Repo can be of significance in understanding glial specific gene expression. Systematic analysis of the *repo* cis-regulatory DNA indicate that a 98 base-pair region recapitulates endogenous *repo* expression with only a single Gcm binding site (Lee and Jones, 2005; Johnson et al., 2011). This 98 bp Epi Repressor fragment was used as “bait” in a Double Interaction yeast screen to identify collaborating factors of Gcm. Fusion proteins recovered in the screen include *gcm*, *groucho*, and three proteins annotated to trichogen cell phenotypes that may function in macrochaetae development. Three confirmed positives may be related to chromatin remodeling: the co-repressor *groucho*, *Rm62*, and *histone 4R*. CG6770 and Groucho, were selected for further study and exhibited *in vivo* interactions with *gcm*.

## DEDICATION

This Dissertation is dedicated to L. L. Branscome, Jr., a cattleman and an applied geneticist whose expertise was acquired by observation and experimentation and whose encouragement and integrity made him an extraordinary instructor in the study of life.

## LIST OF ABBREVIATIONS

CNS	central nervous system
Gcm	Drosophila protein Glial cells missing
<i>gcm</i>	Drosophila gene <i>glial cells missing</i>
PNS	peripheral nervous system
SOP	sensory organ precursor
AC-S	<i>achaete-scute</i> gene complex
ac	Drosophila gene <i>achaete</i>
sc	Drosophila gene <i>scute</i>
Ac	Drosophila protein Achaete
<i>Pyx</i>	<i>polythrix</i> , a <i>glial cells missing</i> allele
Gcm 2	the product of the Drosophila <i>gcm2</i> gene
<i>repo</i>	the drosophila gene <i>reversed polarity</i>
<i>ttk69</i>	Drosophila gene <i>tramtrack 69</i>
<i>pntp1</i>	Drosophila gene <i>pointed P1</i>
Epi Repressor	the 98 bp fragment from cis-regulatory DNA upstream of <i>repo</i>
pAbAi	Clontech plasmid for cloning a “bait” fragment into the yeast genome by homologous recombination
p53AbAi	Clontech plasmid for cloning Drosophila gene <i>p53</i> into yeast genome for use as a positive control
pGADT7-recombinase	Clontech, Inc. plasmid used to create a plasmid library

pGPDgcm	yeast expression vector for expression of glial cells missing
pCasPer 98X2	an engineered cloning vector carrying two copies of the 98 bp epidermal repression fragment
Ura 3-52	yeast gene <i>ura 3</i> with a transposon insertion that renders the yeast incapable of uracil synthesis
ura	uracil
trp	tryptophan
leu	leucine
cDNA	DNA synthesized from messenger RNA
PCI	phenol chloroform/isopropyl alcohol extraction protocol
PCR	polymerase chain reaction by which DNA is copied
Y1HGold	yeast line with Aureobasidin A reporter from Clontech, Inc.
SMART technology	reverse transcriptase with a switching system at the 5'
SD	synthetic dropout media for yeast which has some specific amino acid(s) omitted
YPD	yeast peptone extract media with dextrose
GMR	<i>glass</i> Multimer Reporter, an artificial construct for expression of a gene of interest in the <i>Drosophila</i> eye
ftz <sup>rf10</sup> ry e	a homozygous lethal <i>fushi terazu</i> gene, <i>rosy</i> , and <i>ebony</i>
bp	base pairs

## ACKNOWLEDGEMENTS

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## INTRODUCTION

The *glial cells missing* (*gcm*) gene has been described as a genetic switch in the development of the *Drosophila* central nervous system. Its transient expression controls initiation of glial cell determination in multipotent progenitor cells derived from neuroectoderm (Hosoya et al., 1995; Jones et al., 1995; Vincent et al., 1996). Ectopic expression of Gcm protein results in transformation of presumptive neurons into glial cells whereas *gcm* mutant embryos lack lateral glia (Hosoya et al., 1995; Jones et al., 1995; Vincent et al., 1996). The midline glia originate from the mesectoderm and are not *gcm*-dependent. However, lateral glia are derived from the neurogenic ectoderm and lateral peripheral ectoderm, and *gcm* is required for their cell-fate determination (Bossing et al., 1996; Goodman and Doe, 1993; Jan and Jan, 1993; Schmid et al., 1999). Lateral glia ensheath the longitudinal axons, surround the nerve cell bodies within the cortex, surround the ventral nerve cord, and give rise to the peripheral glia that ensheath the axons of motor and sensory nerves (Lee and Jones, 2005). The glial cells are important in establishing the cellular specifications and connectivity essential for development of a functional central nervous system (Jones, 2001).

In the peripheral nervous system (PNS) *gcm* is expressed in the cells of gliogenic sensory organs (Van De Bor et al, 2002). Nongliogenic sensory organs contain two neurons and no glia. As in the process of delamination of neural precursor cells of the CNS, in the PNS the expression of proneural genes results in neural competence in groups of cells called proneural clusters.

Proteins of the Notch pathway regulate cell communication within the proneural clusters by lateral inhibition so that only one of the cells becomes a sensory organ precursor (SOP) (Doe and Goodman, 1985; Cubas, et al., 1991; Heitzler and Simpson, 1991; Brand et al, 1993; Skeath and Carroll, 1993; Jan and Jan, 1998). The proneural genes of the Achaete-Scute Complex (AS-C) are co-activated by site-specific gene products that bind to enhancer sequences located several kilobases away, and their regulation has been considered the basis of the bristle pattern of the notum (Campuzano et al., 1985; Ruiz-Gomez and Modolell, 1987; Romani et al. 1989; Rodriguez et al., 1990; Cubas et al., 1991; Gomez-Skarmeta et al., 1995). *achaete* and *scute* (*ac* and *sc*) expression in individual proneural clusters confers neural competence and, in combinatorial control with the products of several other genes, is thought to create a “prepattern” of regulator domains. However *pannier* encodes a GATA transcription factor with two isoforms that regulate expression of *wingless* and *achaete-scute* during development of the imaginal disc (Calleja et al., 2002; Fromental-Ramain et al., 2008; Heitzler et al., 1996; Modolell and Campuzano, 1998). Pannier is a direct proneural activator of *ac/sc*; Pannier binds to the dorsocentral enhancers located 4 kb upstream of *ac* and 30kb upstream of *sc*. Heterodimers of Ac and Sc with the protein Daughterless also serve as positive regulators of AS-C transcription while heterodimers of Ac and Sc with Extramacrochaete are negative regulators of AS-C expression. Recent results suggest that a “mosaic of transcription factors” function not only in pre patterning of the notum, but in regulation of other features such as muscle attachment and pigmentation (Calleja et al., 2002).

The sensory organ precursor (SOP) of each proneural cluster undergoes an asymmetric mitotic division to produce cells pIIa and pIIb, which then divide asymmetrically again to produce four daughter cells: the trichogen, tormogen, neuron and thecogen cells. The trichogen

cell gives rise to the shaft of the mechanoreceptor and the tormogen produces the socket from which it extends. In gliogenic sensory organs, the neuron and thecogen develop the neuron and its glial sheath (Furman and Bukharina, 2011). *gcm* expression is required for differentiation of the glial cells (Jones, B. W., 2001; Van De Bor et al., 2002). The asymmetric mitotic divisions of SOP cells are influenced by asymmetry of distribution of the Numb and Neutralized proteins as well as by products of those genes involved in polarization of the cell in two separate phases, and by genes such as *pins* that establish specific orientation of the mitotic spindle (Furman and Bukharina, 2011). Additionally a group of selector genes (including *tramtrack*, *prospero*, and *cut*) regulate further specialization of the cells resulting from these divisions (Furman and Bukharina, 2011). Essentially the daughter cells exit from asymmetric cell division being different from each other and from the parent SOP in their ability to further differentiate and give rise to the sensory organs of the peripheral nervous system of *Drosophila* known as macrochaetae, or bristles (Furman and Bukharina, 2011; Renaud et al., 2002; Van De Bor et al., 2002). The number of the macrochaetae and their position on the thorax is so rigidly determined that they are used as a means of classification of *Drosophila* species. *Drosophila melanogaster* has eleven (11) pairs of macrochaetae (Furman and Bukharina, 2002). Temporal misexpression of *gcm* induces proneural gene expression and results in supernumerary bristles on the notum and scutellum of adult flies (Van De Bor et al., 2002). The supernumerary bristles are associated with the mutation *Polythryx* (*Pyx*), which induces early expression of *gcm* resulting in AS-C expression (Van De Bor et al., 2002). Overexpression of *gcm* by means of an expression vector has also been observed to produce the supernumerary bristle phenotype (Jones, B. W., unpublished data). Although *gcm* regulates development of lateral glial cells in *Drosophila* embryogenesis and is required for development of the glial cells of the PNS, this transcription

factor is also expressed in the type of larval hemocytes called plasmatocytes and is required for their maturation into phagocytic macrophages. Gcm is expressed in tendon cells, and is required for their development (Alfonso and Jones, 2002; Jones, et al., 1995; Hosoya et al., 1995; Soustell et al., 2004). Gcm and its homolog Gcm2 have been shown to function redundantly in differentiation of plasmatocytes (Alfonso and Jones, 2002). In the postembryonic CNS *gcm* is expressed in both glial cells and one neuronal line of cells of the optic lobe lamina (Soustelle and Giangrande, 2007). The early and transient expression of *gcm* in its role in glial differentiation and its expression in other cell lines with different developmental objectives indicate that there must be cofactors that interact with it in bringing about such context dependent outcomes.

Three of the genes that have been identified as targets of Gcm are the transcription factors *reversed polarity (repo)*, *tramtrack isoform 69 (ttk69)* and *pointed p1 (pntp1)* (Lee and Jones, 2005). *gcm* expression promotes glial differentiation through interaction of Repo and Pointed, and, via expression of *tramtrack*, neuronal characteristics are simultaneously repressed (Lee and Jones, 2005; Yausa et al., 2003; Geisen et al., 1997). Essentially, the three act cooperatively to regulate expression of glial-specific genes in *Drosophila* by two different pathways (Geisen et al., 1997). The simultaneous suppression of neuronal differentiation and induction of gliogenesis occurs despite differences in the origin of progenitor cells of the CNS that generate glia and the sensory organ precursors of the PNS that produce neurons and glia (Jones et al, 2004). The homeodomain protein Repo has been shown to be essential for promoting glial differentiation; it has also been shown to act in cooperation with TTK69 to suppress neuronal development (Yausa et al., 2003). The protein Loco is required for morphogenesis of glial cells, and Repo and Pointed act synergistically to regulate expression of *loco* (Yausa et al., 2003).

Because *repo* is expressed exclusively in all lateral glial cells and its protein is essential for appropriate differentiation and migration of glia, defining its transcriptional role can be of significance in understanding glial specific gene expression. *repo* is not expressed in either hemocytes or tendon cells although both are Gcm positive, suggesting that other collaborating factors are important in regulating *repo* expression. The cis-regulatory region of *repo* contains multiple Gcm binding sites (Figure 1). The systematic analysis of these regulatory DNA elements has identified a 98 base pair (bp) epidermal repressor element (Epi Repressor) sufficient for driving glial expression of a *lacZ* reporter while simultaneously repressing epidermal expression (Lee and Jones, 2004; Johnson et al., 2012).

