The *rhinoceros* Gene of Drosophila Restricts Cell Fate Specification in the Developing Eye

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Cell Fate Specification in the Developing Eye

By

Matthew G. Voas

Submitted to the Biology Department on February 3, 2003 in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Biology.

Inductive signaling between cells in the developing eye of Drosophila is very important for establishing the correct number and identity of cell fates. Among the signaling pathways that regulate cell fate determination in the developing eye, RTK/Ras/MAPK is among the most important. One effect of RTK/Ras/MAPK signaling is to downregulate the transcription factor Yan, thus allowing differentiation. A previous genetic screen identified modifiers of Yan\textsuperscript{ACT}, a constitutively active isoform of Yan. From this screen, two alleles of Enhancer of Yan\textsuperscript{ACT}, 3-5 (\textit{EY3-5}) were identified as enhancers. A genetic screen was conducted to isolate new \textit{EY3-5} alleles for genetic and molecular characterization. The conclusion is reached that the defining phenotype of \textit{EY3-5} is a multigenic effect caused by large deletions present in the two founding alleles of \textit{EY3-5}.

These studies led to the investigation of the roles of three genes in signal transduction. The strongest enhancer of Yan\textsuperscript{ACT} among these three genes, called hidden clones, appears to be necessary for growth and is involved in early cell fate decisions in the eye. Also described here is the role of the rhinoceros (\textit{rno}) gene in regulating eye cell fates. Mutation of \textit{rno} causes the overproduction of eye cell fates and inhibits apoptosis. These phenotypes are similar to those seen when EGFR is hyperactivated in \textit{argos} mutants. Tests between \textit{argos} and \textit{rno} alleles show a strong genetic interaction. Furthermore, \textit{rno} mutant tissue shows reduced production of Argos ligand. These data suggest a role for \textit{rno} in the inhibition of EGFR by regulating expression of \textit{argos}. Alleles of \textit{rno} also display a general delay in the expression of differentiation markers in photoreceptors and cone cells. The \textit{rno} gene encodes a nuclear, PHD zinc finger protein, implying that it functions as a transcription factor. Lastly, described here is the genetic analysis of \textit{nur}301, that encodes the largest subunit of the Nucleosome Remodeling Factor complex. In \textit{nur}301 mutants, heat shock transcription and expression of homeotic genes is impaired. Lastly, blood cells are overproduced in \textit{nur}301 larvae. This phenotype and genetic interactions suggest a role for the NURF complex as an antagonist of the JAK/STAT signaling pathway.

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Chapter 1

Introduction:

Ras, Notch and Prepatterened Determinants Specify Cell Fates in the Drosophila Eye
I. An overview of eye development

The Drosophila eye is a highly complex epithelial tissue composed of many neural and non-neural cell types. On a gross anatomical level, one can immediately appreciate the structural beauty of the eye. Several hundred subunits, called ommatidia, occur in a reiterated hexagonal pattern of amazing regularity (Figure 1.1A). This honeycomb-like arrangement is but an intimation of the underlying cellular architecture. At higher magnification, observation of the constituent photoreceptors, cone cells and pigment cells of the eye reveals a mosaic that repeats itself with stunning precision (Figure 1.1, 1.2). This organization belies the need for extensive communication between cells during eye development. Extensive research has demonstrated that almost every known Drosophila signaling pathway, such as Hedgehog (Hh), Wingless (Wg), Decapentaplegic (Dpp), JAK-STAT, Notch and Ras, is necessary to construct a normal eye. In the process of understanding how the Drosophila developmental program makes an eye, researchers have simultaneously learned about cell-cell communication.

