Figure 9. Exposure to epirubicin: 500 µg (*mwh* + *flr3* pattern) SMART analysis.

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Testing gene function in fly head formation using transgenic RNAi.

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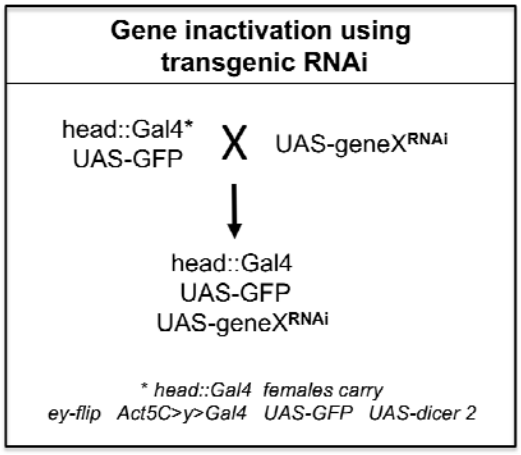
Abstract and Introduction

In this project, we investigated the potential role of 31 genes in eye/head formation in *D. melanogaster*. We tested the function of each gene by using RNAi as a means of knocking-down a single gene at the time within the progenitor tissue that gives rise to the adult head (eye-antennal imaginal disc). The eye/head phenotype was evaluated first at the adult stage and then during development in the larval stage. The range of phenotypes observed included eyeless, reduced eye, bar eye and rough eye, headless, reduced head, and enlarged head. Specific phenotypic categories were also analyzed at the level of imaginal discs in order to understand the nature of the defect better. The mutant phenotypes induced by RNAi-mediated silencing of *Ccn* or *KCNQ* (reduced eye), garz (disorganized neurons), alien or Trn (reduced head), and syx1A, *dock*, *Gdi*, or *drosha* (headless) suggest that these genes play significant roles in the development of the head and/or eye.

Methods

Genes were inactivated by transgenic RNAi (reviewed by Perrimon *et al*., 2010) specifically in the eye-antennal disc by inducing RNAi expression using the Gal4/UAS system (Brand and Perrimon, 1993). Virgin females of the *ey-flip; Actin5C>y>Gal4 UAS-GFP; UAS-dicer-2* genotype, here called "head-Gal4," were crossed to males from UAS-RNAi lines (Figure 1) obtained from the TRiP center collection (see Table 3 for specific TRiP stock numbers; http://www.flyrnai.org/TRiP-TTR.html). All crosses were carried out at 25°C.



Adult F1 progeny were scored using a stereomicroscope. Third instar larvae were dissected and directly mounted to identify eye discs (GFP-positive tissue). To look at neuronal development, anti-ELAV staining was carried out by standard Ab protocol (Sullivan *et al*., 2000) on L3 eye-antennal discs, and images were obtained on a Leica SPE confocal. Images were processed in Adobe Photoshop.

Figure 1. Genetic scheme used to knock-down gene activity specifically in eye/head progenitor cells. Head::Gal4 flies carry *ey-flip* on the X chromosome, *Act>y>Gal4* and *UAS-GFP* on chromosome 2, and *UAS-dicer2* on chromosome 3. All UAS-RNAi transgenes are on chromosome 3.

Results and Discussion

In order to identify genes required for eye and head development, we crossed 31 UAS-RNAi lines with the eye-antennal imaginal disc Gal4 driver line "head-Gal4." The progeny from each cross were first scored at the adult stage and fell into one of the categories shown in Table 1.

The overall summary of the data is shown in Table 2, examples of the HL, EL, and RH phenotypes are shown in Figure 1, and detailed data are provided in Table 3. Among the 31 crosses, 15 gave no viable progeny and 16 did; 12 of the 15 lethals died in the late pupal stage and could be scored from pupal dissection. Out of 31 crosses, 5 crosses gave normal progeny, and 26 led to mutant phenotypes including 16 with abnormal heads (9 HL, 6 RH, and 1 LH), 7 with abnormal eyes (3 Bar, 2 RE, 1 EL, and 1 RR). Three were unscorable due to early death (DD).

