Chapter 2: Echinus Shows Homology to Deubiquitinating Enzymes and is Required for Retinal Cell Death in *Drosophila*

2.1 Abstract

The *Drosophila* eye is a patterned epithelium of repeating units called ommatidia. The ommatidia consist of four cell types, with both cell differentiation and cell death important for the final arrangement of cells. Death occurs early in pupal development and removes the excess cells that surround each ommatidium. Mutants for the *echinus* (*ec*) gene are devoid of this pupal-stage apoptosis and extra cells are left between the ommatidia to create a rough eye in the adult fly. In this study, we characterized several *echinus* mutants and the Echinus protein resembles a deubiquitinating enzyme. The gene possesses a UCH domain, characteristic of deubiquitinating enzymes of the UBP family, though it lacks the necessary catalytic cysteine. Structure-function analysis revealed that the C-terminal poly-glutamine repeat is not important for function. Enhancer trap and *in situ* data corroborated the role of *echinus* in pupal eye development; expression of *echinus* was observed at the time of pupal eye apoptosis. Epistatis analysis suggested that *echinus* acts upstream or independently of the pro-apoptotic genes *hid*, *grim*, and *reaper*.

2.2 Introduction

The *Drosophila* eye is a beautiful hexagonal array of repeating units called ommatidia. The eye consists of approximately 750 ommatidia, which themselves are an ordered set of four cell types. Each ommatidium contains eight photoreceptor (R cells) neurons, four cone cells, and two primary pigment cells. The interommatidial cells (IOCs) surround each ommatidium and help pattern the cellular array. Differentiation of the retinal cells begins when the morphogenetic furrow (MF) progresses through the imaginal eye disc. From a layer of undifferentiated epithelium following the MF, the R8 cell develops first and then recruits the seven other R cells. Simultaneous to the differentiation of the R cells, the remaining cells undergo another round of mitosis to create the final pool of undifferentiated cells (Wolff and Ready, 1991b). The cone cells differentiate next, followed by the primary pigment cells, then finally the secondary and tertiary pigment cells, also known as the interommatidial cells (IOCs). During the time of IOC differentiation, at about 28 hours after pupal formation (APF), a wave of apoptosis occurs to remove excess IOCs, thereby completing the ordered array. How the IOCs commit to cell suicide or to differentiation is not well understood.

The core components of the apoptosis machinery are known to modulate the wave of death during pupal eye development. Active caspases are observed during IOC death (Yu et al., 2002); in addition, overexpression of the *Drosophila* caspase inhibitor

Diap1 causes the formation of excess IOCs (Hay et al., 1995). Likewise, ectopic expression of the pan-caspase inhibitor P35 interferes with cell death and results in the differentiation of extra pigment cells (Hay et al., 1994). Mutants of the F-box/Ubc gene *morgue* have elevated levels of DIAP1 during pupal eye development and have superfluous IOCs (Hays et al., 2002; Wing et al., 2002).

Preliminary studies have also shown that both the *Egfr/Ras* and *Notch* signaling pathways are active during IOC differentiation or death. Activated alleles of both *Ras* and *Egfr* prevented cell loss, while expression of a dominant-negative version of Egfr had too few IOCs (Miller and Cagan, 1998; Freeman, 1996). Additionally, ectopic expression of *argos*, an inhibitor of the EGFR, increased the number of cells undergoing apoptosis (Sawamoto et al., 1998). Antibody studies also showed the presence of the Egfr in the IOCs (Miller and Cagan, 1998). Likewise the *Notch* signaling pathway is active during this stage of eye formation. Notch localizes to the IOCs during pupal development, and the mutant $N^{fa\text{-}g}$ allele shows disrupted primary cell morphology and an abundance of IOCs (Kooh et al, 1993; Cagan and Ready, 1989). It is not known whether either of these signaling pathway work by modulating apoptosis or the differentiation of the IOCs.

Deubiquitinating enzymes (DUBs) are ubiquitin-specific proteases that cleave ubiquitin at its C-terminus and have been shown to play many cellular roles (reviewed in Wilkinson, 1997). DUBs are categorized into two classes, the small-protein UCH family and the large-protein UBP family. A *Drosophila* member of the

UCH family, UCH-L3, localizes to the PA700 regulatory region of the proteosome and is thought to remove ubiquitin moieties from proteosome-bound proteins (Holzl et al., 2000; Wojcik and DeMartino, 2002). The proteins of the UBP family of deubiquitinating enzymes have little sequence similarity outside their UCH domain and are involved in a wide range of cellular processes. Two of the best characterized deubiquitinating enzymes are *fat facets* and HAUSP. The *Drosophila* gene *fat facets* (*faf*) mediates photoreceptor internalization by removing ubiquitin from *liquid facets*, the *Drosophila* epsin homolog (Cadavid et al., 2000). The UBP HAUSP opposes the ubiquitin ligase Mdm2 by hydrolyzing Ub from p53 (Li et al., 2002).

In this report, we characterize and clone the *Drosophila* gene *echinus*. *echinus* was originally isolated as a spontaneous mutant with rough eyes and subsequently used as a marker for the X-chromosome (Bridges, 1916, reiterated by Lindsley and Zimm, 1992). Loss-of-function alleles are deficient for apoptosis in the developing pupal eye and contain extra IOCs. *echinus* was cloned and found to show homology to deubiquitinating enzymes of the UBP family; however, Ec lacks residues thought to be critical for deubiquitinating activity. Homologous genes in humans, mice, and *Arabidopsis* show similarity only in the UCH domain. Enhancer-trap staining and *in situ* hybridizations show that *echinus* is expressed in the cone cells, the primary pigment cells, and the IOCs at developmental stages prior to and after the wave of cell death in the pupal eye. Genetic interactions with components of the death machinery show that *echinus* works upstream or independently of the core *Drosophila* apoptotic machinery.

2.3 Materials and Methods

Drosophila lines and genetics

Drosophila strains and crosses were performed at 25 ^{o}C. Pupal timing is expressed in hours, with the white prepupal stage defined as 0 hours after pupal formation (APF). Pupal dissections were performed at 42 hrs APF unless noted otherwise. The following strains were used: ec^{1} (Bloomington Stock Center, Indiana University), ec^{PlacZ} , GMR-hid, GMR-reaper, GMR-grim, GMR-dronc, GMR-strica. Generation of the series of *ec* deletion mutants were performed by imprecise P-element excision on the line EP(X)1343. RH68894 was obtained from Research Genetics/Invitrogen (Carlsbad, CA) and cloned into pGMR-1N. Interommatidial cell counts were made by counting the IOCs, minus the bristles, that surround two primary pigment cells. Three separate areas were counted per pupal eye discs, and at least five eye discs were counted for each genotype.