A. Allocation of eye tissue

The presumptive eye and antennal tissues of the fly are referred to as the eye-antennal imaginal discs. The eye-antennal discs are allocated late in embryogenesis as two bilateral groups of approximately 20 cells each that express the retinal determination genes eyeless (ey), twin of eyeless (toy) and eyegone (eyg) (34, 61, 84). During the larval stages, each eye-antennal disc is composed of two parallel epithelial layers joined at the edges. The columnar epithelium that gives rise to the adult eye and antennal tissue is known as the disc proper, while the peripodial epithelium (or peripodium) is a squamous epithelium that plays important roles in the development of the disc proper (28, 62, 63). Little is known about eye-antennal disc development before the second instar larval stage except that cellular growth and proliferation is
continuous. Beginning in the second larval instar stage, cells become restricted to either antennal or eye fates (from here on, the eye component of the eye-antennal disc will be referred to as the eye disc). This process utilizes the Notch and Ras signaling pathways to modify the activity of the retinal determination genes, with Notch signaling promoting eye specification and Ras signaling antagonizing it (90).

B. Dorsal/ventral patterning of the eye

Division of the eye imaginal disc into dorsal and ventral compartments can be visualized as early as the second larval instar (28). Expression of Iroquois Complex genes in the dorsal half of the eye restricts fringe expression to the ventral half of the eye (42, 108, 172). This patterning depends upon Wg expressed at the dorsal margin (68) and expression of the JAK/STAT pathway ligand, Unpaired (Upd) near the optic stalk (175). As a result of this compartmentalization, the Notch receptor becomes activated along the dorsal/ventral (D/V) boundary (29, 42, 121). Evidence suggests that this activation of Notch is partly dependent upon signaling ligands produced by the peripodium. For instance, Hh in the peripodium is necessary to restrict expression of the Notch ligand, Serrate (Ser), along the D/V boundary of the disc proper (28). In the third instar larval eye disc, Wg secreted from the dorsal and ventral edges combines with Upd secreted from the D/V midline to pattern ommatidial orientation (163, 175). These complementary Wg and Upd gradients are believed to regulate production of an unknown diffusible ligand that is most concentrated at the D/V midline (163, 175). In turn, this hypothetical ligand is thought to activate the Frizzled receptor, creating a gradient of Frizzled activation that imparts polarity by differential activation of Notch within the ommatidium (30, 48, 177).
C. The initiation of cellular differentiation

Cellular differentiation does not begin simultaneously throughout the eye disc. Rather, it initiates at the posterior edge during the middle of the third instar stage and sweeps across the eye disc towards the anterior edge. This wave of differentiation is observable as an apical constriction of the epithelium, and thus it is called the morphogenetic furrow (MF, (125). The MF begins at the posterior extreme of the D/V midline through the combined activities of the Notch, Ras, Hh, Dpp and Wg pathways (recently reviewed (73, 98). The genetic events that then propagate the MF are essentially a chain reaction of differentiation. The Hh ligand diffuses from differentiating photoreceptors in an anterior direction to activate expression of \textit{dpp} and the proneural gene \textit{atonal} immediately anterior to the MF (41, 64, 71, 106). Secreted Dpp diffuses further ahead of the MF and induces cell cycle arrest in G1 (75). Dpp signaling also establishes a “pre-proneural state” (64). The pre-proneural state is characterized by expression of Hairy, a basic-Helix-Loop-Helix (bHLH) transcription factor, and Emc, an HLH protein without the DNA-binding basic region. In the absence of Hairy and Emc, differentiation in the eye occurs much faster than normal (19). Hairy slows neural differentiation by repressing transcription of \textit{atonal}, which encodes another bHLH protein (19, 64). On the other hand, Emc appears to antagonize Atonal by competing for binding to the bHLH protein Daughterless (Da), because Atonal normally activates transcription as a heterodimer with Da (22, 81, 157). Near the dorsal and ventral edges of the MF, Wg also holds the MF in check by preventing transcription of Daughterless (23, 105, 153). As mentioned above, Hh relieves \textit{atonal} repression just anterior to the MF. Hh also induces much stronger expression from \textit{atonal} by stimulating production of the Notch ligand Delta. Activation of Notch leads to enhanced \textit{atonal} transcription and
downregulation of *hairy* and *emc*. This step is essential for neurogenesis in the eye. Loss of Notch function at this timepoint completely prevents differentiation in the eye (9, 103).