The twelve crosses, scored DD (3) and HL (9), raised the question of whether the progeny simply lacked discs, thus making it impossible to form heads and eyes, or whether the discs were present but unable to join and form the head during metamorphosis. Because of this and since in all cases death occurred at the pupal stage, these two categories were selected for analysis at the larval stage.

Third instar larvae were dissected and eye-antennal discs were stained for the ELAV protein to visualize neurons and for the EYA protein to mark eye progenitor cells. In four cases (*brm, dock, Gdi, Syx1A*), no discs (GFP-positive) were found, suggesting that discs were either absent or extremely small in size (not shown). Six other crosses (*skpA, N, Smr, nct, mam, drosha*) showed extremely small discs (not shown), whereas two crosses (*neur, garz*) produced L3 discs that when

stained for ELAV showed plenty of neurons but somewhat abnormal clusters (Figure 3B and not shown).

Table 1. Phenotypic Categories.

CODE	ADULT HEAD/EYE PHENOTYPE
BAR	Bar-like eye
DD	dead (some could be scored as late pupae)
EL	eyeless
HL	headless
LH	larger head than normal (including eyes)
NSS	normal shape and size
RE	reduced eye, smaller eye
RH	reduced head
RR	rough eyes

Table 2. Overview Cross Results.

Crosses with normal progeny	5/16
Crosses with abnormal progeny	26/31
Lethals	15/31
Lethals scored as late pupae	12/15 (3/15 DD RH, 9/15 DD HL)
Lethals unscorable	3/15 (3 DD)
Viable with abnormal heads	16/31 (9/16 HL, 6/16 RH, 1/16 LH)
Viable with abnormal eyes	7/31 (3/7 Bar, 2/7 RE, 1/7 EL, 1/7 RR)

We also analyzed at the larva stage the knock-down crosses resulting in the Bar phenotype: *ato, csw,* and *spitz*. As expected, silencing of the *ato* gene resulted in strong bar-eyed flies and, based on anti-ELAV antibody staining, it had only a few neurons (not shown). The *csw* knock-down also resulted in an extreme bar-eye phenotype at the adult stage, and only a thin line of ELAV-positive cells were seen in the discs (Figure 3C). Spitz-RNAi adults were also bar-eyed; however, at the L3 stage, the discs were large in size and displayed plenty, but disorganized, neuronal clusters along the posterior portion of the disc (Figure 3D). Therefore, in the case of *Spitz*, neuronal development did begin to produce neurons (though abnormally as shown by the disorganization of the neuronal clusters) and then it probably stopped before all ommatidia were formed (hence the bar-like eye).

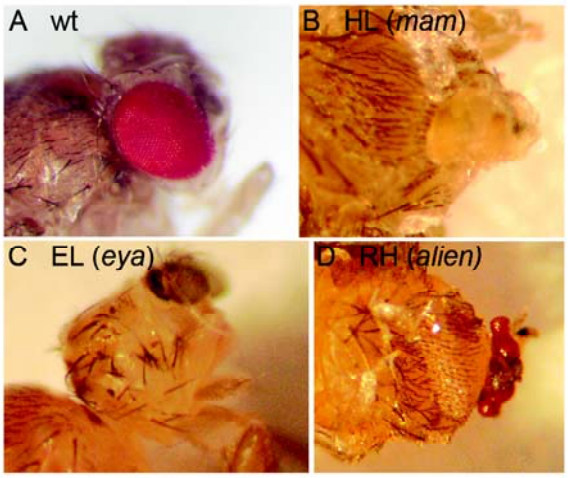


Figure 2. Examples of adult phenotypes. A) wt fly head; B) *ey-flip; Act>y>Gal4 UAS-GFP; UAS-dicer2/UAS-mam*^{RNAi}; C) *ey-flip; Act>y>Gal4 UAS-GFP; UAS-dicer2/UAS-csw*^{RNAi}; D) *ey-flip; Act>y>Gal4 UAS-GFP; UAS-dicer2/UAS-alien*^{RNAi}.

