Characterization of *inxs*

a gene involved in programmed cell death in the developing *Drosophila* retina

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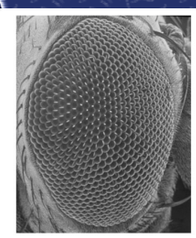
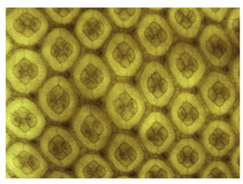
1. Abstract

Selective programmed cell death (PCD), or apoptosis, plays a critical role in controlling cell populations and in sculpting the shape of developing organs and tissues. In addition, PCD is implicated in disease pathogenesis and is associated with several human diseases including cancer, neurodegenerative disorders, AIDS, and autoimmunity. We are using the *Drosophila* retinal epithelium to study the molecular mechanisms of PCD during development. The retina consists of 750 identical repeating units called ommatidia. The ommatidia are initially separated by numerous interommatidial cells - some differentiate to become pigment cells and the excess cells undergo PCD. Inhibition of PCD leads to supernumerary cells between ommatidia and a consequent disruption of the normally precise ommatidial pattern. This disruption is evident as a rough eye phenotype in the adult. Using a genetic approach, we identified a new gene, *inxs* ("in excess"), involved in the retinal PCD process. Loss-of-function mutations in *inxs* demonstrate dominant enhancement of the rough eye phenotypes conferred by mutations in *irregular chiasm C-roughest* (*irreC-rst*) and *echinus* (*ec*), two genes implicated previously in retinal cell death. The *inxs* mutant phenotype on its own includes a rough eye in the adult and a cellular organization in the pupal retina similar to that observed in transgenic animals expressing the baculovirus caspase inhibitor p35. Acridine orange staining and TUNEL labelling confirmed that excess cells are due to a reduction in cell death. We have identified two additional alleles of *inxs*. However, flies homozygous for these alleles die prior to retinal PCD. In order to examine the retinal cell death pattern for these alleles, we used the FLP/FRT recombination system to induce somatic clones homozygous for *inxs*. The pattern of cell death in homozygous *inxs* embryos is also being investigated, using TUNEL labelling together with a marker to distinguish heterozygotes. We used deficiency mapping to localize *inxs* to polytene chromosome interval 64F, a region that does not correspond to previously characterized cell death genes in *Drosophila*. Using deficiency breakpoint mapping, we showed that *inxs* must lie in a 100 kb interval which contains 20 known or predicted genes. To identify *inxs* among the candidate transcripts, we are using sequencing and real-time RT-PCR. Given the highly conserved nature of PCD, these studies will contribute further insights into PCD regulation in other animals, including humans, and provide new avenues for investigation into this crucial process.

2. The *Drosophila* Eye is sculpted by PCD



Scanning EM of Adult *Drosophila* eye



Apical surface of pupal late stage retina
Stained with cobalt sulphide (labels membranes)

PCD organizes the interommatidial lattice by removing excess precursor cells

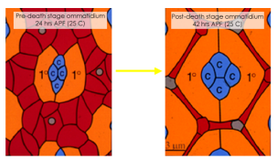
Blue: Cone cell

Orange: 1° pigment cell

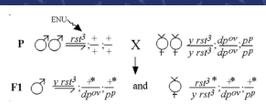
Red:

pre-death = interommatidial precursor cells

post-death = 2° or 3° pigment cells

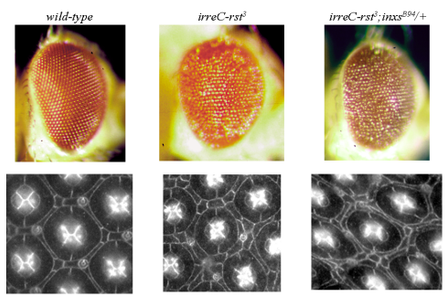


3. Isolation of *inxs*



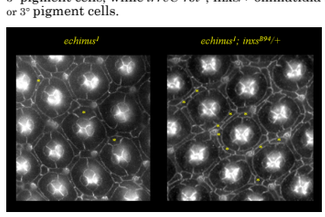
Two *inxs* alleles (designated $inxs^{B94}$ and $inxs^{P2}$) were isolated from a genetic screen for modifiers of *irreC-rst*^{3}, a gene required for normal retinal PCD. Following mapping of inxs (see below), a third allele ($inxs^3$) was identified through complementation analysis of stocks available from that region.