Constructs and transgenes

For RNAi, gene fragments were placed between two UAS control element on opposing strands, as previously described (Giordano, 2002). ec^{RNAi} (coding region amino acid 1237–1505) and $CG2901^{RNAi}$ (coding region amino acid 1–171) were made using this technique. A third *echinus* fragment (coding region 232–398) was

also introduced into this vector. Flies carrying this construct also phenocopy *echinus* loss-of-function (data not shown).

Full-length cDNA of *ec* was isolated from a larval-pupal cDNA library. A probe was generated against the UCH domain of CG2904. Several clones were isolated and sequenced. The longest cDNA was used to generate GMR and UAS constructs. Transformants were made by using microinjection into <1 hour w^{1118} embryos. Several transgenic lines were prepared for each construct.

GMR-*ec* (-polyQ) encodes the first 1389 amino acids of coding sequence and was generated by PCR amplification from *ec* cDNA. GMR-*ec* (UCH) was PCR amplified from *ec* cDNA and encodes the first 400 amino acids. The QuikChange protocol (Stratagene) was used to make a C134S mutation. GMR-USP53 was derived from a pGEX vector and placed into EcoRI-NotI site of pGMR-1N.

Microscopy, immunocytochemistry, and antibodies

Scanning electron microscope images were produced on a Hitachi machine. Flies were dehydrated in an ethanol series, incubated in hexamethyldisilazane (Sigma) overnight, and dried prior to use. Confocal microscopy was performed on a Leica (Deerfield, IL) DM IRBE microscope.

Pupal eye discs were dissected and immunostained according to standard procedures. Eye discs were fixed (20 minutes, room temperature) in 4% formaldehyde in 1x PBS

(pH 7.2). Antibodies were used at the following concentration: mouse anti-Dlg (1:150) (DSHB, University of Iowa, Iowa City, IA), mouse anti-β galactosidase (1:100) (Promega). Alexa Fluor 488 mouse secondary antibodies were used (Molecular Probes). Eye discs were mounted in VectaShield medium (Vector, Burlingame, CA).

2.4 Results

Characterization of *echinus* mutants

A previous study on *echinus* has shown that the rough eye in the adult was due to the presence of excess interommatidial cells and a loss of apoptosis (Wolff and Ready, 1991a). Cell counts of the pupal eye disc and BrdU analysis showed no increase in cell number or proliferation (Wolff and Ready, 1991a). Based on these considerations, it was concluded that *echinus* is involved in the death of the interommatidial cells in the eye.

A new allele of *echinus*, caused by a P-element insertion, was isolated and mapped to the 3F3 region of the X-chromosome (Fig. 1). $ec^{P\ lacZ}$ did not complement the original echinus allele (ec^{1}). Excisions of the $ec^{P\ lacZ}$ P-element reverted the rough eye phenotype, confirming the role of this P-element in the echinus phenotype. This P-element is between the predicted genes CG2901 and CG2904. dsRNA targeted to CG2904 caused a rough eye phenotype in the adult, mimicking *echinus* (Fig. 3C, 3H).

Pupal eye dissections of the RNAi line revealed about five excess IOCs per ommatidium.

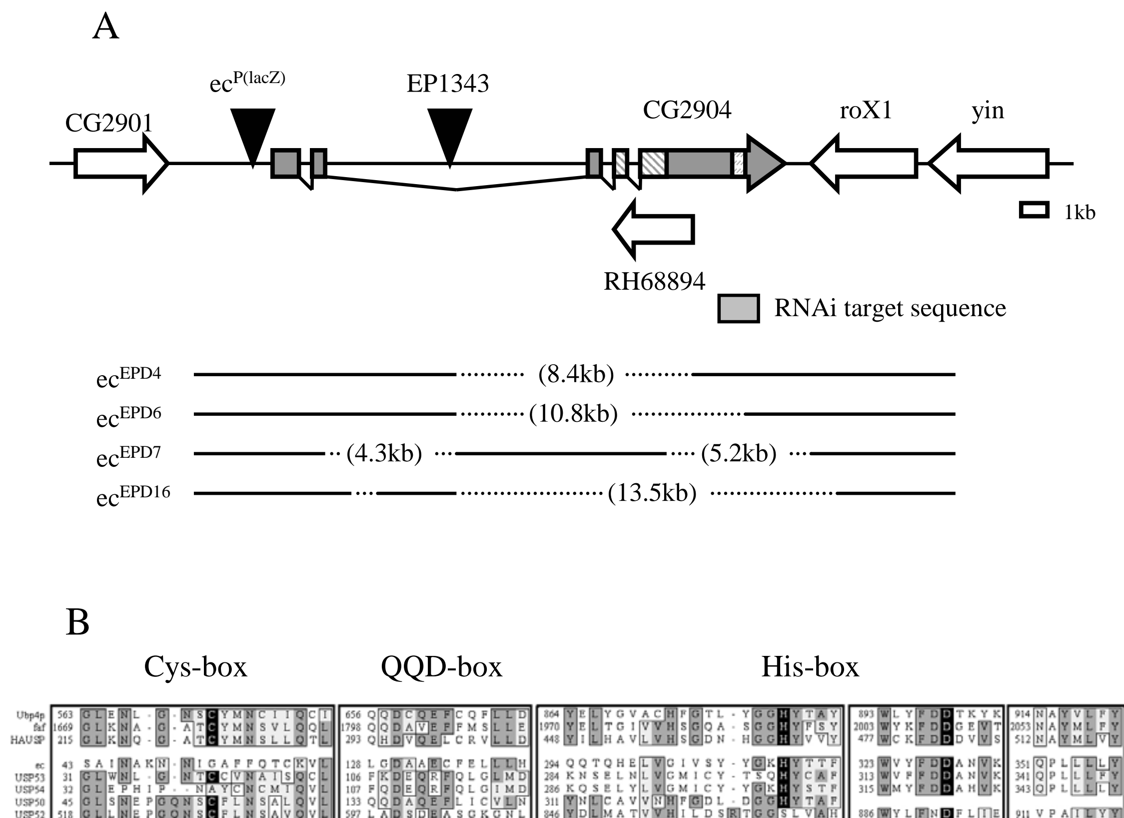


Figure 1. *echinus* encodes a protein with homology to deubiquitinating enzymes. A) Shown is the genomic region surrounding *echinus*, CG2904. The UCH domain is shown in diagonal stripes while the poly-glutamine stretch is shown in hatch marks. P-element insertions are represented by black triangles. Note that *ec*PlacZ is an allele of *echinus*, while EP1343 is not. Shown below the genomic structure is a series of deletion alleles. Missing DNA sequences are shown as dotted lines with the parentheses indicating the size of the deletion. B) Sequence alignment of echinus to other deubiquitinating enzymes. The first three proteins, above the space, encode functional enzymes, while the five below, including Echinus are non-functional. The human Echinus orthologs, USP53 and USP54, are just below the Echinus protein sequence. Catalytic residues are shown in black, with grey representing conserved residues. Note the lack of catalytic cysteine in Ec.