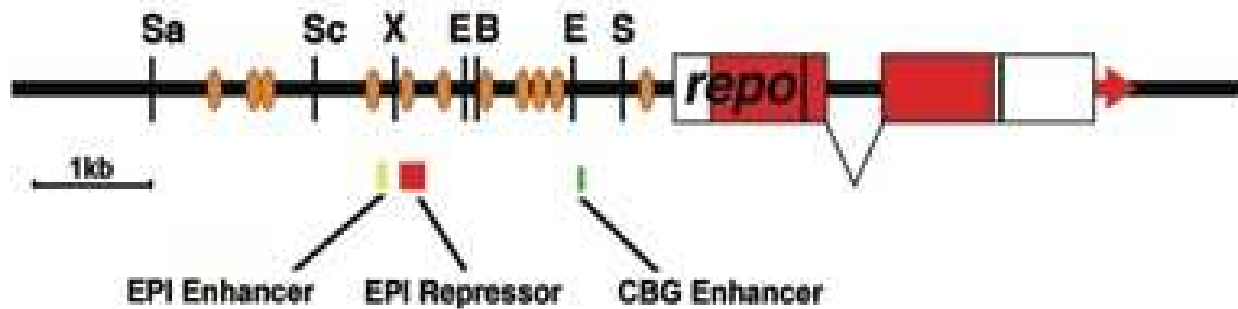


Figure 1. Diagram of cis-regulatory DNA of *repo*. The proposed location of the 98 bp epidermal repressor element is within the region of cis-regulatory elements upstream of the *repo* gene. Gcm binding sites are designated by orange ovals; three DNA regions conferring specific expression activities are represented by bars below the map. Restriction enzyme sites: Sa, Sall; Sc, ScaI; X, XhoI; E, EcoRI; B., BamHI; S., SpeI. The repressor fragment contains one Gcm binding site and is capable of recapitulation of the *repo* expression pattern in a *Drosophila* line carrying a *lacZ* reporter construct. Adapted from Johnson, R., Wood, J., and Jones, B. 2011. Reproduced by permission of the authors.



In an effort to identify molecules that serve as co-factors with Gcm in regulating expression of *repo*, a Double Interaction Yeast Screen (Yu et al., 1999) was performed using the 98 bp Epi Repressor sequence as “bait”. Because *repo* is not expressed in Gcm-positive hemocytes or tendon cells, collaborating factors acting with Gcm in this context afford an opportunity to study Gcm control of its target genes.

The Double Interaction Screen is an adaptation of a one-hybrid yeast screen for identifying protein-DNA interactions. Tandem repeats of the target/bait DNA were cloned into a pAbAi vector which was then integrated into the yeast genome upstream of a reporter gene by homologous recombination (Clontech 2009). The *Aureobasidin A resistance* reporter gene (*AbAi*) carried by the *pAbAi* vector confers the ability to grow in the presence of the antibiotic Aureobasidin A. A *Drosophila* cDNA library of Gal4T7 plasmids was constructed in the yeast reporter strain (Y1HGold/*AbAi*) by homologous recombination. Proteins expressed from the *Drosophila* library are expressed as fusions to the yeast Gal4 activation domain. (Gal4AD prey proteins). When a fusion protein binds to the “bait” sequence its Gal4 activation domain drives expression of the *AbAi* reporter gene ( Clontech, 2009), conferring the ability to grow on the selective media containing Aureobasidin A.

A yeast expression vector carrying the *gcm* gene was then introduced by transformation into the Y1HGold/*AbAi* yeast reporter strain, adding a second dimension to the screen. Proteins interacting with Gcm as co-factors or binding partners should also be identified by their interaction in the reporter system (Figure 4) as well as those transcriptional regulators capable of binding to the “bait” DNA individually (Yu et al., 1999). This Double Interaction Screen which included the *gcm* expression vector pGAD/*gcm* was performed simultaneously with a classic yeast one-hybrid screen (Figure 5).

## 2. EXPERIMENTAL PROCEDURES

### 2.1 Yeast Plasmid Construction

The Matchmaker Gold One Hybrid Yeast kit (Clontech, Inc., 2009) was used for the modified one-hybrid screen. Materials provided include (A) the plasmid pABAi for insertion of the bait construct and its integration into the yeast genome; (B) the p53AbAi plasmid for constructing the positive control; (C) the pGADT7-Recombinase plasmid for generating the library of prey plasmids; and (D) the p53 plasmid which serves as a positive control during transformation.

The two copies of the 98 base pair fragment of *repo* cis-regulatory DNA referred to as the epidermal repressor (cgaatcctctccctcggtgcaatccttgaagccagaccacataattggcacat  
tggctaatgcaaaatactgtctgattattcacacgcaacgag) were synthesized as an oligonucleotide by Integrated DNA Technologies, Inc. and inserted into pCasPeR between Not1 and BamH1 sites in the multiple cloning sites. For the bait in the Double Interaction Screen, a set of four tandem repeats was constructed by excising two copies from plasmid pCasPeR- 98X2 (containing two tandem copies of the 98 bp fragment). The restriction enzyme Not1 was used to linearize the CasPeR 98 X 2 plasmid. Klenow was used to blunt the overhanging ends of that restriction, then XhoI was used to restrict the other end of the 98 bp fragment. The same pCasPer-98X2 plasmid was restricted with BamHI, the overhanging ends blunted with Klenow, and PCI extracted. The linearized plasmid was then restricted with XhoI to create compatible ends for directional cloning and again was PCI extracted. With directional cloning the two-copy fragment was

inserted into the prepared vector using 40ng vector to 200ng insert in a reaction with T4 DNA ligase. The new construct carrying four copies of the 98 bp fragment was transformed into *E. coli* DH5 alpha (Invitrogen, Inc.) and the presence of the four copies was confirmed by gel analysis. CasPer-98X4 DNA was isolated and the four tandem copies were harvested. The fragment containing four tandem copies of the 98bp cis-regulatory DNA was excised from CasPer using EcoR1, blunted with Klenow, extracted by phenol chloroform isopropyl alcohol (PCI), and restricted a second time with XhoI. The pAbAi vector was also digested with EcoR1, treated with Klenow, and digested with XhoI. The fragment containing four tandem copies of the Epi Repressor “bait” was transformed into pAbAi. The pBait-*AbAi* plasmid was digested with BbsI to linearize it and transformed into Y1HGold yeast. Introduction of *pAbAi* into the yeast genome repaired the transposon-induced disruption in the yeast *ura 3-52* gene by homologous recombination with the wild type *ura-3* gene carried in the *pAbAi* plasmid so that yeast cells transformed with pAbAi-bait have the capacity to synthesize uracil. The transformation was plated on synthetic dropout media deficient for uracil (SD-ura) to select for the transformed *pAbAi-98X4* yeast line. Colony transformation was verified using the PCR Insert Check Kit (Clontech, Inc., 2009) and correct integration into the yeast genome was confirmed.

The *p53AbAi* plasmid was linearized with BstB1 and transformed into Y1H Gold. This plasmid provides a positive control “bait” sequence for interaction with the p53 plasmid as well as compatible ends in the Ura-3 gene for homologous recombination with the Y1H Gold genome so that the bait-carrying DNA enables the control line to synthesize uracil. The Y1HGold yeast was transformed with *p53AbAi* to produce a positive control line, Matchmaker *p53AbAi*, and was plated on SD-Ura to select for yeast colonies carrying the positive control “bait”.

Using *Drosophila melanogaster* 8-12 hour mRNA (provided by B. Jones) as the template molecules, cDNAs with SMART end sequences were produced by PCR following the protocol of Clontech, Inc. Briefly, for synthesis of cDNA from the mRNA, *Moloney* Murine Leukemia Virus reverse transcriptase (MMLVRT) and a system with a switching mechanism at the 5' end of RNA transcripts (SMART technology) was used (Clontech, Inc., 2009). In a reaction with CDSIII/6 random primers, the mRNA library, MMLV reverse transcriptase, and 10mM *deoxynucleotide* triphosphate (dNTP) mix, single strand cDNAs were generated with a CCC sequence added by the transcriptase to the 3' end of each transcript. The reaction was treated with *RNaseH* to remove the mRNA. The single stranded cDNAs with uniform end sequences were then amplified by PCR using Advantage 2 PCR Kit (Clontech, Inc., 2009) and the double stranded cDNA products of the reaction were purified by ChromaSpin-400 column (Clontech, Inc., 2009). The ends of the double stranded cDNA fragments were then consistent and homologous to the cloning sites in the pGADT7-Recombinase vector. When the library of double-stranded cDNAs were co-transformed into the competent cells of the yeast line pAbAi-98X4 along with the pGADT7-Recombinase vector, the cDNA library fragments were homologously recombined into the linearized vector, resulting in a library of pGADT7 expression vectors.

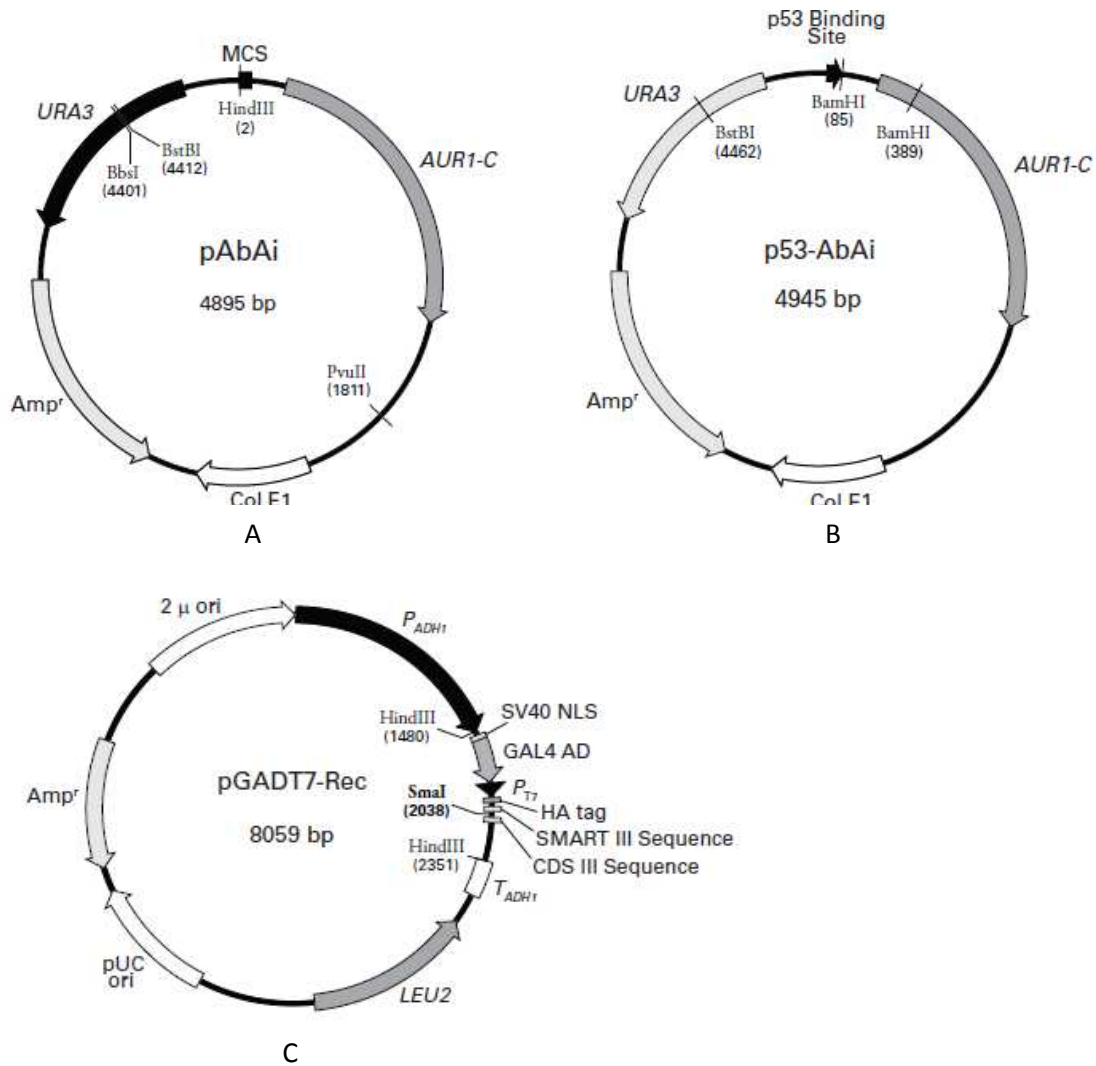


Figure 2. Yeast screen plasmids. Plasmid vectors for insertion of bait fragment into yeast genome(A); P53AbAi for positive control (B); and PGADT7-Recombinase for construction of *Drosophila* cDNA plasmid library (C). Clontech, Inc. 2009