4. *inxs* is a dominant enhancer of *irreC-rst*^{3} and *echinus*



Top Adult eye phenotype

Bottom Apical surface of post-death stage pupal retina (fluorescent antibody staining). *irreC-rst*^{3} ommatidia demonstrate some excess 2° or 3° pigment cells, while *irreC-rst*^{3}; inxs/+ ommatidia show many excess 2° or 3° pigment cells.



Echinus is another gene required for retinal PCD. (Refer also to the poster by Bosdet et al.) Some excess cells are labelled with *

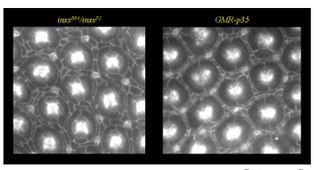
An enhancement of the mutant phenotype is observed in flies which also carry *inxs*

Cell membranes outlined by immunostaining with anti-Armadillo antibody

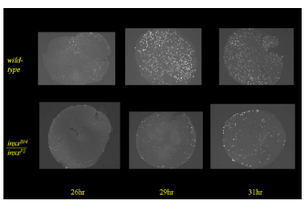
5. Phenotypes of *inxs* alleles

genotype	lethal stage	
$inxs^{P2}$ / $inxs^{P2}$ $inxs^{P2}$ / $inxs^{B94}$ $inxs^{P2}$ / $inxs^{3}$	pharate adult	
$inxs^{B94}$ / $inxs^{B94}$	late larva/early pupa	
$inxs^{B94}$ / $inxs^{3}$	larva	
$inxs^{3}$ / $inxs^{3}$	embryo	

Comparison of the lethal stage for flies homozygous or heteroallelic for the three alleles of inxs. The three alleles can be ranked for severity as follows: $inxs^{P2} < inxs^{B94} < inxs^{3}$

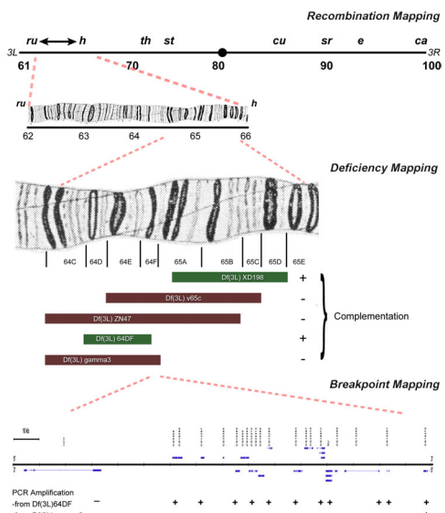


Comparison of pupal retinae from $inxs^{B94}$/ $inxs^{P2}$ and *GMR-p35* transgenic animals carrying the baculovirus inhibitor-of-apoptosis-protein,driven by a retina-specific promoter. Excess 2° or 3° pigment cells are apparent in both phenotypes.



Dissected Drosophila pupal retinae treated with TUNEL (TdT-mediated X-dUTP nick end labeling), showing peak cell death in wild type flies at 29hr and reduced levels of cell death in $inxs^{B94}$/ $inxs^{P2}$.

6. Genetic Mapping

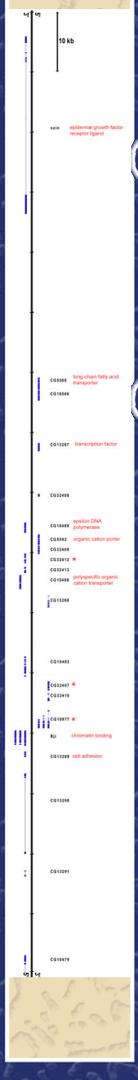


Recombination mapping, using a multiply-marked 3rd chromosome, showed that *inxs* lies between the markers ru and h. *inxs* was then tested over available deficiencies from the region and further localized to a region of approximately 100 kb. The boundaries of this region were determined using PCR amplification of gene-specific fragments from embryos homozygous for the deficiencies Df(3L)gamma3 and Df(3L)64DF.

There are approximately 20 genes in the candidate region. We have amplified genomic sequence for these genes from embryos homozygous for the most severe allele, *inxs*^{3}. Sequencing of these genes, using Mu transposon insertions into pooled genomic PCR products, with subsequent transfer into Gateway vectors, is in progress. (See the sidebar for details of the candidate region).

Real time quantitative PCR analysis of transcripts from the candidate region is also underway. Preliminary results show that three transcripts are found at lower levels in $inxs^{P2}$/$inxs^{3}$ pupae than in wild type. (marked with * in the sidebar).

Candidate Region



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