Adult eyes were wild type in RNAi-mediated CG2901 knockdown lines (data not shown).

To generate additional alleles of *echinus*, imprecise P-element excision using the Δ2-3 transposase was performed. The EP line, EP1343, which lies in between the *ec* P-element and the predicted CG2904 gene, has a normal, wild-type eye. A series of *ec* alleles were generated that removed significant regions of the gene CG2904 by excising EP1343 (Figure 1). The $ec^{EP\Delta 4}$ allele was the smallest of these deletions, removing approximately 8kb of the predicted gene CG2904 (Fig. 2). Several EMS-induced point mutations were also isolated in the course of this study. ec^{3c3} is a loss-

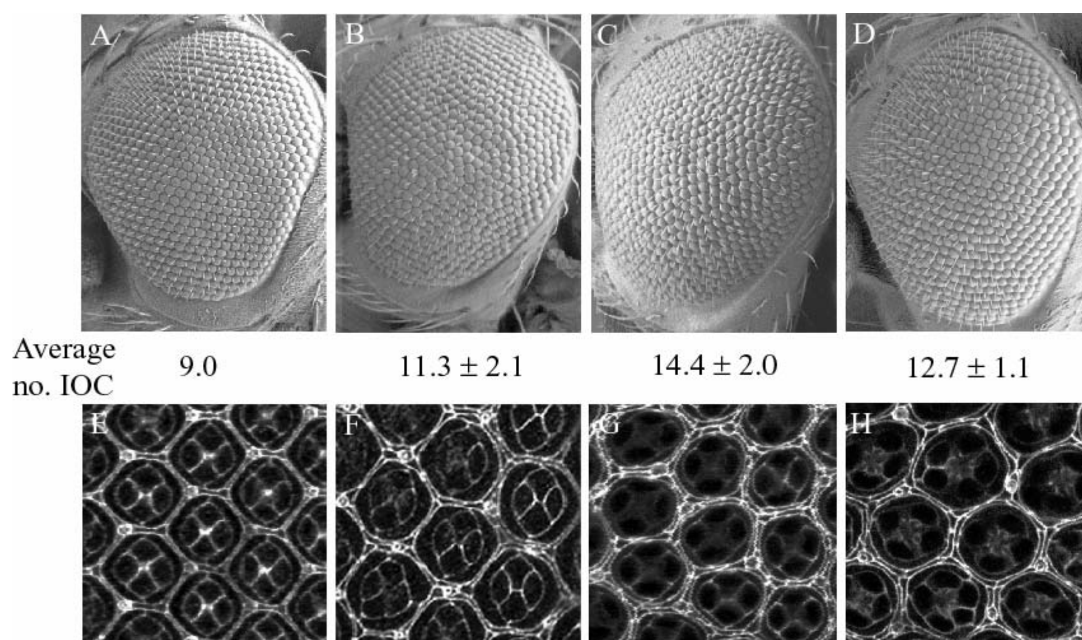


Figure 2. *echinus* mutants have rough eyes due to extra intraommatidial cells. Scanning electron micrographs of adult *Drosophila* eye (A-D) and confocal imaging of 42 hr APF pupal retinas (E-H). Cell borders are marked with anti-Discs large (Dlg) in confocal images. (A,E) Wild type. (B,F) *echinus*(1). (C,G) *echinus*(PlacZ). (D,H) *echinus*(EPΔ4).

of-function mutant carrying a lysine to missense mutation at amino acid 758. Later experiments showed that the original *echinus* mutant (ec^1) is a transposon insertion in the 5' UTR of CG2904 (I. Bosdet, personal communication). In all cases, each of the loss-of-function alleles gave adult eyes that were rough and disorganized. Upon dissection, the pupal eyes contained excessive interommatidial cells, and occasionally contained extra primary pigment cells.

echinus shows homology to deubiquitinating enzymes

These results demonstrate that CG2904 encodes *echinus*, a putative deubiquitinating enzyme. To isolate a full-length cDNA of this gene, a pupal-larval library was screened. Using a probe for the ubiquitin C-terminal hydrolase (UCH) domain, several cDNAs were isolated, all having a close resemblance to the predicted gene CG2904. The longest fragment was sequenced, and was shown to be a 6747 bp fragment, covering roughly 15 kb of the genomic region (GenBank accession number AY576488). Importantly, the 5' exon of the isolated cDNA lies 500 bp downstream of the mutant $ec^{P\ lacZ}$ allele, thus providing an explanation for how this allele disrupts Echinus function.

The sequenced cDNA encodes a predicted 1712 amino acid protein with an N-terminal UCH motif and a C-terminal poly-glutamine stretch. UCH domains are the defining characteristic of deubiquitinating enzymes (DUBs), a large family of proteins involved in many biological processes. Like other members of the UBP

family of DUBs, Echinus is a large protein (188 kDa) and shares little resemblance to other deubuiquitinating enzymes outside its UCH domain.

DUBs are cysteine proteases that remove ubiquitin from ubiquitin-conjugated proteins (Wilkinson, 1997). Catalytic activity required three key resides: a cysteine, a histidine, and an aspartate. Based on homology to other DUBs, the catalytic histidine of Echinus is proposed to be at residue 310 and the aspartate at 328. The putative catalytic cysteine is difficult to pinpoint, as the sequence alignment to homologous proteins show that Echinus has a glycine in place of the active site cysteine. The alignment suggests either that Echinus encodes a non-functional deubiquitinating enzyme or that the putative catalytic cysteine lies elsewhere in the UCH domain. Four other putative catalytic cysteines exist within the UCH domain, at positions 79, 84, 87, and 134, though each shows only weak similarity to the consensus UCH motif.

The other noticeable feature of the Echinus protein is a stretch of glutamines near the C-terminus. This stretch of 156 amino acids contains 77 glutamines, or about 49%. One region contains a 34 amino acid poly-glutamine repeat. The significance of this region in Echinus and in other proteins is still unclear.