The cDNA for the *Drosophila* gene *gcm* was excised from 8ug *Pgcm1* plasmid DNA (Jones et al., 1995) by restriction with XbaI, treatment with Klenow to blunt the overhanging ends, PCI extraction, and a second restriction with ClaI. The insert was harvested by gel extraction. Five ug of p414GPD DNA was restricted with XhoI and treated with the Klenow fragment to blunt the overhanging ends. PCI extraction was followed by a second restriction with ClaI. The linearized plasmid was dephosphorylated with CIP and PCI extracted. The fragment

containing *gcm* was directionally cloned into yeast expression vector p414GPD (Figure 3). After the ligation reaction the plasmid DNA was transformed into *E. coli* and plated on luria-bertani media with ampicillin (LB amp). Insertion and proper orientation of the gene was confirmed by restriction analysis and by sequencing (Macrogen USA). The p414GPD-*gcm* expression vector was then transformed into the Y1HMatchmaker Gold yeast line (Y1H Gold) carrying the 98X4 “bait” construct in its genome. Y1H Gold is auxotrophic for tryptophan. The p414GPD-*gcm* plasmid confers the ability to synthesize tryptophan, so that the yeast line was plated on synthetic dropout media deficient for uracil and tryptophan to maintain selective pressure on both the plasmid and the bait insert in the genomic DNA. Standard molecular biology methods were used for all procedures (Maniatis et al. 1992).

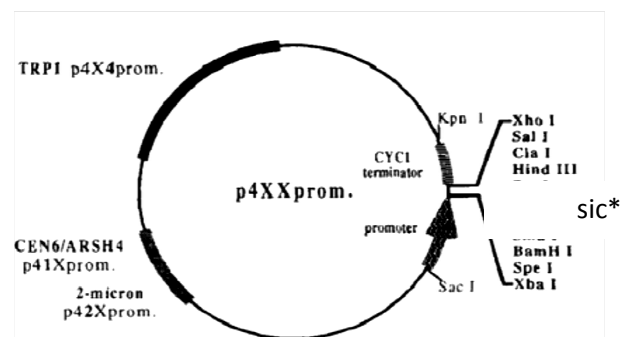


Figure 3. Yeast expression vector p414GPD (Mumberg, D. et al. 1995. *Gene* 156, 119-122)

\*It was determined by sequencing that the restriction sites PstI, EcoRI and EcoRV are in reverse order to the published image.

## 2.2 Yeast Screen

*Saccharomyces cerevisiae* Y1H Gold was grown on media made from yeast extract, peptone, dextrose, and agar and supplemented with 15 ml of a 0.2% adenine hemisulfate solution per liter of medium. For the yeast screens chemically defined synthetic dropout (SD) media were made by addition of specific amino acids to a nitrogen base without amino acids. 250ng/ml

Aureobasidin A was added to the media for the screens after a titration procedure to establish the minimum concentration necessary for suppression of growth of the yeast containing the “bait”. The appropriate amino acids were added for selection of the particular yeast line. 10X dropout supplements were made for SD-Uracil, SD-leucine, and SD-tryptophan as shown in Table 1.

<u>Nutrient</u>	<u>10X Concentration</u>
L-Adenine hemisulfate	200 mg/L
L-Arginine HCl	200 mg/L
L-Histidine HCl monohydrate	200 mg/L
L-isoleucine	300 mg/L
L-Leucine	1000 mg/L
L-Lysine HCl	300 mg/L
L-Methionine	200 mg/L
L-Phenylalanine	500 mg/L
L-Threonine	2000 mg/L
L-Tryptophan	200 mg/L
L-Tyrosine	300 mg/L
L-Uracil	200 mg/L
L-Valine	1500 mg/L

Table I. 10X dropout media. For selective media, uracil was omitted for culture of pAbAi-98X4 yeast (“bait”) and positive control line p53AbAi. Leucine was omitted for SD-leucine for the yeast screen. Both leucine and tryptophan were omitted to make SD-Leu-Trp for the Double Interaction Yeast Screen.

All Synthetic Dropout media were adjusted to a pH of 5.8 and supplemented with sterile glucose to 2%. YPD agar and Synthetic Dropout agar plates were made by the addition of 20g agar/L of media.

The gene for *Aureobasidin A resistance (AURI-C)* is the reporter on the bait vector, pAbAi. The minimal inhibitory concentration of Aureobasidin A was determined for the bait strain by selecting healthy colonies from freshly grown plates of the pBait-AbAi yeast line and re-suspending them in sterile 0.9% saline. The concentration of cells was adjusted to an optical density of 0.002 at 600nm (OD<sub>600</sub>), yielding a cell density of approximately 2000 cells/ul. One hundred microliters of this suspension was then plated on Synthetic Dropout media deficient for Uracil (SD-Ura) with varying concentrations of the antibiotic. The series of SD-Ura media included SD-Ura; SD-Ura + 100 ng/ml Aureobasidin A; SD-Ura + 150ng/ml Aureobasidin A; SD-Ura + 200 ng/ml Aureobasidin A; and SD-Ura + 250ng/ml Aureobasidin A. 150ng/ml Aureobasidin A was adequate to suppress growth of the pBait-AbAi strain when the cells were diluted to 2000/ul. However, for screening the library of cDNAs the Aureobasidin A concentration was increased to 250ng/ml so that weak positives and background interactions from the presence of the plasmid expressing *gcm* in the Double Interaction portion of the screen were adequately suppressed.

YPD liquid media was used for growing fresh Y1H gold colonies to log-phase (OD<sub>600</sub> between 0.4 and 0.6) to make competent cells for small scale transformation reactions of the pAbAi bait plasmid and the p53AbAi positive control plasmid into the yeast and integration of those constructs into the yeast genome. One ml of YPD was inoculated with a large colony of Y1Hgold from fresh plates of growth. The cells were dispersed by vortexing and the inoculum was transferred into 50 ml of YPD for incubation at 30°C for 16-18 hours with shaking at 250



rpm. 30 ml of the stationary phase culture was transferred to 300 ml of YPD and incubated at 30°C for 3 hours with shaking or until the OD<sub>600</sub> reached 0.4-0.6. Cells were centrifuged at 1000 x g for 5 minutes, washed in sterile distilled water and re-suspended in 1.5 ml of sterile 1X lithium acetate (LiAc). The competent cells were held on ice.

For the transformation reactions, 0.1 ug of plasmid DNA and 0.1 mg of carrier DNA were combined in a 1.5 ml tube. 0.1 ml of yeast competent cells was added to each tube and mixed by vortexing. 0.6ml of sterile polyethylene glycol (PEG)/lithium acetate (LiAc) solution was added and the mixture was vortexed. The transformation mixture was incubated for 30 minutes at 30°C with shaking. 70 ul of dimethyl sulfoxide (DMSO) was added and the reaction was mixed by inversion. The transformation mixture was then heat shocked for 15 minutes at 42°C after which the cells were placed on ice for 2 minutes. The treated cells were centrifuged at 14,000 rpm for 5 seconds, the supernatant removed and the cells re-suspended in sterile 1X TE. 100 ul of the transformed were plated on SD-ura agar plates. Dilutions of 1:10, 1:100, and 1:1000 of the transformation were made and plated on selective media for evaluation of transformation efficiency.

For transformation of the cDNA library plasmids, competent yeast cells were prepared of both the pAbAi-98X4 yeast line and the pAbAi-98X4/GPD-*gcm* yeast line. For each, fresh colonies were vortexed into 5ml of sterile SD liquid media and then used to inoculate 300 ml of the appropriate SD dropout media. For the pAbAi98X4 line SD-ura liquid media was used to grow the culture. For the Double Interaction Screen, SD-ura-trp liquid media was used for incubation of the pAbAi-98X4/GPD-*gcm* yeast line. Competent cells were harvested when the cultures reached OD<sub>600</sub> of 0.4-0.6. Growth rate of the pAbAi-98X4/GPD-*gcm* yeast was up to three hours slower than the yeast growing in SD-ura only. However, co-transformation of the

p414 GPD-*gcm* plasmid along with the library of plasmids reduced transformation efficiency by an order of magnitude, so the Y1Hgold yeast line harboring both the 98X4 bait and the GPD-*gcm* expression vector was maintained and transformed separately with the cDNA library plasmids. Competent cells of both yeast lines were washed and re-suspended in lithium acetate (1X LiAc).

For library scale transformations of the yeast competent cells, 20 ul of SMART-amplified cDNA, 6 ul of SmaI linearized pGADT7-Recombinase plasmid DNA, 20 ul denatured Carrier DNA were combined in a pre-chilled 15 ml. tube. 600 ul of competent cells and 2.5 ul of PEG/LiAc were added. The mixture was incubated for 45 minutes at 30 degrees C. 160 ul DMSO was added and the mixture was incubated at 42 degrees C for 20 minutes with mixing every 10 minutes. The simultaneous co-transformation reactions were centrifuged at 700 g for five minutes and the supernatant removed. Cells were re-suspended in 3 ml of YPD Plus and incubated 90 minutes. The cells were centrifuged, the supernatant discarded and a 0.9% NaCl suspension was made for plating of the cells.

The transformation reactions were prepared in duplicate. Competent cells of the pAbAi-98X4 yeast line and the pAbAi 98X4/GPD-*gcm* line were added to separate large scale transformation mixtures. For the positive control, a small scale transformation mixture containing the p53 plasmid DNA and denatured carrier DNA was prepared, and the p53AbAi competent cells were added to it. This transformation mixture was plated on SD-leu+100 ng/ml Aureobasidin A because the control is only capable of growth up to 100 ng of Aureobasidin A. The pAbAi98X4 yeast library transformation was plated on 100 mm plates of SD-leucine agar. The pAbAi98X4/GPD-*gcm* yeast transformation was plated on 100 mm plates of SD-leucine-tryptophan agar. The entire transformation reaction was plated for both screens. All plates were

incubated at 30<sup>0</sup>C. Fifty of the largest colonies from each screen were chosen for re-streaking after 5 days of incubation. The colonies from the pAbAi98X4/GPD-*gcm* Double Interaction transformation were labeled A1-A50 and the colonies from the pAbAi98X4 transformation were labeled B1 through B50.