**D. Selecting the first photoreceptor**

As the MF moves forward, changes in the Atonal expression pattern reflect the two-step process that specifies the R8 photoreceptor (Figure 1.3). The first step is the restriction of Atonal to clusters of about twelve cells, referred to as the proneural clusters (PNCs, 82). The high level of Atonal expression in the PNCs results from the activation of Notch within the MF during “proneural enhancement,” as described above. More uncertain is how *atonic* expression is downregulated between the PNCs. The PNCs are staggered relative to newly born ommatidia, reflecting the ultimate honeycomb arrangement of ommatidia in the eye. This suggests that a secreted ligand from new ommatidia radiates posteriorly to block *atonic* expression between the PNCs. So far, such a ligand has not been conclusively identified (77).

After proneural enhancement, the next phase of Atonal pattern refinement is Notch-mediated lateral inhibition within each PNC (2, 5, 7). The lateral inhibition mechanism that selects R8 cells amongst the eye disc epithelium is essentially the same as the one that selects neuroblasts in the embryonic epidermis. Initially, Notch and its ligand are expressed at consistent levels throughout the epithelium. Changes in a cell’s ligand concentration, either by random fluctuation or intrinsic bias, lead to unequal Notch activation within the epithelium. These differences are reinforced by a feedback system that increases ligand production in cells with low Notch activation while suppressing ligand production in cells with high Notch activation. Eventually, neural fate is assigned evenly throughout the epithelium. In the case of the eye,
exclusion of Notch signaling from a presumptive R8 cell can be seen by the gradual loss of Atonal from cells of a PNC as it matures (82). If Notch is somehow inhibited during this process, then the opposite is true, resulting in a massive increase in neural marker expression (25, 103, 122).

It is interesting to note that over a very short developmental time period, Notch pathway activation produces opposite results. Within the MF, during proneural enhancement, Notch strongly upregulates atonal expression. Just posterior to the MF, during lateral inhibition in the PNC, Notch eliminates atonal expression from all cells but one. Such dramatic differences in Notch mutant phenotypes, in this case from absence of R8 cells to extra R8 cells, is a common theme in the analysis of Notch during ommatidial assembly. This behavior perfectly demonstrates that timing and context play very important roles in the way a cell interprets a signal during development.

**E. R8 initiates ommatidial assembly**

Each newly specified R8 cell behaves as a founder cell for one ommatidium by initiating the sequential recruitment of other cells (3, 97) (Figure 1.3). From detailed morphological studies, the exact time at which each cell joins has been established (24, 149, 166). Shortly after the R8 cell is specified, photoreceptors R2 and R5 are added, followed by R3 and R4 to form the five-cell precluster. At this point, all undetermined cells complete a coordinated S-phase, and then undergo mitosis. This coordinated cell cycle is referred to as the “second mitotic wave” and it is the last round of cell division for the vast majority of eye cells. Following the second mitotic wave, the R1 and R6 photoreceptors are recruited, followed by R7.
After the photoreceptors are specified, non-neural cell types join the ommatidium. First the cone cells are added, followed by the primary pigment cells, then secondary and tertiary pigment cells (1°, 2° and 3° PCs). Finally, unused cells are eliminated by apoptosis, at which point the fly has completed about half of pupal development. The anterior to posterior movement of the MF, combined with the gradual assembly of the ommatidia, results in a gradient of maturity in the late third instar larval eye disc. Cells anterior to the morphogenetic furrow are growing and dividing. In the region of the MF, cells arrest at G1 and expression of Atonal is refined to single cells. Posterior to this, ommatidia can be observed in various stages of completion, with the most mature residing along the posterior edge. Thus, in one moment of eye disc development, a broad range of stages can be observed.