Of the 31 genes tested, 14 of them were previously known to affect imaginal discs and/or eye/head development. In these cases, our findings were consistent with previous reports (as recorded in Flybase). These loci include *Notch, mam, neur,* and *nct* (components of Notch signaling), *spi, Cbl,* and *csw* (components of EgfR signaling), *dpp, eya, ato, trr, SkpA, Pten, and Mib1* (see Table 3 for details of phenotype and molecular function).

Conversely, *SesB* and *burs* were not expected to affect head/eye size or shape based on previous data and/or molecular function, as reported in Flybase (see Table 3), and no abnormalities were observed in our test as well. Knock-down of *SNF4Ag, raptor,* and *dnd* also did not result in any obvious defects. However, in these cases, we cannot exclude that gene silencing simply did not occur because of lack of relevant data. Nonetheless, these results generally support the validity of our test.

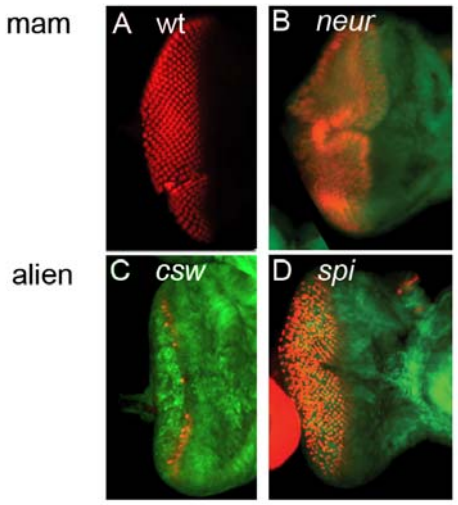


Figure 3. Examples of L3 eye disc phenotypes. L3 eye discs were stained for Elav to mark neurons (red) in all panels and for GFP to mark RNAi expressing cells (green) in panels B-D. A) wt eye disc stained for Elav to mark neurons (red); B) HL fly with proboscis directly attached to thorax, *ey-flip; Act>y>Gal4 UAS-GFP; UAS-dicer2/UAS-neur*^{RNAi}; C) EL fly, *ey-flip; Act>y>Gal4 UAS-GFP; UAS-dicer2/UAS-csw*^{RNAi}; D) RH fly with small collapsed head, *ey-flip; Act>y>Gal4 UAS-GFP; UAS-dicer2/UAS-spi*^{RNAi}.

We report here novel head and/or eye phenotypes for 12 loci. Among these, the biological processes affected by *brm*, *smr*, and *Pp4-19C* (which include chromatin-level regulation of gene expression and control of mitotic cell cycle) agree with the observed phenotypes of DD with no discs (*brm*), HL with small discs (*Smr*), and RH (*Pp4-19C*). The mutant phenotypes induced by knock-down of *Ccn* and *KCNQ* (RE), *garz* (DD with disorganized neurons), *alien* and *Trn* (RH), *syx1A*, *dock*, and *Gdi* (HL with no discs), and *drosha* (HL with small discs) are reported here for the first time and implicate these genes in the formation of the eye (*Ccn*, *KCNQ*, *garz*) and/or head (*alien*, *Trn*, *Syx1A*, *dock*, *Gdi*, *drosha*).

Based on their reported function (Flybase), processes such as vesicle-mediated transport (*garz* and *Gdi*), insulin receptor signaling (*dock*), primary micro-RNA processing (*drosha*), potassium ion transport (*KCNQ*), phagocytosis and/or nuclear import (*Trn*) may play significant roles in the development of the eye and head tissue. Confirmation of these phenotypes using a second, different RNAi, or other reagent, will have to be carried out prior to further studies.