Colonies 1-50 from the yeast screen and the double interaction screen were re-streaked onto fresh SD agar two times or more in an effort to segregate the plasmid producing the interaction with the bait sequence, since yeast are capable of harboring more than one plasmid. The extra generations of growth were used to promote loss of any plasmids not necessary for the Aureobasidin A resistance. Colony PCR of the inserts in the colonies that survived repeated transfers onto selective media made it possible to identify colonies that contained more than one plasmid and to select representative plasmids for further analysis. The plasmid inserts were analyzed by restriction with HaeIII and/or HindIII and compared on electrophoresis gels. By sequencing of the selected inserts (Advantage PCR Insert Kit, Clontech, Inc.; Macrogen USA), colonies were chosen for rescue of the pGADT7 library plasmids.

For plasmid rescue, yeast cells from a 10mm square of fresh growth were suspended in a solution with lyticase (5 units/ul) and incubated with glass beads at 37 degrees C. 10ul of 20% SDS was added to the yeast mixture and it was vortexed for one minute to disrupt the cell walls. The supernatant was extracted with PCI and the DNA was precipitated with 10 M ammonium acetate and 100% ethanol. The pellet was resuspended in 20ul of distilled deionized H<sub>2</sub>O. The DNA was transformed into *E. coli* HB101 competent cells and plated on M9 media augmented with proline, leucine and thiamine. The plasmid DNA was isolated from the *E. coli* HB101 cells and re-transformed into competent yeast pAbAi98X4 and/or pAbAi98X4/GPD-*gcm* with plating

on the appropriate synthetic dropout media to confirm the positive interaction of the fusion protein produced by each rescued, purified plasmid with the 98 base pair bait fragment.

## 2.3 Bioinformatics

Databases at the National Center for Biotechnology Information (N.C.B.I) National Institute of Health were accessed for analysis of the sequenced pGADT7 plasmid inserts. All sequencing was by Macrogen USA. The text files of sequenced inserts were submitted for BLAST searches with the *Drosophila* genome and identified by those alignments. The Ref Seq program at NCBI and the Interpro database were accessed for information concerning the protein domains of the genes recovered.

## 2.4. *Drosophila melanogaster* stocks

*Drosophila* line  $y^1 w^{67c23}; P\{EPgy2\}CG6770^{EY00294} (CG6770^{EY00294})$  from Bloomington *Drosophila* Stock Center (stock number 14827) was used to study the CG6770 yeast screen isolate. This line contains a P element insertion within the reading frame of CG6770 and is either a null or a hypomorph for the encoded protein. The line was crossed with  $w; gcm^{\Delta P1}/CyO;$  ( $gcm^{\Delta P1}$ ), a *gcm* null ;  $ry^{506} P\{PZ\}repo^{03702}/TM3; ry^{RK} Sb^1 Ser^1 (repo^{3702})$ , a *repo* null line;  $P\{XP\}CG6770^{d07430} (CG6770^{007430})$ , a second line with a P element insertion in CG6770;  $w; Ki^{P\Delta 2-3}$ , a line with transposase for excising the P element in CG6770;  $gro^{C105}/TM3, Sb^1$ , a *groucho* line balanced by TM3; and with *yw*, a wild type. *Drosophila* wild type lines Canton S and *yw* were used to image the wild type eye and thorax.

The *groucho* deletion line *gro*<sup>C105</sup>/*TM3 Sb*<sup>1</sup> from Bloomington Stock Center (stock number 2124) was used to study the yeast screen pGADT7 isolate A7F which contains an insert of ~700bp of the *Drosophila groucho* gene. The line was crossed with *w; gcm*<sup>ΔP1</sup>/*CyO*; *ry*<sup>506</sup> (*gcm*<sup>ΔP1</sup>), a *gcm* null; *Df(2L)200;CyO*, which is null for both *gcm* and *gcm2*; *ry*<sup>506</sup> *P{PZ}repo*<sup>03702</sup>/*TM3, ry*<sup>RK</sup> *Sb*<sup>1</sup> *Ser*<sup>1</sup> (*repo*<sup>3702</sup>), a *repo* null line; *y*<sup>1</sup> *w*<sup>67c23</sup>; *P{EPgy2}CG6770*<sup>EY00294</sup> the *CG6770* null line; and with *yw*, a wild type line of *Drosophila melanogaster*.

The *glass* Multimer Reporter targets expression of the gene of interest in the *Drosophila* compound eye in all cells in and posterior to the morphogenic furrow (Mishra-Gorur, K. et al., 2002). *GMR-gcm* flies were generated by cloning the *gcm* cDNA into the *glass* Multimer Reporter (*GMR*) P element vector and introducing the construct into the *Drosophila* germline using standard techniques (Jones, B. W., unpublished data). The line, *ftz*<sup>rf10</sup> *ry e/TM3,Sb P[w<sup>+</sup>GMR-gcm]*, was crossed with *CG6770*<sup>EY00294</sup> (*y*<sup>1</sup> *w*<sup>67c23</sup>; *P{EPgy2}CG6770*<sup>EY00294</sup>) to achieve misexpression of the *gcm* gene localized to the eye in the *CG6770* null background. The *Drosophila* line carrying the same construct (*ftz*<sup>rf10</sup> *ry e/TM3,Sb P[w<sup>+</sup>GMR-gcm]*) was also crossed with the *groucho* deletion line *gro*<sup>C105</sup>/*TM3, Sb*<sup>1</sup> to evaluate *gcm* misexpression in the eye in a background deficient in *groucho*. The *Drosophila* eye is made up of ommatidia, each of which has eight receptor cells in addition to rods and cones in a highly ordered arrangement. Expression of gene products that produce cell death or interfere with developmental pathways by their interactions create disorder among cells of the ommatidia and result in an easily recognizable rough eye phenotype. The Canton S wild type *Drosophila* line was crossed with *ftz*<sup>rf10</sup> *ry e/TM3, SbP[w<sup>+</sup>GMR-gcm]* flies as a negative control for the system.

## 2.5 Immunohistochemistry

With the exception progeny of the genetic cross of *CG6770<sup>EY00294</sup>* with *w;Ki P<sup>PA2-3</sup>*, embryos were collected from each of the genetic crosses described above, and horseradish peroxidase (HRP) immunohistochemistry protocols were carried out as described in Patel, 1994, for detection of proteins in the embryos. Anti-Repo and BP102 primary antibodies were prepared at a 1:300 dilution. Rabbit anti- $\beta$ -galactosidase (anti  $\beta$ Gal) antibodies were prepared at a 1:10,000 dilution. HRP-conjugated secondary antibodies HRP anti- mouse and HRP anti-rabbit (Jackson Immunoresearch) were prepared at a 1:300 dilution.

### 3. RESULTS

#### 3.1. Double Interaction Yeast Screen

A modification of yeast one and two hybrid screens, a Yeast Double Interaction Screen utilized as “bait” four copies of the previously characterized 98 base pair cis-regulatory element of the gene *repo* called the “Epi Repressor” (Figure 1) (Johnson et al., 2012). This 98 base pair element was chosen because it exhibits two properties. The Epi Repressor has been shown to be sufficient for inhibiting epidermal expression of *repo* in a *lacZ* reporter. The fragment also is sufficient to drive reporter expression in the lateral glia via a single Gcm binding site (Johnson et al, 2012). The design of the yeast screen makes possible identification of DNA binding *trans*-regulators of *repo* gene expression as well as cofactors that interact with the Gcm protein and influence gene expression either by that interaction alone or by cooperative DNA binding (Figure 4). Four tandem copies of the 98 base pair fragment were transformed into the Matchmaker Gold yeast line in a plasmid (pBait-AbAi) carrying the wild type gene *Ura 3*. Homologous recombination of the plasmid with the bait and *Ura 3* gene into the yeast chromosome made the yeast capable of growth on SD uracil deficient media.

To express *Drosophila* proteins in yeast, a cDNA library was made using 12-18 hour *Drosophila* mRNA. The cDNAs were amplified by PCR with ends compatible for cloning into the pGAD T7-recombinase yeast expression vector. This vector also conferred the ability to grow on leucine deficient media so that synthetic dropout media deficient for leucine (SD-leu)

selected for growth of yeast cells carrying the prey plasmid. When expressed in yeast the plasmids produce *Drosophila* proteins fused to a Gal4 Activation domain (GAD). Any fusion protein produced from the plasmids and binding to the “bait” fragment activated the AbAi reporter, whether or not the protein is naturally an activator *in vivo* (Figure 4).

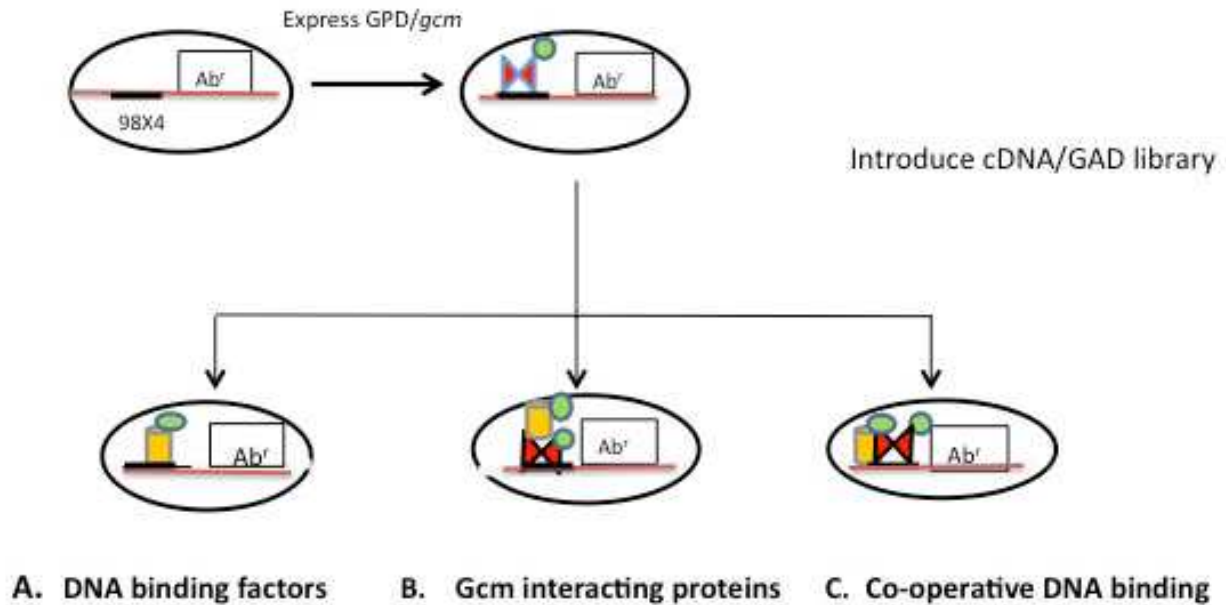


Figure 4. Double interaction yeast screen. The 98 bp epidermal repressor fragment (black line) was cloned into the Y1H Gold genomic DNA (red line). The expression vector p414GPD*gcm* was transformed into the yeast line. The plasmid PGADT7 recombinase was transformed into the yeast with a 12 hour cDNA library of the *Drosophila melanogaster* genome. The activation domain of fusion proteins generated by the PGADT7 library plasmids activated the Aureobasidin A resistance reporter (rectangle Abr) when bound to the 98bp “bait” fragment. Yeast genomic DNA is represented by a red bar; Abr by white rectangle; Gcm protein by red hourglass; activation domain by blue circle; cofactor and Gal4AD by yellow cylinder and blue circle.