Echinus most closely resembles the two human genes USP53 and USP54 and two *Arabidopsis* genes At3g47900 and At3g47890. Specifically, the UCH domain of Echinus is 42% identical and 45% identical to its counterpart in USP53 and USP54,

respectively. Likewise, the two predicted plant genes (At3g47900 and At3g47890) are common to Echinus only within their UCH domain, both being 26% identical.

A bacterial assay has been used to show that the *Drosophila* deubiquitinating enzyme Fat Facet can cleave ubiquitin from a β-galactosidase protein conjugate (Huang et al., 1995). Bacterially expressed Echinus, however, failed to remove ubiquitin from the protein conjugate, unlike the control protein Ubp2p, a *S. cerevisiae* deubiquitinating enzyme (Table 1). Of interest, both human orthologs USP53 and USP54 also failed in this *in vitro* assay (Quesada et al, 2004).

Plasmid	Colony Color
pUb-Arg-β-Gal	Blue
pRB105	White
pRB-ec	White
pUb-Arg-β-Gal; pRB105	White
pUb-Arg-β-Gal; pRB-ec	Blue

Table1. Results of an in vitro assay to test for deubiquitination of a ubiquitin-β-galactosidase protein fusion. In pUb-Arg-β-gal ubiquitin is tied to β-galactosidase by the amino acid arginine. Cleavage of ubiquitin from β-gal exposes an N-terminal arginine residue on β-gal and promotes β-gal instability. Colony color is a measurement of β-gal activity of the X-gal substrate. Blue indicates an active β-gal enzyme. pRB105 encodes the yeast deubiquitinating enzyme Ubp, while pRB-ec encodes echinus.

CG2904 rescues *echinus*

Expression of the *echinus* cDNA under the control of the GMR enhancer looks wild type (Fig. 3D, 3I). All the pupal eye disc cells are present in their correct

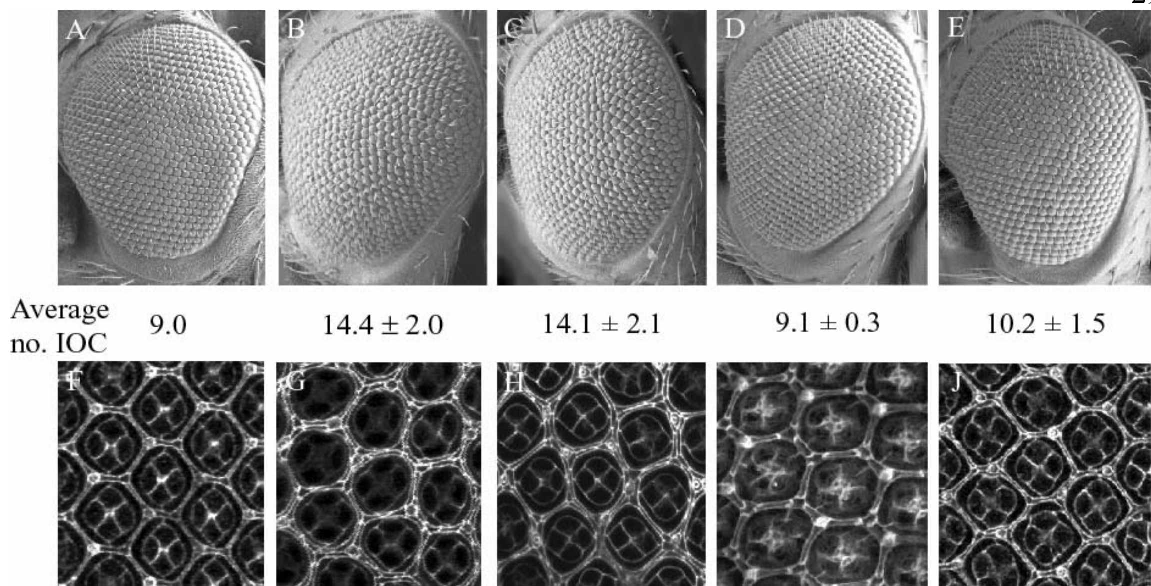


Figure 3. *echinus* resembles a deubiquitinating enzyme. Scanning electron micrographs of adult *Drosophila* eye (A-E) and confocal imaging of 42 hr APF pupal retinas (F-J). Cell borders are marked with anti-Discs large (Dlg) in confocal images. (A,F) Wild type. (B,G) *echinus*(PlacZ). (C,H) RNAi of the deubiquitinating enzyme CG2904. (D,I) Eye specific expression of CG2904 using the GMR enhancer. (E,J) GMR-CG2904 in an *echinus* loss-of-function background.

numbers and positions. When placed into an *echinus* loss-of-function background, GMR-CG2904 nearly rescues the rough eye phenotype (Fig. 3E, 3J). The adult eye is indistinguishable from wild type, and pupal eye dissections reveal about one extra IOC above wild type.

Several cDNAs have been isolated that overlap with the *echinus* coding region, though in the antisense direction (Fig. 1). To verify that these cDNAs do not mediate *echinus* function, we tested their ability to modulate the echinus phenotype. A representative of these cDNAs, RH68894, failed to rescue or enhance any *echinus* mutants (data not shown). Based on these observations, we conclude that *echinus*

encodes a protein with homology to a deubiquitinating enzyme. The roles, if any, of the antisense gene, RH68894 is unknown.

Echinus is expressed in pupal interommatidial cells

Both an enhancer trap line and *in situ* hybridizations were used to determine the