II. The mechanisms of cell fate specification

An early attempt to explain the mechanism of eye cell fate specification in another insect, the ant, hypothesizes that the eight photoreceptors of the ommatidium are clonally descended from one precursor cell that divides three times to produce the necessary cells (13). According to this model, the lineage and time of birth of a given cell would determine its fate. Recent research has shown that a similar mechanism is indeed used to assign fates to daughter cells of a dividing neural stem cell in Drosophila (80). However, the generation of marked clonal lineages early in eye development has conclusively demonstrated that the eight photoreceptors of the Drosophila ommatidium are not necessarily clonally derived (125). Instead, all available evidence supports a model whereby cell fates of the eye are specified by inductive interactions between neighbors. The next part of this introduction will review the literature that helps explain how the
ommatidium is assembled, taking as a developmental starting point events that occur after the R8 cell is specified. Attention will be focused on how these events are governed by cell-cell signaling, particularly signaling via the Ras and Notch pathways.

A. The RTK/Ras/MAPK pathway

Signaling through the Ras GTPase is integrated with a number of pathways (33, 159). "The Ras pathway," as it is called here, refers to the Receptor Tyrosine Kinase (RTK)/Ras/Mitogen Activated Protein Kinase (MAPK) signaling pathway (Figure 1.4). The important RTKs for eye development are the Drosophila Epidermal Growth Factor Receptor (EGFR) and Sevenless (Sev). EGFR is required for all stages of eye development, so it will be discussed here in detail. Sev has the very specific task of inducing the R7 cell fate (150). Other well-studied RTKs in Drosophila include Torso and the Fibroblast Growth Factor (FGF) Receptor homologs Breathless, and Heartless (140, 165, 176). EGFR has a number of extracellular ligands, all of which include an EGF domain (137). Gurken is similar to mammalian TGF-α and interacts with EGFR in the egg chamber during oogenesis (116). Vein is a neuregulin-like ligand that is used for patterning of the pupal wing (134).

The most widely used ligand for EGFR is Spitz, which also resembles TGF-α, a known ligand for the vertebrate EGFR (133). Spitz is used in a vast number of developmental processes, from embryogenesis to adulthood, including virtually every stage of eye development (55, 137). Expression of Spitz is ubiquitous, but it cannot function as a ligand in the absence of Star and Rhomboid proteins (136). Star is necessary for extracellular transport of Spitz, while Rhomboid proteins, of which there are at least seven in Drosophila, proteolytically remove Spitz's
transmembrane domain, allowing it to diffuse (99, 155, 162). Recently, a second Spitz-like ligand has been reported called Keren (128, 156). Structurally, Keren is very similar to Spitz and can activate the receptor in vivo, but currently its genetic functions are unknown. Another EGFR ligand is Argos, which is the only known naturally occurring inhibitory ligand for an RTK (135). Developmentally, Argos is used in many, if not all, of the same times and places as Spitz (26, 46, 59, 141, 161). This tandem ensures restricted local activation of EGFR.

Upon binding to a ligand, RTKs dimerize, allowing the cytoplasmic tails to transphosphorylate each other (RTK activation reviewed in (78). This phosphorylation provides a binding surface for downstream proteins via a Phospho-Tyrosine Binding (PTB) or Sarc Homology 2 (SH2) domain (138). For EGFR, the most important direct interactor is Drk, an SH2-SH3 adaptor protein. Downstream of receptor kinase(Drk) also binds to the Guanine Nucleotide Exchange Factor, Son of Sevenless (Sos) (120, 132, 139). When Drk binds the phospho-tyrosine of EGFR, it allows Sos to activate membrane-tethered GDP-Ras by stimulating the exchange of GDP for GTP (16, 85). Activated GTP-Ras in turn recruits Raf, a MAPKKK, to the plasma membrane, which sets off the MAPK cascade (20, 100, 142). Raf phosphorylates MEK (a MAPKK, (38, 94), which then phosphorylates ERK (MAPK, 63). The activated form of MAPK then translocates into the nucleus, where it phosphorylates several protein targets, including the ETS DNA binding domain transcription factors, PointedP2 (PntP2) and Yan (21, 117). Phosphorylation of PntP2 increases its ability to activate transcription, while phosphorylation of Yan targets it for proteolysis (117, 127). PntP2 and Yan are known to bind to the same DNA sequence, the ETS Binding Site (117). The net result of MAPK phosphorylation of these two proteins is that downstream transcriptional targets that had been repressed by Yan are activated
by PntP2 (117). In combination with other extracellular signals and the differentiation state of
the receiving cell, an appropriate transcriptional response is generated.