In order to express Gcm protein in yeast strains, the cDNA of *gcm* was cloned into the yeast expression vector p414GPD, and this construct was transformed into the yeast strain carrying four copies of the 98 bp Epi Repressor “bait” fragment. The p414 plasmid confers the



ability to grow on tryptophan deficient media. Because the Epi Repressor contains a Gcm binding site, the 98x4 “bait” has four Gcm binding sites. To insure complete background suppression of the interaction of Gcm with the “bait” Aureobasidin A was added to the dropout media in a concentration of 250ng/ml; high Aureobasidin A concentration was needed to suppress activation by Gcm.

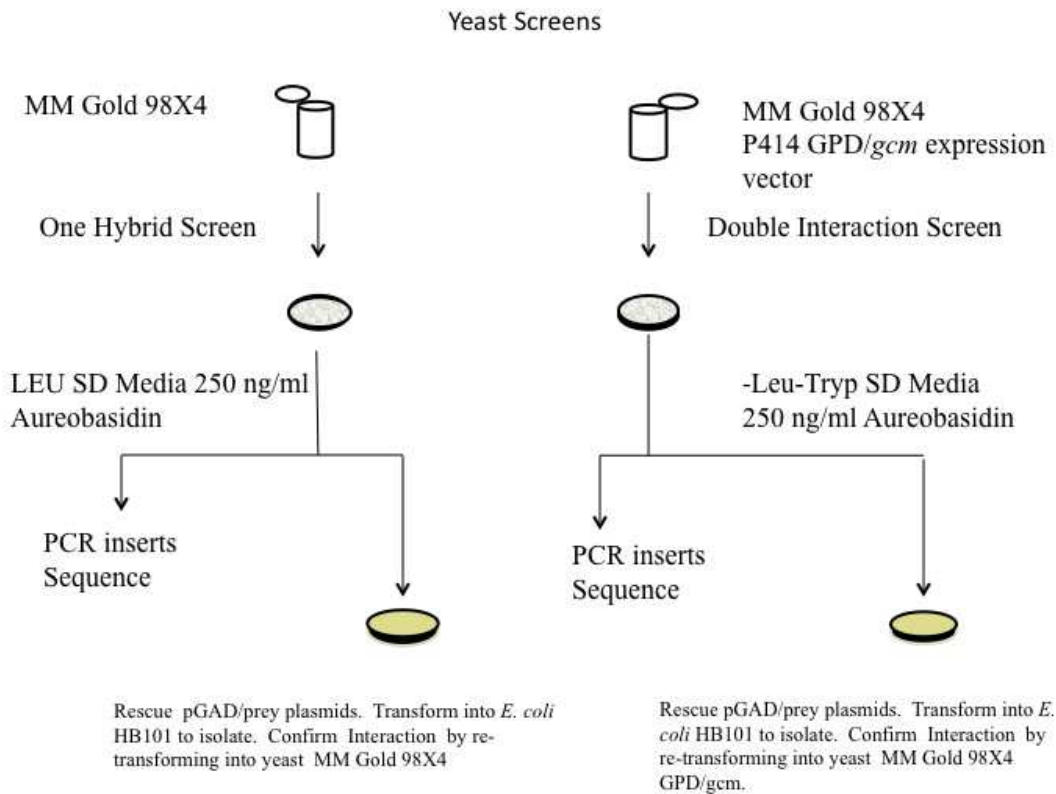


Figure 5. Concomitant yeast screens. Yeast screen using Y1H Gold (Clontech, 2009) for a Double Interaction Screen was carried out simultaneously with a standard one hybrid screen.

The plasmid library was transformed into competent yeast cells of both the yeast line carrying only the 98X4 “bait” sequence and the yeast carrying the “bait” plus the *GPD/gcm* plasmid. The yeast line carrying p414GPD/*gcm* was plated on SD-leucine-tryptophan media with 250ng Aureobasidin A/ml (Figure 5).

Fifty colonies were selected for vigorous growth in the absence of leucine and presence of 250 ng/ml Aureobasidin A for the one-hybrid screen conducted without the p414GPD/*gcm* plasmid and designated “B” along with a colony/strain number. Fifty colonies were also selected for their growth in the absence of both leucine and tryptophan and in the presence of 250ng/ml Aureobasidin A for the Double Interaction part of the yeast screen and designated “A” with a colony number. All colonies grew within 3-5 days at 30 degrees C. Single colonies were re-streaked to new SD/-leu (or SD/-leu-try) multiple times for confirmation of the positive phenotypes from the screen. Colony PCR and sequencing were then used for identification of the fusion protein producing the Aureobasidin A resistance. To recover the inserts for each positive, total DNA was recovered from the yeast and transformed into *E. coli* HB101 cells that were plated onto M9 minimal media containing ampicillin. HB101 isolates from the M9 media transform with only one plasmid so that isolation and identification of each of the pGADT7 inserts was possible. Re-transformation of the recovered, purified plasmids into Y1HGold/ 98X4 yeast confirmed positive interactions.

Of the plasmids rescued, there were five containing cDNA inserts identified by sequencing that encoded non-nuclear proteins which include the proteasome beta subunit and ribosomal proteins. These are likely to be false positives. Also, histone 4R was recovered twice. Six plasmids recovered and confirmed to produce positive interactions with the Aureobasidin A resistance gene supported growth in 250 ng Aureobasidin A/ml of SD-leu or SD-leu-try. None of the six were dependent upon co-expression of Gcm protein. However it appeared that *groucho* interacted synergistically with *gcm*. Isolate A7 encodes a 1.2 kb cDNA of the protein Groucho, a co-repressor previously described as a factor in many developmental pathways, including Notch, Ras, Decapentaplegic, and Epidermal Growth Factor Receptor (Cinnamon et

al., 2008; Hasson and Paroush, 2006). The remaining cDNAs recovered include an unknown zinc finger with a nucleic acid binding domain, three unknown nuclear genes and a nuclear phosphoprotein of unknown function (Table 2).

Interestingly, a pGADT7 plasmid carrying *gcm* cDNA was discovered to have been harbored within the same yeast line with a pGADT7 plasmid carrying *groucho* cDNA. The preliminary colony PCR had identified the *groucho* cDNA, but efforts to recover the plasmid DNA from HB101 cells and confirm it by sequencing revealed the second plasmid in colonies from the positive yeast line. Although yeast can tolerate the presence of more than one plasmid, the sub-culturing of this line had occurred for more than 12 months without loss of either plasmid. The two purified plasmids were confirmed by electrophoresis and sequencing, then each was transformed (separately) back into the yeast line carrying only the 98X4 bait, with the plasmid carrying *groucho* producing growth on SD-leu+250ng AbA/ml. The pGADT7 library plasmid carrying *gcm* produced small colonies on SD-leu+250ng AbA/ml, due possibly to a stronger interaction of the GAD activation domain than that which occurred with the p414GPD/*gcm* plasmid product. However, when both the *gcm* and *groucho* pGADT7 plasmids were co-transformed, growth was more robust than with *groucho* alone.

Since *groucho* is not known to bind DNA, the affinity of the fusion protein for the 98 bp epidermal repressor fragment was questioned. The pGADT7-R plasmid carrying *groucho* DNA was transformed into a yeast line carrying no DNA bait sequence, in an attempt to demonstrate a false positive due to yeast proteins. This yielded no growth. The plasmid carrying the *groucho* fragment was then transformed into the MM Gold positive control line which carries a DNA bait fragment provided for giving a positive control reaction with the p53 control plasmid. Results were negative (no growth). The *groucho* plasmid was transformed into a yeast line carrying two

copies of the 98 bp repressor fragment (pbj173), producing strong growth. An effort was made to quantitate the positive interaction of the Groucho and Gcm fusion proteins by making media containing varying concentrations of Aureobasidin A.

The pGADT7*gcm* plasmid transformed into yeast containing 98X4 as bait produced growth on media containing 150ng of Aureobasidin A/ml, and small colonies on media containing 200ng Aureobasidin A/ml. The pGADT7 *groucho* isolate produced growth on SD-leu media up to 300 ng Aureobasidin A/ml. pGADT7 *gcm* plus pGADT7 *groucho* co-transformed into yeast with 98X4 as bait produced growth on SD-leu+350ng Aureobasidin A/ml. No other yeast colonies were capable of growth on media containing such levels of the antibiotic. This may be evidence of a synergistic interaction between *gcm* and *groucho*.

<u>Plasmid</u>	<u>Gene</u>	<u>Description*</u>
A7E	CG12245	<i>gcm</i> ; binary switch in glial development
A7F	CG8384	<i>groucho</i> ; repressor essential for neurogenesis bristle phenotype, postalar macrochaetae
A13	CG10681	expressed in CNS; KXDL domain
A22	CG12268	unknown
A24	CG12730	Marvel domain; nuclear gene
A36	CG6769	Zn ion binding; nucleic acid binding
A45	CG10279	DEAD box; RNA helicase Rm62; Trichogen cell phenotype at dorsocentral and scutellar macrochaetae
B12	CG6770	nuclear phosphoprotein P8; phenotype annotated to Trichogen cell
B14	CG12730	Marvel domain; nuclear gene
B21	CG6583	nuclear; phenotype annotated to Trichogen cell
B41	CG3379	histone 4R
B44	CG6770	nuclear phosphoproteinP8; phenotype annotated to Trichogen cell

\*References: Jones et al., 1995; Fisher and Caudy, 1995; Mummery et al., 2009; Gonzales et al., 2005; Bourbon et al., 2002; Marinho et al., 2013; Akhmanova et al., 1996; Stofanko et al., 2008.

Table 2. PGADT7R plasmids recovered, sequenced, and confirmed as positives in second transformation into Y1HGold/98X4 yeast.

### 3.2. *Drosophila melanogaster* In Vivo Interactions

Two of the genes recovered in the screen, *groucho* and *CG6770*, were selected for further study in an effort to demonstrate an interaction *in vivo* with either *gcm* or *repo*. Using the *Drosophila* line *groucho*<sup>C105</sup> and *CG6770*<sup>EY00294</sup> genetic crosses were made to alter the copy number, or dosage, of the genes for the transcription factor *gcm* and each of the genes of interest. “Transcription factors that must form complexes or that compete for DNA sites with other such factors may be sensitive to dosage. If dosage alteration causes no overt phenotypic change it can often sensitize the fly to dosage changes at other loci,” (Greenspan, 1997).

The cross was set up between *groucho* null females (*gro*<sup>C105</sup>/*TM3, Sb*<sup>1</sup>) and males from *gcm* null *gcm*<sup>AP1</sup> as well as between the *gcm*<sup>AP1</sup> virgins and *groucho* null males. Embryos collected and stained with anti-Repo were observed for phenotypic changes. The progeny of the cross of the *groucho* null female exhibited severe abnormalities since there is a significant maternal contribution of *groucho* protein. Because this protein serves as a co-repressor in a variety of cell fate choices and interacts in neurogenesis and fundamental axis patterning pathways, the reduction of maternal *groucho* alone causes profound developmental disturbances in the CNS and lethality (Turki-Judeh and Courey, 2012). Reduction in the dosage of both Gcm and maternal Groucho resulted in very significant early lethality and many of those embryos surviving to Stage 12 had such severe abnormalities that the Repo positive cells were scattered, disorganized, and unidentifiable as to position in the CNS. The genetic cross of Gcm deficient females and Groucho deficient males produced more viable embryos but no clearly identifiable phenotype was observed to be unique to the simultaneous reduction in dosage of Gcm and Groucho proteins. In the Y1H Gold yeast line, the plasmid harboring the cDNA of *gcm* and the plasmid carrying the cDNA from *groucho* produced yeast growth at a higher level of the

inhibitor Aureobasidin A when co-transformed than either plasmid was capable of producing individually. Also, the yeast line into which the p414GPD/*gcm* plasmid had been transformed enabled their growth at slightly greater concentrations of Aureobasidin A. These results suggested the possibility of a synergistic interaction between the proteins which may not have been made obvious by reduction of the dosage of both proteins. A second *in vivo* study was undertaken to pursue alteration of the copy number (dosage) of the *gcm* gene and the *groucho* gene in which *gcm* expression levels were increased in the eye as mediated by the *glass* Multimer Reporter .