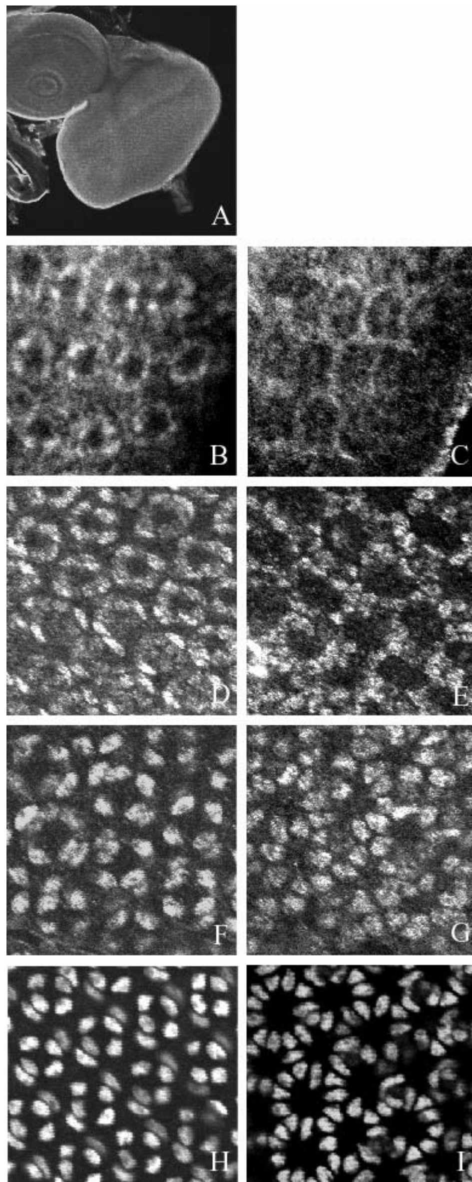
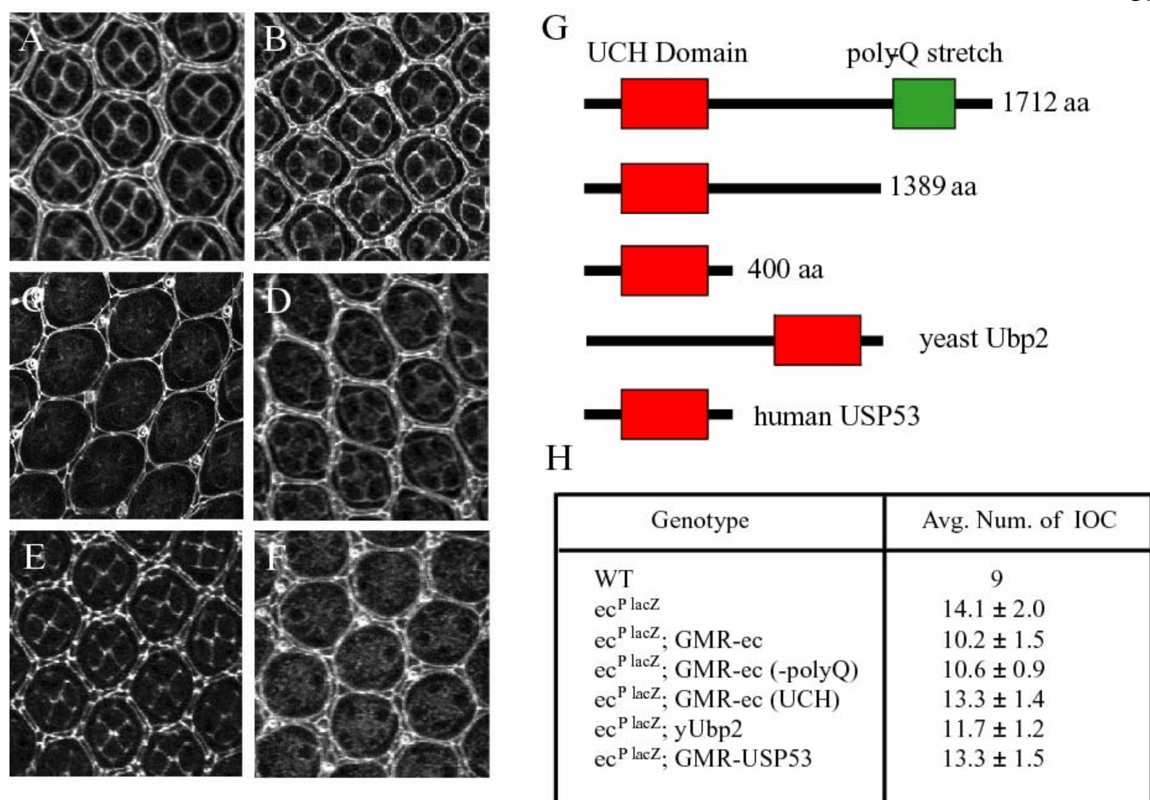


Figure 4. *echinus* is expressed in cone cells and pigment cells prior to and after during the onset of apoptosis in the *Drosophila* pupal eye. Echinus is visualized by anti-β-gal staining in $ec^{P\ lacZ}$/+ flies. A) No expression of *echinus* is seen in white prepupal stage (0 hr APF). (B, C) 20 hr APF. Faint expression is seen in cone cells and primary pigment cells (B) and IOCs (C). (D,E) 30 hr APF. Clearer echinus expression is seen in cone cells and primary pigment cells (D) and in IOCs (E) duringthis time of apoptosis. (F, G) 45 hr APF. echinus is still expressed in cones and primaries (F) and IOCs even after time of pigment cell removal (30 hr APF). (H,I) Enhancer trap staining using anti-β-gal antibody in the LX37 line. Cone cells, primary pigment cells (H) and IOCs (I) are visualized at 45 hr APF.

expression patterns of *echinus*. Flies heterozygous for the enhancer trap P-element, $ec^{P\ lacZ}$, were stained with a β-galactosidase antibody to detect *echinus* expression. At the white pre-pupal stage (0 hour APF), no β-galactosidase staining was seen (Fig 4A). Antibody staining was first detected at 20 hours APF in the cone cells, primary pigment cells, and the IOCs (Fig. 4B, C). Mutant analysis supports the notion that *echinus* acts within these cells, as *echinus* mutants have extra cells from each of these cell types. *ec* expression was detected in cone cells, primary pigment cells, and interommatidial cells at the time of cell death (30 hours APF) and afterwards (45 hours APF) (Fig 4). *In situ* hybridizations show the same expression pattern as the enhancer trap line (data not shown). Because *echinus* is expressed in the non-dying cone cells and primary pigment cells, it appears that *echinus* is not sufficient to induce death in the cells where it is expressed, but it is necessary for cell death as its absence prevents the loss of IOCs.

Structure-function analysis

Several mutant constructs of the *echinus* gene were made to determine which domains and amino acid residues were necessary for function. Of particular interest was the UCH domain, and in particular, the catalytic residues involved in ubiquitin C-hydrolysis. All *echinus* constructs were placed under the control of the GMR enhancer and tested for their ability to rescue the loss-of-function allele $ec^{P\ lacZ}$.