1. After R8, EGFR is required for all eye cell fates

EGFR is essential for embryonic development long before the major events of ommatidial
assembly take place. This early requirement presents an obstacle for genetic analysis of Egfr in
the adult eye. To circumvent this problem, studies of Egfr loss of function have used
temperature sensitive alleles, expression of a dominant negative isoform (EGFR\textsuperscript{DN}), or
generation of homozygous null eye tissue in an otherwise heterozygous animal by stimulating
mitotic recombination with the FLP/FRT system. These experiments have revealed that EGFR
stimulates proliferation in undifferentiated cells (171), specifies cells to antennal vs. eye fate
(90), helps initiate (but not propagate) the MF (91), helps determine the spacing of R8 founder
cells (8), specifies multiple cell fates (57), and suppresses apoptosis posterior to the MF (43).
The last two roles, specifying multiple cell fates and suppressing apoptosis, will be the primary
focus of this discussion of ommatidial assembly.

By following expression of neural antigens in a developing third instar larval eye disc, one can
observe the sequential addition of photoreceptors to the ommatidium. In eye clones that are
homozygous for a null allele of Egfr, R8 photoreceptors express the markers Atonal, Boss and
ELAV normally (4, 8, 43). Thus, specification of R8 founder cells is unaffected. Similar results
are seen by expression of EGFR\textsuperscript{DN}, or by shifting a temperature sensitive allele to the non-
permissive temperature (57, 92). However, loss of Egfr function impairs the later steps of
ommatidial assembly. By following expression of neural antigens like ELAV and HRP, one
finds that none of the other photoreceptors are added to the ommatidium (4, 8, 43, 171). The
same is true of the non-neural cell types as well. EGFR\textsuperscript{DN} can interfere with the recruitment of
cone cells and all three types of pigment cells if it is expressed ubiquitously at the later
timepoints when these cells normally join the ommatidium (57).

The gain of function analysis of Egfr is perfectly complimentary to the loss of function analysis.
Transgenic expression of a constitutively activated isoform of EGFR (EGFR\textsuperscript{ACT}) results in the
overproduction of all cell types in the ommatidium, except R8 (57). The exact time of
expression also proves to be an important determinant for the types of cells produced.
Expression of EGFR\textsuperscript{ACT} during late larval stages produces additional photoreceptors and cone
cells, while early and mid-pupal expression produces extra primary and 2\textsuperscript{nd}/3\textsuperscript{rd} P.C.s, respectively
(57). Genetic analysis of two important EGFR ligands, Spitz and Argos, are consistent with
these data. Eye clones that are null for spitz almost exactly phenocopy Egfr null eye clones
(147). Conversely, overexpression of a secreted (and thus, activated) form of Spitz results in the
overproduction of all retinal cell types (except R8) in the same time-dependent manner as
EGFR\textsuperscript{ACT} (57). Argos, on the other hand, functions as an inhibitory ligand for EGFR. Loss of
arginos function in the eye phenocopies expression of EGFR\textsuperscript{ACT} while transgenic overexpression of
arginos phenocopies Egfr null clones (56, 59).