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Table 3 follows:

Table 3. Head/eye phenotypes for 31 genes tested by transgenic RNAi. * entries based on flybase (*http://flybase.org*)

CODE	ADULT HEAD/EYE PHENOTYPE	CODE	ADULT HEAD/EYE PHENOTYPE
BAR	Bar-like eye	NSS	normal shape and size (more subtle defects may have been missed)
DD	dead (some could be scored as late pupae)	RE	reduced eye, smaller eye
EL	eyeless	RH	reduced head
HL	headless	RR	rough eyes
LH	larger head than normal (including eyes)		

TRiP #	CG #	Gene Name	Head/Eye phenotype	Phenotype code	Disc phenotype	Reported eye function*	Molecular function*	Biological process*
HM05119	CG9556	*alien*	Dead (pupa); reduced eyes; reduced head; collapsed head and eyes	DD RH	Not Determined	NONE	ligand-dependent nuclear receptor binding; transcriptional repressor co-factor	protein stabilization
JF02089	CG7508	*ato - atonal*	Strong Bar eyes	BAR	Only a couple of neurons	photoreceptors are completely absent; required for R8	transcription factor	eye morphogenesis; R8 photoreceptor cell fate commitment; neuronal fate and differentiation
HM04019	CG5942	*brm brahma*	Dead (pupal stage)	DD	No discs recovered	Formation of larval head structures is abnormal but the nature of the defects is undetermined.	protein binding, DNA binding, general RNA polymerase II transcription factor activity, regulation of transcription	chromatin-mediated maintenance of transcription; positive regulation of EGFR signaling pathway; cell differentiation
JF02260	CG18419	*burs bursicon*	Normal shape and size	NSS	Not Determined	NONE	hormone activity	chitin-based cuticle tanning
JF02650	CG7037	*Cbl*	Rough eyes	RR	Not Determined	Rough mosaic eyes, larger than wt, with larger ommatidia. block of apoptosis; over-recruitment of all eye cell types	protein binding; signal transducer activity	sensory organ development; regulation of EgfR; neuron differentiation; regulation of cell proliferation

TRiP #	CG #	Gene Name	Head/Eye phenotype	Phenotype code	Disc phenotype	Reported eye function*	Molecular function*	Biological process*
HM04023	CG32183	*Ccn*	Reduced eyes (in some)	RE	Not Determined	NONE	growth factor activity	unknown
HMS00012	CG3954	*csw corkscrew*	Extreme Bar eye, very narrow and oblong	BAR	a thin line of ELAV positive neurons in the disc	loss of photoreceptor R7, & occasional loss of outer (R1-R6) photoreceptors	protein binding; protein tyrosine phosphatase activity	EGFR signaling pathway; cell signaling; cell fate commitment; mitotic cell cycle
JF02638	CG6560	*dnd dead end*	Normal shape and size	NSS	Not Determined	NONE	GTPase activity; GTP binding	cilium assembly; signal transduction
JF02809	CG3727	*dock dreadlocks*	Dead (pupa); headless	DD HL	No discs recovered	photoreceptor cell axons targeting defects	insulin receptor binding	insulin receptor signaling pathway, axon guidance
JF01090	CG9885	*dpp decapentaplegic*	Dead (pupal stage) - reduced head, small head	DD RH	Not Determined	small and/or roughened eyes, consistent with a failure of normal eye morphegenesis	ligand; cell signaling	regulation of organ morphogenesis
JF02784	CG8730	*drosha*	Dead (pupa); headless	DD HL	Small discs	NONE	double-stranded RNA binding; ribonuclease III activity	primary microRNA processing
JF03160	CG9554	*eya eyes absent*	Eyeless	EL	Not Determined	eya mutants have reduced eyes or are eyeless	ser/thr phosphatase; tyr phosphatase; transcriptional activator non-DNA-binding co-factor	regulation of cell fate specification; eye progenitor cells specification
JF01603	CG8487	*garz gartenzwerg*	Dead (pupal stage)	DD	Plenty of neurons but disorganized	NONE	ARF guanyl-nucleotide exchange factor activity	ER to Golgi and intra-Golgi vesicle-mediated transport
JF02617	CG4422	*Gdi GDP dissociation inhibitor*	Dead (pupa); headless	DD HL	No discs recovered	NONE	Rab GDP-dissociation inhibitor activity	neurotransmitter secretion; regulation of Rab GTPase activity; vesicle-mediated transport