Genotype	Avg. Num. of IOC
WT	9
ec$^{P\ lacZ}$	14.1 ± 2.0
ec$^{P\ lacZ}$; GMR-ec	10.2 ± 1.5
ec$^{P\ lacZ}$; GMR-ec (-polyQ)	10.6 ± 0.9
ec$^{P\ lacZ}$; GMR-ec (UCH)	13.3 ± 1.4
ec$^{P\ lacZ}$; yUbp2	11.7 ± 1.2
ec$^{P\ lacZ}$; GMR-USP53	13.3 ± 1.5

Figure 5. Structure-function analysis of Echinus. A-F show pupal eye discs at 42 hr APF. Cell borders are visualized with anti-Dlg antibody. (A) *echinus* mutant (B) *echinus* mutant rescued by GMR-ec. (C) *echinus* mutant rescued by GMR-ec (-polyQ). (D) GMR-ec (UCH) fails to rescue *echinus*. (E) GMR-yUbp2 partially rescues *echinus*. (F) GMR-USP53, which overexpresses the UCH domain of USP53, fails to rescue *echinus*. (G) Structure of genes used in A-F. UCH domains are red, while the Echinus poly-glutamine stretch is green. Top three constructs are *echinus*, fourth is the *S. cerevisiae* gene Ubp2, and fifth is the human USP53 gene. (H) IOC number were counted and averaged over at least six different pupal eye discs. Standard deviations are shown.

The poly-glutamine stretch does not appear to be important for Echinus function. GMR-ec transgenic flies were generated that contained the first 1389 amino acids and lacked the C-terminal poly-glutamine stretch. This construct, GMR-ec(-polyQ), rescued *echinus* mutants to the same degree as full-length GMR-*ec* clone (Fig. 5C). Rescued adult eyes are wildtype in appearance, with the ommatidia again in an

ordered array. The pupal eye discs occasionally show an extra IOC, at levels similar to those seen in the GMR-Ec rescue. In a wildtype background, GMR-ec(-polyQ) did not alter the adult eye morphology (data not shown). The UCH domain is not sufficient for rescue of *echinus* mutants. Transgenics of either the *echinus* UCH or the USP53 UCH domain, placed under the control of GMR, failed to rescue (Fig. 5D, 5F).

echinus acts upstream or independently of the core apoptotic machinery

echinus seems to be a pro-apoptotic gene, as recessive mutants show impaired apoptosis during pupal eye development. To test whether *echinus* is involved with the core components of the apoptosis machinery, crosses were made of *echinus* to the GMR driven pro-apoptotic genes. Specifically, either the recessive allele $ec^{P\ lacZ}$ or the GMR-ec construct was crossed to overexpressed *hid, grim, reaper*, *Dronc*, *Drice*, *Strica*, and *dcp-1*. Loss of *echinus* did not suppress any of these genes, suggesting that *echinus* acts upstream of these genes or is not directly involved in their activation (Fig 6). GMR-ec fails to activate apoptosis in the eye; the cell number is the same as that of wildtype. GMR-ec also did not enhance the death in response to the expression of the apoptotic machinery (Fig. 6). Crosses to other genes implicated in apoptosis, such as *morgue*, *ubcD1*, *hpo*, *wts*, and *sav*, did not change any of the phenotypes associated with *echinus*.

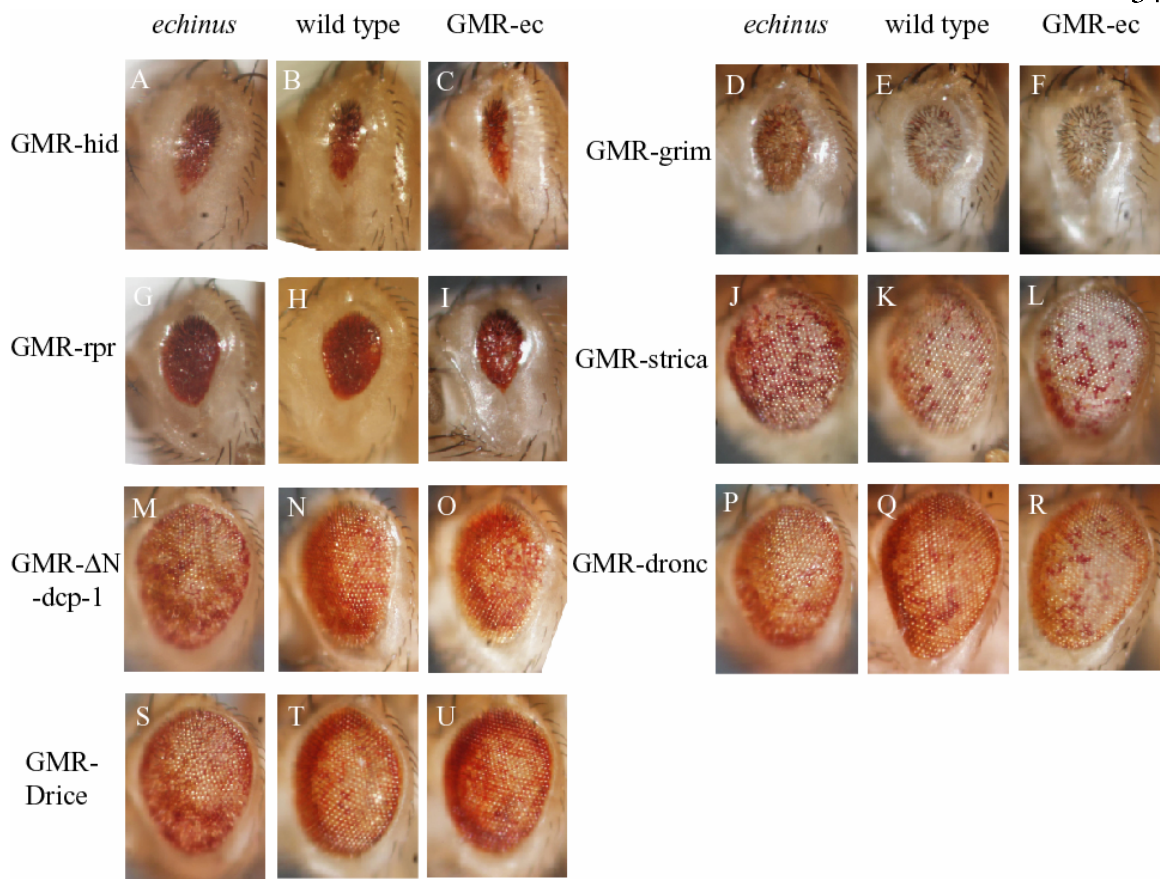


Figure 6. *echinus* acts upstream or independently of the core apoptotic machinery in *Drosophila*. *echinus* mutants (A, D, G, J, M, P, S), wildtype (B, E, H, K, N, Q, T) and GMR-*ec* (C, F, I, L, O, R, U) were crossed to GMR-*hid* (A-C), GMR-*grim* (D-F), or GMR-*rpr* (G-I). Overexpression of the caspases Strica (J-L), ΔN dcp-1 (M-O), Dronc (P-R), and Drice (S-U) were also tested.