The *glass* Multimer Reporter (*GMR*), an artificial construct for expressing a gene of choice in the eye of *Drosophila*, was used to misexpress *gcm*. A cross between the Groucho deficiency line and the *GMR-gcm* misexpression line (*gro*<sup>C105</sup>/*TM3*, *Sb*<sup>1</sup> x *ftz*<sup>rf10</sup> *ry e*/*TM3Sb P[w+ GMR-gcm]*) produced offspring with rough eyes (Figure 6) . Twenty-five percent (25%) of the progeny exhibited an extreme rough-eye phenotype, indicating an interaction *in vivo* in flies with a deficiency of *groucho* and increased expression of *gcm*. Overexpression of *gcm* alone in the *ftz*<sup>rf10</sup> *ry e*/*TM3*, *Sb P[w+ GMR-gcm]* line exhibits a mildly rough eye. Canton S flies were crossed with the flies of genotype *ftz*<sup>rf10</sup> *ry e*/*TM3*, *Sb P[w+ GMR-gcm]* as a control and the mild disorganization of their eyes was comparable to *ftz*<sup>rf10</sup> *ry e*/*TM3*, *Sb P[w+ GMR-gcm]* .

The same line of flies carrying the *glass* Multimer Reporter construct for expressing *gcm* in the eye was used to investigate the interaction of the protein product of CG6770 and *gcm* *in vivo*. Flies of the *ftz*<sup>rf10</sup> *ry e*/*TM3*, *Sb P[w+ GMR-gcm]* line were crossed with *P{EPgy2}CG6770*<sup>EY00294</sup> (the CG6770 null line). The genetic crosses were set up both

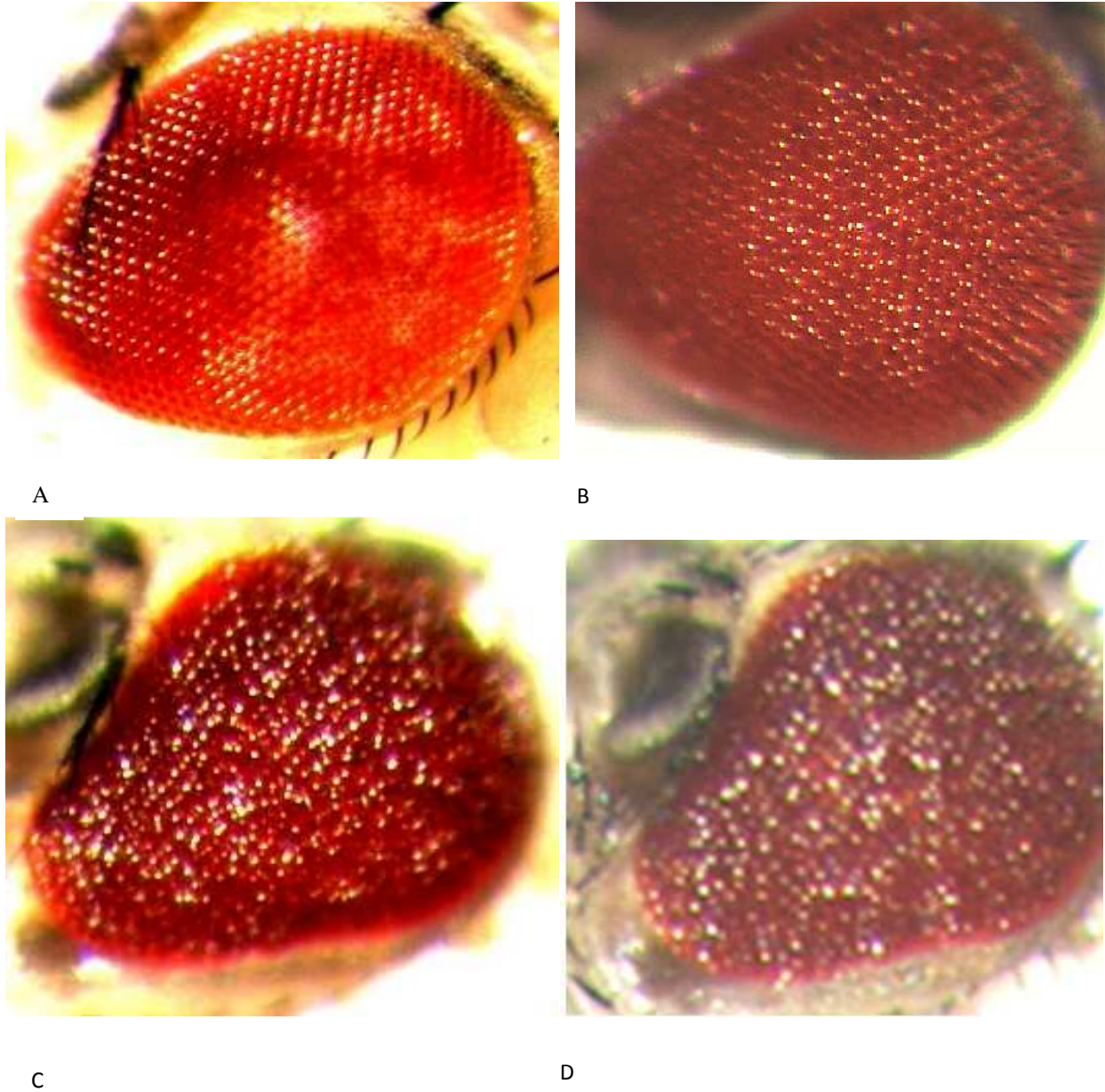


Figure 6. Comparison of *GMR-gcm* eye phenotypes.

(A) Canton S wild type;

(B)  $ftz^{rf10} ry e/TM3, SbP[w^+GMR-gcm]$  ;

(C)  $CG6770^{EY00294}/TM3, SbP[w^+GMR-gcm]$  ( progeny of genetic cross  $CG6770^{EY00294} x ftz^{rf10} ry e/TM3, SbP[w^+GMR-gcm]$  ) ;

(D)  $gro^{C105}/TM3, SbP[w^+GMR-gcm]$  (progeny of genetic cross  $gro^{C105} x ftz^{rf10} ry e/TM3, SbP[w^+GMR-gcm]$  ).

using the virgins from CG 6770<sup>EY00294</sup> (*P{EPgy2}CG6770<sup>EY00294</sup>*) x males from *ftz<sup>f10</sup> ry e/ TM,SbP[w<sup>+</sup> GMR-gcm]* and a second genetic cross was set up using the females from the line *ftz<sup>f10</sup> ry e/ TM3,Sb P[w+GMR-gcm]* X males from *P{EPgy2}CG6770<sup>EY00294</sup>*. Both crosses produced slightly more than 45 % (45.4% and 46.1% respectively) rough-eyed offspring close to the expected 50%, confirming an interaction between Gcm and the CG6770 proteins.

Two *Drosophila melanogaster* lines carrying insertion elements in CG6770 were used to investigate possible interactions of the product of CG6770 with Gcm and Repo. Both lines produced individuals with extra macrochaetae on the notum and/or scutellum, but the phenotype was weaker in the CG6770<sup>d07430</sup> line with the p[XP] element insertion upstream of the open reading frame of the gene at 12,045,945 on *Drosophila* chromosome 2L. Bloomington line 14827 (*P{EPgy2}CG6770<sup>EY00294</sup>*) carries EYO294, and the site of insertion is at 12,045,925 on chromosome 2L. Thirty-one per cent (31%) of the *P{EPgy2}CG6770<sup>EY00294</sup>* individuals exhibited extra bristles, or macrochaetae, on either the notum or scutellum, and some had multiple extra bristles (N=500). There were also examples of individual flies with missing bristles at the same positions on the notum or scutellum in both CG6770 lines. Most frequently the supernumerary macrochaetae were located at either the anterior or posterior dorsocentral positions on the notum. However there were frequent extra bristles on the scutellum and occasional extra bristles at the anterior and posterior postalar positions of the notum. The presence of one or more supernumerary macrochaetae at any position on the notum or scutellum was considered evidence of the aberrant bristle number phenotype for purposes of enumeration of individuals affected. The number of flies exhibiting missing bristles was not considered as a part of the computations for phenotypic penetrance in part due to the difficulty of visualizing



“empty” sockets and the likelihood of inaccuracy in such a count. In one vial of 60 flies such a count was undertaken and seventeen individuals exhibited extra macrochaetae (two flies of which were also missing one or more macrochaetae) while 12 were missing one or more of the macrochaetae from either the notum or scutellum. All enumeration was performed using flies from colonies 7-10 days old grown at 18-20 degrees C.