TRiP #	CG #	Gene Name	Head/Eye phenotype	Phenotype code	Disc phenotype	Reported eye function*	Molecular function*	Biological process*
JF02562	CG33135	*KCNQ potassium channel*	Abnormal head & very small eye	RE	Not Determined	NONE	voltage-gated potassium channel	potassium ion transport
JF02881	CG8118	*mam mastermind*	Dead (pupa) - headless	DD HL	Small discs	mediates N signaling; Notch pathway required for disc growth	transcription coactivator activity	compound eye development; nervous system development; Notch signaling pathway
JF02629	CG5841	*mib1 mind bomb 1*	Reduced head	RH	Not Determined	small wing and eye imaginal discs	protein binding; ubiquitin-protein ligase activity	compound eye morphogenesis, positive regulation of Notch signaling
JF01356	CG3936	*N Notch*	Dead (pupa) - headless	DD HL	Small discs	required for proliferation of eye disc epithelium	cell-cell signaling receptor; regulator of transcription	sensory organ development, neurogenesis; regulation of cell proliferation
JF02648	CG7012	*nct nicastrin*	Dead (pupa); headless	DD HL	Smaller and deformed discs	formation of supernumerary neurons; Notch pathway; required for disc growth	processing of Notch signaling receptor	cytoskeleton organization; Notch signaling pathway; photoreceptor cell morphogenesis; cell proliferation
JF02048	CG11988	*neur neuralized*	Dead (pre-pupal stage)	DD	Plenty of neurons but disorganized	ommatidia poorly defined, rhabdomeres and lenses severely disrupted, eye bristle missing	ubiquitin-protein ligase activity	regulation of Notch signaling pathway
JF02807	CG32505	*Pp4-19C Protein phosphatase 19C*	Reduced head	RH	Not Determined	NONE	protein serine/threonine phosphatase activity	microtubule-based process; regulation of mitotic cell cycle
JF01859	CG5671	*Pten*	Big head; big eyes	LH	Not Determined	larger eyes than normal when eyes mosaic for mutant	serine/threonine phosphatase activity	insulin receptor signaling pathway; negative regulation of growth, cell size, proliferation, cell cycle
JF01088 - strong	CG4320	*raptor*	Normal shape and size	NSS	Not Determined	NONE	protein binding	response to DNA damage

TRiP #	CG #	Gene Name	Head/Eye phenotype	Phenotype code	Disc phenotype	Reported eye function*	Molecular function*	Biological process*
JF01810	CG16944	*sesB stress-sensitive B*	Normal shape and size	NSS	Not Determined	normal eyes	ATP: ADP antiporter	synaptic vesicle transport
HM05185	CG16983	*skpA*	Dead (pupa); headless; mouth parts directly on shoulders	DD HL	Small discs	imaginal discs are either entirely absent or rudimentary, extensive apoptosis	protein binding	centrosome duplication, chromosome condensation, positive regulation of mitotic cell cycle
JF02413	CG4013	*Smr Smrter*	Dead (pupa) - headless	DD HL	Small discs	NONE	protein and DNA binding; transcription corepressor activity	regulation of mitotic cell cycle
JF02060	CG17299	*SNF4Aγ SNF4/AMP-activated protein kinase gamma subunit*	Normal shape and size	NSS	Not Determined	NONE	AMP-activated protein kinase component	positive regulation of cell cycle; cholesterol homeostasis
JF03322	CG10334	*spi spitz*	Bar eyes	BAR	Discs with disorganized neuron clusters along posterior only	mitotic clones have reduced numbers of photoreceptors and loss of whole ommatidia	epidermal growth factor receptor binding	EGFR signaling pathway; cell signaling; mitotic cell cycle; cell fate commitment
JF01829	CG31136	*Syx1A Syntaxin 1A*	Dead (pupa); headless	DD HL	No discs recovered	disrupted ommatidial array	SNAP receptor activity	cytokinesis; cellularization; exocytosis; neurotransmitter secretion; vesicle-mediated transport
JF02697	CG7398	*Trn Transportin*	Reduced head	RH	Not Determined	NONE	protein transmembrane transporter activity	protein import into nucleus; phagocytosis, engulfment
JF03242	CG3848	*trr trithorax-related*	Dead (pupal stage) - small head	DD RH	Not Determined	morphogenetic furrow progression defects, disorganized ommatidial array	histone methyltransferase activity; transcription coactivator binding	cell differentiation