2.5 Discussion

Echinus resembles a deubiquitinating enzyme, but lacks a catalytic cysteine

Mutants of *echinus*, produced by P-element mutagenesis or RNAi, have excess IOCs in the eye, while eye-specific expression of the CG2904 cDNA rescued the mutant echinus phenotype. Echinus looks like a deubiquitinating enzyme, except that it lacks

a critical catalytic cysteine residue. The UBP family of deubiquitinating enzymes is a loose organization of proteins united only by their UCH domain and ability to remove ubiquitin from protein conjugates. An *in vitro* assay in bacteria revealed that Echinus could not act as a deubiquitinating enzyme, much like its human orthologs USP53 and USP54. Structure-function analysis shows that a poly-glutamine stretch of the gene is not necessary for rescuing the mutant phenotypes, but the UCH domain is.

Predicting the correct active-site cysteine for Echinus is difficult. Sequence alignment of yeast deubiquitinating enzymes shows a rough consensus sequence surrounding the active site cysteine (GLV**N**MG**N**T**C**YM**N**SIL**Q**CL) (Wilkinson, 1997). The crystal structure of the deubiquitinating enzyme HAUSP shows that the surrounding asparagines and a following glutamine are important for activity (Hu, et al, 2002). Recently, additional human deubiquitinating enzymes were isolated and the alignment of the human DUBs revealed conservation of additional residues (Quesada, et al, 2004). Specifically, the active-site cysteine is always followed by a phenylalanine, tryptophan, or tyrosine. When Echinus was compared to its human orthologs, however, no cysteine within Echinus stood out as the catalytic residue. The best sequence alignments reveal a non-catalytic glycine (amino acid 52) in place of the catalytic cysteine.

Four other cysteines (C79, C84, C87 and C134) are potential alternative active-site residues. Cysteine 134 fits best within the criteria, as it is followed by a phenylalanine, and mutation of this residue prevents *echinus* from fully rescuing the

mutant alleles (data not shown). Cysteine 134, however, might be a part of the ubiquitin-binding region of *echinus*, based on similarity to the crystal structure of HAUSP. The general importance of this cysteine is also supported by the fact that the *echinus* orthologs share remarkable sequence similarity to cysteine 134 and the surrounding region. Thus, while the lack of rescue underscores the importance of this residue, it is not sufficient to conclude a catalytic role.

Whether Echinus is a deubiquitinating enzyme is still in question. Unlike the deubiquitinating enzyme *fat facets*, *echinus* loss-of-function mutants were not modified by heterozygous mutants of the proteosome or *ubcD1* (data not shown; Wu et al., 1999). Echinus, as well as the human orthologs USP53 and USP54 could not remove ubiquitin from a β-galactosidase reporter gene, though only the UCH domains of USP53 and USP54 were tested and this may have disrupted enzyme activity (Table 1; Quesada et al, 2004). The yeast gene Ulp1 has been shown to remove the ubiquitin-like gene Smt3 from protein conjugates, opening the possibility that Echinus encodes de-SUMOlytating or de-Neddylating enzymes (Li, 1999). Phenotypes of either the loss of *echinus* or its overexpression driven by GMR-gal4, however, were not modified by SUMO or Nedd8 mutants *Smt3*, *Cul1*, *Cul3*, and *Nedd8* (data not shown).

Several cDNAs have been isolated that overlap with *echinus* – however, in the opposite orientation. These cDNAs do not play role in Ec function. Eye-specific expression of RH68894, a representative of these cDNAs, has no phenotype and fails

to rescue the mutant phenotype. Furthermore, overexpression of the cDNA fails to enhance any of the adult eye phenotypes, suggesting that the cDNA doesn't act as an antisense inhibitor to *echinus*. No obvious gene or miRNA is predicted within this cDNA, making it unlikely that they play an important role in the echinus phenotype.

The role of *echinus* in apoptosis

Our results confirm that *echinus* plays a role in activating apoptosis in the developing *Drosophila* eye, though the context in which it acts is unresolved. This situation is quite similar to the genes *irregular chiasmC-roughest* (*irreC-rst*) and *nemo*. Like *echinus*, an *irreC-rst* mutant was isolated because it lacked pupal eye apoptosis (Wolff and Ready, 1991). *IrreC-rst* encodes a transmembrane protein of the immunoglobulin superfamily and the protein is localized to the cell membrane contacts (Ramos et al., 1993; Reiter et al., 1996). While *morgue*, an ubiquitin-conjugase-related protein modifies the *irreC-rst* mutant phenotype, and *Delta*/*Notch* perturb IrreC-rst protein localization, it is not clear how *IrreC-rst* acts to promote apoptosis or antagonize cell differentiation (Gorski et al., 2000; Hays et al., 2002). Viable *nemo* mutants have extra interommatidial cells and a decrease in apoptosis and in the developing eye (Mirkovic et al., 2002). *nemo* encodes a protein kinase, and homologues have been shown to be activated by dTak1, a component of the JNK signaling pathway (Choi and Benzer, 1994; Ishitani et al., 1999; Rocheleau et al., 1999). All three of these genes, *echinus*, *irreC-rst*, and *nemo*, remain somewhat mysterious.

Several signaling pathways and protein cascades have been shown to be mediated by ubiquitination. DIAP1 has been shown to protect cells by ubiquitinating the pro-apoptotic machinery (Wilson et al., 2002; Yan et al., 2004). However, our results show that *echinus* does not mediate apoptosis by inhibiting DIAP1's function. *echinus* mutants alleles fail to suppress ectopic expression of *hid*, *grim*, or *reaper*, and *echinus* overexpression fails to enhance these genes as well (Fig. 6). Furthermore, loss of *diap1* specifically in the eye induces cell death resulting in a small eye. Loss of *echinus* or its overexpression did not attenuate this small-eye phenotype (data not shown).

While *echinus* does not appear within the core components of the apoptotic machinery, it might act as an upstream activator. The *Notch* signaling pathway has been implicated in promoting apoptosis in the developing retina (Miller and Cagan, 1997). *neur*, LNX, sel-10, and Su(dx) encode E3 ligases and have been shown to mediate protein degradation of components of the Notch signaling pathway, establishing the role of ubiquitination within the *Notch* pathway (reviewed in Lai, 2002).