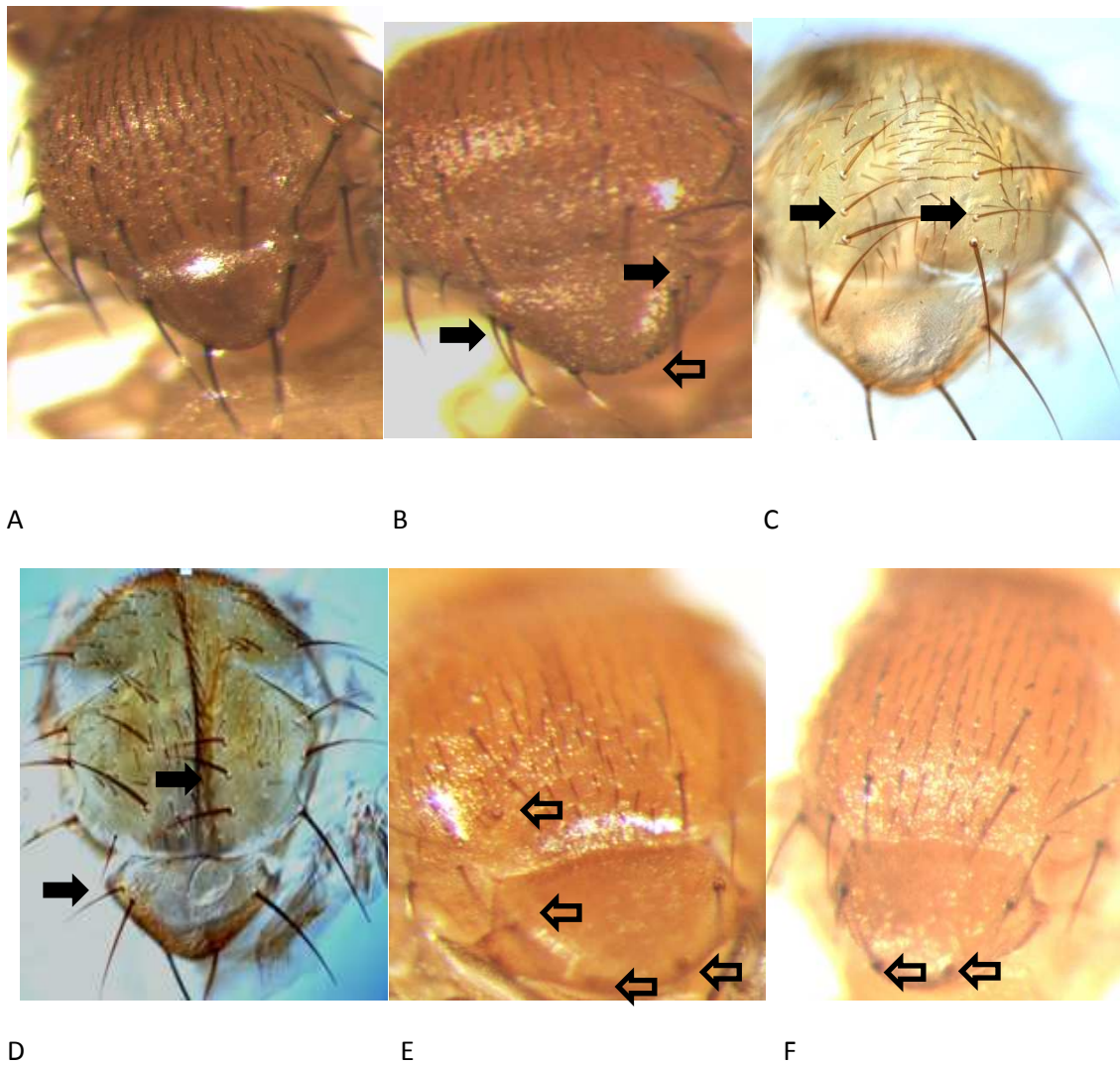
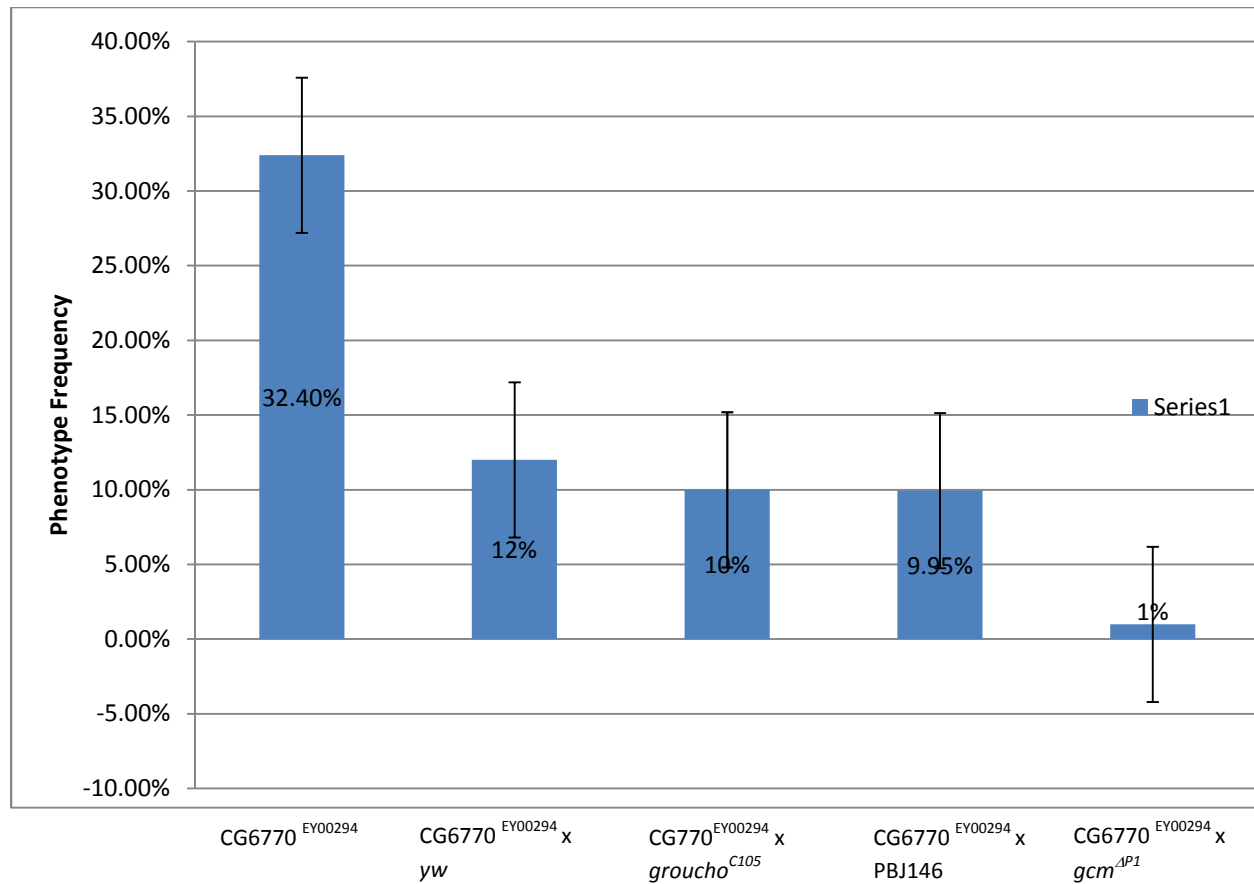


Figure7. Supernumerary and missing macrochaetae phenotype. Phenotype of P{EPGY2} CG6770<sup>EY00294</sup> showing comparison to bristle pattern of wild type Canton S (A); supernumerary macrochaetae and missing macrochaetae on scutellum (B); supernumerary macrochaetae at posterior dorsocentral notum (C); supernumerary macrochaetae at anterior dorsocentral and scutellum (D); missing macrochaetae at posterior dorsocentral (E) and missing macrochaetae at anterior dorsocentral and posterior scutellum (F). Filled arrows indicate supernumerary bristles; un-filled arrows indicate missing bristles.

The supernumerary bristle phenotype was used to evaluate potential interaction of *groucho*, *gcm*, *repo*, and CG6770. Each of the lines were crossed with twenty (20) P{EPgy2}CG6770<sup>EY00294</sup> females and their progeny were evaluated for supernumerary bristles. *yw* wild type and PBJ146 flies were also crossed with the P{EPgy2}CG6770<sup>EY00294</sup> line to serve as controls. The offspring of each genetic cross were counted for abnormal bristle number and/or juxtaposition. The frequency of supernumerary macrochaetae in the F1 progeny of the P{EPgy2}CG6770<sup>EY00294</sup> x *yw* cross was 12%; frequency for P{EPgy2}CG6770<sup>EY00294</sup> x PBJ146 was 10%; the frequency of abnormal bristle numbers in P{EPgy2}CG6770<sup>EY00294</sup> x *gcm*<sup>ΔP1</sup> was 1% (Figure 4). The two-tailed Student's T Test was used to statistically evaluate the results. The difference of the calculated values exceeded the chosen threshold of 0.05, and the null hypothesis that there is no difference in frequency of the occurrence of the phenotype was rejected. There is a statistically significant difference between the percentage of progeny of the *gcm*<sup>ΔP1</sup> x CG6770<sup>EY00294</sup> cross exhibiting the supernumerary macrochaetae phenotype and the percentage of progeny of the *yw* x CG6770<sup>EY00294</sup> cross exhibiting the supernumerary macrochaetae phenotype. Imprecise excision of the transposon of P{EPgy2}CG6770<sup>EY00294</sup> resulted in establishing the CG6770<sup>EX1</sup> line, which had a phenotype of white eyes. The phenotype of white eyes is consistent with loss of the P element.



Frequency of Supernumerary Macrochaetae in CG6770<sup>EY00294</sup> Progeny

Figure 8. Frequency of phenotype in the progeny of CG6770<sup>EY00294</sup>. Comparison of the frequency of individual flies exhibiting supernumerary macrochaetae in CG6770<sup>EY00294</sup>; frequency of supernumerary macrochaetae phenotype in offspring of a genetic cross of CG6770<sup>EY00294</sup> x *yw*; frequency of supernumerary macrochaetae in offspring of a genetic cross of CG6770<sup>EY00294</sup> x *groucho*<sup>Δ105</sup>; frequency of supernumerary macrochaetae phenotype in offspring of a genetic cross of CG6770<sup>EY00294</sup> x PBJ146; and frequency of the supernumerary phenotype in the offspring of a genetic cross of CG6770<sup>EY00294</sup> x *gcm*<sup>ΔP1</sup>.

## 4. DISCUSSION

The regulatory DNA upstream of the *gcm* target *repo* provides an opportunity to search for co- factors interacting with Gcm to drive context dependent outcomes of expression. A modification of the yeast one and two hybrid screens was used to identify DNA- binding regulators of the *repo* epidermal repressor and cofactors that interact with the Gcm protein. A simple one-hybrid screen lacking the p414GPD/*gcm* plasmid was performed concomitantly for purposes of comparison. The 98 bp fragment is known to be necessary and sufficient for repression of *lacZ/ repo* reporter construct expression in the epidermis while simultaneously producing a wild type *gcm* pattern in the central nervous system using anti-*repo* and horseradish peroxidase staining techniques.

### 4.1. Yeast screen isolates predicted to have roles in repression and epidermal specification

*gcm* cDNA and that of *groucho*, a known co-repressor, were recovered from the same yeast colony capable of growth on leucine deficient media in the presence of Aureobasidin A, indicating that the cDNAs were carried by the plasmids of the *Drosophila* library.

Electrophoresis confirmed that the recovered plasmid carrying *gcm* was the 8 kb pGADT7 vector of the library and not the 5kb p414GPD vector. Growth of the Y1HGGold98X4 yeast co-transformed with both pGADT7 containing *gcm* and pGADT7 *groucho* was Aureobasidin A

resistant at concentrations up to 350ng/ml, which is greater than that with either single plasmid. Although the plasmid pGADT7R *groucho* fusion protein produced Aureobasidin A resistance without the presence of *gcm* suggesting that *groucho* may interact with endogenous factors, the resistance increased when both fusion proteins were simultaneously expressed, which strongly suggests a synergistic interaction between *groucho* and *gcm* in the yeast.

The 98 bp element referred to as the “epidermal repressor” is likely to have binding sites that function in assembly of regulatory complexes. It was observed that some of the largest colonies recovered in the yeast screen contained multiple plasmids, which is relatively unremarkable. However, attempts to eliminate one or more of the plasmids for recovery and sequencing resulted in complete loss of that particular yeast strain and consequently loss of the opportunity to identify the plasmid inserts. Yeast screen recovery line A1 is an example of a positive yeast line with an insert that exhibited multiple peaks when sequenced. The results were consistent with the presence of more than one plasmid insert. We hypothesize that two or more of the fusion proteins interacted to trigger the positive (Aureobasidin A resistance) and that loss of either resulted in immediate loss of viability of the strain. The implication is that cooperative binding of the fusion proteins is necessary, and such regulatory complexes have been fully characterized for *groucho* and *polycomb/trithorax*.

Three of the cDNA inserts recovered and confirmed to have produced positive interactions with the *Aureobasidin A resistance* reporter gene may function in transcriptional and post-transcriptional gene silencing. These include *groucho*, *Rm62*, and *histone 4R*, each of which has been characterized in *Drosophila* and may relate to regulation of gene expression and long term repression (Boeke et al, 2011; Courey and Jia, 2001; Lamiable et al., 2010; Patel et al, 2012). These proteins are described as promoting chromatin remodeling activity and are

known to act in multimeric complexes. Groucho repression is achieved by epigenetic mechanisms (Courey and Jia, 2001; Patel et al., 2012). The product of *grg4*, a *groucho* ortholog, is known to function in repression of *pax 2* by recruiting an arginine methyl transferase to DNA binding sites as part of a complex that displaces the binding adaptor protein PTIP, inhibiting *pax* transcription. Like Repo, Pax 2 is a homeodomain protein. The molecular mechanism by which Grg/Tle repression of *pax* genes occurs is via the symmetrical dimethylation of histone 4 arginine 3 (H4R3) (Patel et al., 2012). Evidence indicates that the symmetric dimethylation of H4R3 leads to the recruitment of Polycomb genes, which is described as “a critical step towards gene silencing” (Patel et al., 2012). Although this mechanism has not been described in *Drosophila*, the Polycomb and Trithorax proteins are important in regulation of homeobox gene expression in flies and also in maintenance of the epigenetic status established by interactions of maternal and zygotic factors early in fly development (Schuettengruber et al., 2011).

“Heterochromatin-like structures are involved in the stable inactivation of developmental regulators such as the homeotic gene clusters in *Drosophila* and mammals, and the mating-type genes in fungi,” (Grewal and Mooazed, 2003). Rm62, an Enhancer of Polycomb and Trithorax, (Lamiable et al., 2010) was also isolated in the yeast screen. Rm62 has been identified as a binding partner of the Dorsal Switch Protein 1, and is the first RNA helicase to be characterized as having a role in the regulation of homeotic genes (Lamiable et al., 2010). *Drosophila* Rm62 has been shown to interact with histone modifying SU(VAR)3-9 in mechanisms likely to regulate gene expression or to re-establish repression of inducible genes (Boeke et al., 2011). The PGADT7 screen plasmid encoding a fusion protein from the *Drosophila histone 4 R* gene was recovered twice in the yeast screen, and was initially dismissed as a false positive. The *Drosophila histone H4* replacement gene has arginine at the identical position as that of

vertebrate H4R3 which was shown to be an essential part of the mechanism by which Grg4/TLE establishes repressive activity. Therefore each of the three proteins, histone 4R fusion protein, the Groucho fusion protein, and the Rm62 fusion protein, are functionally linked to Polycomb, a well characterized repressive complex that is responsible for long-term silencing of homeotic genes (Czermin et al., 2002). Attraction of the three fusion proteins individually to the 98 bp “bait” fragment is unlikely to be coincidental and may suggest that components of a repression complex assemble on this site. This is in keeping with the previously published finding that the fragment is sufficient to repress epidermal expression of the *repo/LacZ* reporter construct (Johnson, et al., 2011). Because misexpression of *repo* is lethal, long term repression in cells other than glial cells is essential and epigenetic mechanisms are recognized as a means of gene silencing (Courey and Jia, 2001; Lee and Jones, 2005).

The *in vivo* interaction of *gcm* with *groucho* described in this study in *Drosophila* could suggest a role in such repressive activity at the cis-regulatory DNA upstream of the gene *repo*. The 98bp element used in the yeast screen is just a portion of a regulatory element, different fragments of which confer different activities and expression levels of the *lacZ* reporter construct (Lee and Jones, 2005). Mutation of the binding sites for Gcm in this DNA reduced the intensity of the specific activities and it was concluded that Gcm may act synergistically with other factors to bring about such specificity (Lee and Jones, 2005). One possibility is that the Gcm protein could recruit the Groucho co-repressor under certain conditions and limit the expression of its target gene *repo*. Both Dorsal and Runx proteins have the capacities either to initiate transcription of target genes or to recruit Groucho as a co-repressor (Flores-Saaib et al., 2001). Dorsal’s ability to both activate and repress transcription is dependent upon the context of the binding sites and its interaction with the Groucho protein is via a motif that has partial homology

to the 10 amino acid factor from Engrailed (eh1 motif) (Flores-Saaib et al, 2001). It is hypothesized that the imperfect eh1 motif may prevent the recruitment of Groucho without other co-repressors (Flores-Saaib et al, 2001). This reasoning could apply to the *gcm-groucho* interaction as well. A second possibility is that Gcm interacts with Groucho in a manner that could relieve repression, allowing transcription of genes necessary for patterning sensory organs of the PNS after which RM62, Groucho, His4R3, and other factors re-establish the chromatin repressive structure established in the early embryo.

Because Rm62 is recognized as an Enhancer of Polycomb and Trithorax, there is a possibility that the Epi Repressor has a role as a Polycomb response element. Polycomb specifically competes with the Gcm protein for target sites and downregulates *gcm* autoregulation during gliogenesis (Popkova et al, 2012). The regulatory role of Polycomb and Trithorax on transiently expressed genes may be important in maintaining a balance between repression of *repo* and the expression of genes essential for patterning of sensory organs of the peripheral nervous system. Since six of the twelve genes recovered have trichogen or bristle phenotypes, the role of the Gcm protein may be speculated to be relief of Polycomb repression. .

A second group of fusion proteins recovered are each annotated to trichogen cell phenotypes. These gene products may be related in function to *gcm* and other factors that are expressed in the asymmetrical cell divisions necessary for development of epidermal precursors into the sensory organs of the peripheral nervous system. The products of CG10279, CG6583, and CG6770 are nuclear, and perturbations in their expression are reported to result in abnormalities in the trichogen cells, the external projections of the macrochaetae of the peripheral nervous system (Buszczak and Spradling, 2006; Lamiable et al., 2010; Bellen, et al., 2004 ). Gcm misexpression gives rise to abnormal macrochaetae, and Groucho mutants have



abnormal bristles above the eyes and at the postalar macrochaetae on the notum. Rm62 mutants also have abnormal macrochaetae numbers at the dorsocentral positions of the notum and on the scutellum. Many genes have a role in morphogenesis of the macrochaetae including those of the Notch, Numb, Pannier, and AC-S pathways. The transcription of proneural genes *ac* and *sc* is regulated by complexes that have regional enhancers and repressors, with combinations of proteins providing the positional information that activate specific enhancers of these genes. Pannier drives bristle development in the most dorsal region of the thorax by activating the proneural genes of the *achaete-scute* complex, binding to specific enhancers for appropriate patterning of the bristles (Ramain et al., 1993; Heitzler et al., 1996). Domain specific repressor elements and co-activators are predicted to influence bristle patterning (Ramain et al., 2000). In gliogenic sensory organs, the SOP divides to produce PIIa and PIIb. The PIIb cell then divides to produce PIIIb and a glial precursor (GP) cell. *gcm* is expressed in the GP cell and is required for the differentiation of glial cells to which it gives rise. The PIIIb sister cell divides to produce the neuron and its tormogen. AS-C and Notch signals influence both the early initial designation of the SOP (the cell with the highest proneural level due to Notch signals) and the later asymmetric cell divisions that give rise to the four cells of the bristle. Notch is known to negatively regulate expression of *gcm* in gliogenic sensory organs, but in another context (in non-gliogenic lineages) Notch is inactive in the IIIb cell as well, where its inactivity produces a neuron (Van De Bor and Giangrande, 2001). The context dependent outcomes may suggest interaction with common co-factors that influence the pathways. *gcm* is reported to initiate Ac expression when it is temporally misexpressed, demonstrating an interaction between the two (Van De Bor et al., 2002). The Gcm target, *tramtrack P69*, is expressed in the same PNS precursor cells suppressing neuronal characteristics as it does in the CNS, and here it is said to

act downstream of *notch* (Okabe, 2001; Furman and Bukharina, 2011; Lee and Jones, 2005). Many of the same gene products that are present in patterning and development of the sensory bristles interact in roles reminiscent of their roles in the developing CNS. Since five of the fusion proteins recovered in the double interaction screen are encoded by genes that cause abnormal trichogen cell phenotypes, they may have roles as either enhancers or repressors in limited domains wherein combinatorial interactions with other patterning factors result in wild type bristle patterns. Previously described Gcm interactions with both Achaete-Scute and Notch and its demonstrated interaction with CG6770 in this study suggest that the phenotype of irregular bristle number and position that result from CG6770 deficiency may indicate a role in a common pathway involved in bristle patterning.

The same proteins also interact in development of other systems. In a misexpression screen overexpression of the CG6770 protein was shown to reduce the number of sessile hemocytes in *Drosophila* larvae, reduce the total hemocyte number, and disrupt the dorsal hemocyte compartment (Stofanko et al., 2008). This would appear to be characteristic of a protein with repressor function over some aspect of hemocyte development. The disruption of the dorsal compartment is associated with defects in proteins necessary for cell structure and adherence (Stofanko, 2008). Hematopoiesis in *Drosophila* occurs in two phases, one during development of the embryo when cells of the procephalic mesoderm give rise to hemocytes and a later phase during larval development in the lymph gland. Pannier, a gene previously described in bristle patterning, is required for maturation of hematocytes (Minakhina et al., 2011). Gcm and Gcm2 are also required for the maturation of plasmatocytes and their ability to function as macrophages in the embryo. These embryonic hemocytes migrate throughout the embryo replicating themselves in the hemolymph and persist into the adult fly (Alfonso and Jones,

2002). The Stofanko misexpression screen (2008) identifying CG6770 as a protein which reduces hemocyte numbers does not distinguish between hemocytes that are of embryonic origin and those produced during larval hematopoiesis. Thus, the reduction in hemocyte numbers may be driven by defects in gene expression in either or both processes. CG6770 overexpression may influence the same pathway of hematocyte differentiation in which Gcm and Gcm2 are essential for maturation and functional competence of macrophages. Evidence of interaction between Gcm and the CG6770 protein in *Drosophila* was confirmed in this study with the *glass* Multimer *Reporter* construct and by the genetic crosses between CG6770<sup>EY00294</sup> and *gcm*<sup>ΔP1</sup>.

#### 4.2. Summary of recovered plasmids

In addition to the co-repressor *groucho*, *gcm*, and CG6770, the double interaction screen identified four candidate regulators, and a possible transcription factor. This modified screen relied upon a well-characterized cis-acting element that served as a target for the Gcm protein and is capable of driving *lacZ* reporter expression that recapitulates the typical pattern of lateral glial expression. Recovery of the PGADT7 library plasmid carrying the cDNA of *gcm* lends validation to the selection method of the Double Interaction Yeast Screen. The recovered cDNAs are currently being investigated.

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Major: Biology

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Degree: B. S., Cum Laude

Honors: Mortar Board, Honors Program 1964-65, Beta Beta Beta Honor Society, Blacklist  
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1967

2. Druid City Hospital School of Medical Technology, Tuscaloosa, Alabama

Certificate in Medical Technology; M.T. (A.S.C.P.) National Certification

1968

3. Delta State University, Cleveland, Mississippi

Major: Biology

Degree: M. S.

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#### Professional Positions

1. Medical Technologist, Clinical Laboratory, Biochemistry Department

Druid City Hospital, Tuscaloosa, Alabama

1968-1969

2. Medical Technologist, Clinical Laboratory, Hematology Department

St. Vincent's Hospital, New York, New York

1969-1970

3. Medical Technologist, Clinical Laboratory, Biochemistry Department

Grenada Lake Medical Center, Grenada, Mississippi

1972-1973 and 1999-2000

4. Instructor of Biology

Kirk Academy High School, Grenada, Mississippi

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5. Medical Technologist, Clinical Laboratory, Generalist

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