A potential role for *echinus* in the *EGFR/ Ras* pathway is also possible, but is more remote. The EGF receptor is known to promote differentiation of R cells, cone cells, and primary pigment cells. Ectopic expression of a dominant-negative version of the receptor suggests a role in IOC formation as well (Freeman, 1996). *D-cbl* and orthologs have been shown to mediate ubiquitin-dependent degradation of receptor

protein-tyrosine kinases (Miyake et al., 1998; Joazeiro et al., 1999). The possibility that the Egfr is a target of Echinus is slight as *echinus* mutants would lead to decreased Egfr levels and fewer cells in the developing pupal eye. Another possible target is the negative regulator Ttk88. Ttk88 protein levels have been shown to be mediated by the actions of either *ebi* or *phyl* and *sina* (Dong et al., 1999; Li et al., 1997; Tang et al., 1997). However, *echinus* loss-of-function and ectopic expression fail to modulate ectopic expression of Ttk88 (data not shown).

The search for such potential targets of Echinus is limited by the fact that the gene is specific to the eye. Enhancer trap staining using the ec^{PlacZ} alleles failed to stain embryos and imaginal discs (data not shown). Yeast 2-hybrid screens would be ineffective since the *Drosophila* libraries are generated from embryos, ovaries, and imaginal discs.

Determining the enzymatic target of Echinus and its role is important because little is known of how the apoptotic machinery is activated in a developmental context. Echinus potentially lies at the junction of several signaling pathways and the activation of apoptosis. We have ruled out several possibilities, and for now the puzzle is unsolved.

2.6 Conclusions

echinus is necessary for cell death of the interommatidial cells in the developing *Drosophila* retina. All *echinus* mutants examined have excess IOCs due to the lack of apoptosis. CG2904 encodes *echinus* as mutants for the CG2904 phenocopy *echinus* and its overexpression rescues the mutant phenotype. Echinus belongs to an inactive subclass of deubiquitinating enzymes; Echinus and its orthologs share homology to other deubiquitinating enzymes, though they lack catalytic activity. *In situs* show that *echinus* is expressed in both dying and non-dying pupal eye cells suggesting that *echinus* is not sufficient to drive apoptosis.

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2.8 Supplementary Figure

Genetic crosses of *echinus* with signaling pathway component involved in *Drosophila* eye development. – refers to change in dominant phenotype.

***echinus* crosses with echinus and RH68894**

	$ec^{P\ lacZ}$	GMR-ec	GMR-gal4, UAS-ec
ec^{1}	fails to complement		
$ec^{P\ lacZ}$		rescue	same phenotype as GMR-gal4, UAS-ec
GMR-RH68894	-	-	-
UAS-RH68894(RNAi)			-

***echinus* crosses with the genes involved in protein ubiquitination**

	$ec^{P(lacZ)}$	GMR-LP2	GMR-gal4, UAS-LP2
$morgue^{19}$	-		
$ubcD1^{\Delta 173}$	-	-	-
l(3)73Ai	-	-	-

***echinus* crosses with the apoptotic machinery**

	$ec^{P(lacZ)}$	GMR-LP2	GMR-gal4, UAS-LP2
GMR-Hid	-	-	-
GMR-lg Hid	-	-	-
GMR-Reaper	-	-	-
GMR-lg Reaper			-
GMR-Grim	-	-	-
GMR-lg Grim			-
GMR-Debcl	-	-	-
GMR-Dronc	-	-	-
GMR-Dream	-	-	-
GMR-ΔN Dcp1	-	-	-
GMR-Drice	-	-	-
GMR-P35	no enhance.		-
GMR-diap1	no enhance.		-
H99 Deficiency	-		-
th^5		-	-
GMR-gal4, UAS(RNAi)-diap1	-	-	

***echinus* crosses with the genes involved in the JNK pathway**

	$ec^{P(lacZ)}$	GMR-LP2	GMR-gal4, UAS-LP2
GMR-gal4, UAS-dTraf1		-	
GMR-Eiger	-		
GMR-dTak	-		
GMR-Hep	suppresses lethality no change in eye size		
hep^{r75} (hep fem)	-	-	-
GMR-Rac1	-		
$nemo^{j147-1}$	-	-	-
Jra^{IA109}	-	-	-
puc^{E69}			
bsk^{1}	-	-	-
GMR-gal4, UAS-bsk			
GMR-gal4, UAS-bsk^{dn}			-
msn^{102}	-		
GMR-gal4, UAS-msn			
peb^{EP55}	-	-	
peb^{1} (peb fem.)	-	-	

***echinus* crosses with the EGF pathway**

	ec$^{P(lacZ)}$	GMR-LP2	GMR-gal4, UAS-LP2
Egfr^{E1}	-	-	
Egfr^{f24}	-	-	-
GMR-gal4, UAS-Egfr^{dn}	-	-	-
argos^{W11}	-		-
Dsor1^{LH110}	-		
rl^{1}	-	-	-
rl^{SEM}	very slight enhancement in females	-	-
GMR-ras^{V12}	-	-	lethal
elav^{C155}	-		
GMR-gal4, UAS-ras^{N17} (on X)	slight suppression in ec/+ female	slight suppression	slight suppression
ebi^{k16213}			-
GMR-ttk	-	-	-

***echinus* crosses with the *Notch* pathway**

	$ec^{P(lacZ)}$	GMR-LP2	GMR-gal4, UAS-LP2
GMR-gal4, UAS-Dl	-	-	(combination of 2 phenotypes)
Dl^{RF}	-	-	-
DL^{6B}	-		
GMR-gal4, UAS-N^{ICN}	-	-	-
GMR-gal4, UAS-N^{dn}	stronger suppression in males	slight supp. more black in eye	pupal lethal
sev-N^{ICN}	-	-	
N^{fa-g}	- (in double het. females)	-	btw the 2 phenotypes; female = nothing
N^{spl-1}	-	-	-
$neur^{11}$	-	-	-

echinus* crosses with *hippo, salvador, warts

	$ec^{P(lacZ)}$	GMR-LP2	GMR-gal4, UAS-LP2
GMR-hpo	-	black spots in anterior region of eye	-
GMR-gal4, UAS-hpo	-		-
GMR-gal4, UAS-hpo(^{KD})	rougher eyes		same phenotype as GMR-gal4, UAS-ec
hpo^{MGH3}	-	-	-
hpo^{42-47}	-	-	-
GMR-sav		-	-
sav^{3}	-	-	-
GMR-wts	-	-	variable roughness

***echinus* crosses with genes involved in cell cycle regulation**

	$ec^{P(lacZ)}$	GMR-LP2	GMR-gal4, UAS-LP2
dap^{4}	-	-	
$CycE^{AR95}$	-	-	
stg			-