2. The source of cell fate specificity

These data have led to a model of ommatidial assembly whereby stimulation of EGFR at specific
timepoints produces a particular cell fate. First the R8 founder cell is specified by a mechanism
independent of EGFR. Next, R8 activates EGFR in neighboring cells by producing its ligand,
Spitz. The first two cells to be stimulated in this way are the precursors for R2 and R5. Together, these three R cells activate EGFR in the presumptive R3 and R4 cells. Next, precursors for R1 and R6 are stimulated the same way, followed, in order by the precursors for R7, the cone cells and three types of pigment cells. Consistent with this picture of sequential EGFR activation is the observation that expression of Star and Rhomboid is first observed in R8, then in R2, R5 and R8 (58, 69, 145). Thus, the production of active Spitz ligand occurs sequentially, as might be required to cause sequential activation of EGFR.

By this model of cell fate specification in the ommatidium, the response of an undifferentiated precursor cell to EGFR depends upon the time point of activation. Early stimulation generates R cells while late stimulation produces pigment cells. The ability of undifferentiated cells to respond differently to the same signal indicates that the there must exist some form of code that combines EGFR signaling with other factors to specify all the necessary cell types (55, 67, 89, 115). Since the larval eye imaginal disc contains a gradient of ommatidial maturity, perhaps the particular factors that make up the code are expressed at different positions relative to the MF. Using this logic, researchers have constructed combinatorial models that account for the specification of each cell type in the eye, and these will be discussed further on.

3. Does EGFR do more than prevent apoptosis?

The realization that the EGFR signal is permissive rather than truly instructive raises the possibility that perhaps the only requirement for EGFR during ommatidial assembly is to prevent apoptosis. It is well established that EGFR signaling inhibits apoptosis in the eye. First, no cells from Egfr null clones survive to adulthood, presumably because these clones undergo
extensive programmed cell death soon after the MF has passed (43, 171). Also, temperature sensitive inactivation of Egfr in pupae increases apoptosis in the eye, apparently because the choice between 2⁰/3⁰ PC fate or apoptosis in the mid-pupal retina hinges upon EGFR activation (174). Thus the EGFR ligand Spitz serves as a survival signal that is available in limited quantity. A similar function for Spitz has been seen in midline glial cells during embryogenesis. In this case, Spitz made by the midline glia acts as a trophic factor for neighboring CNS neurons (12). A crucial link between EGFR and apoptosis is the pro-apoptotic gene hid. EGFR activation inactivates Hid by MAPK phosphorylation and by transcriptional repression of the hid locus (11, 93).

In order to dissect the Egfr null eye phenotype, it is necessary to separate cell death from differentiation. This analysis can be performed using the bacculovirus caspase inhibitor, p35. When expressed in the developing eye, p35 almost completely prevents cell death (66). The resulting adult flies show excess pigment cells, but normal numbers of photoreceptors (except on the periphery of the eye, where incomplete ommatidia are usually removed by apoptosis). The same result is seen in eye tissue that is mutant for the cell death gene hid, or by simultaneous loss of the cell death genes hid, reaper and grim (93, 158). This outcome makes sense, because the majority of programmed cell death in the eye occurs during the stages when pigment cells are being specified. If an undifferentiated cell is not told to become a photoreceptor or cone cell and is not told to die, it becomes a pigment cell. In contrast to these survival phenotypes in which only pigment cells are overproduced, quite different phenotypes are seen when EGFR is ectopically activated. Expression of EGFR<sup>ACT</sup> or loss of argos function in the larval eye causes overproduction of all eye cell types, including photoreceptors. Clearly, the extra R cells received
some cell fate instruction from EGFR, or they would have become pigment cells. Also, when
Egfr null clones are made in a p35-expressing eye, R8 cells differentiate normally, but the other
photoreceptor types do not(4, 8). In these Egfr eye clones cell death is completely blocked by
p35, but this is not enough to allow normal differentiation. This outcome clearly demonstrates
that in addition to blocking programmed cell death, EGFR is necessary to provide cell fate
instructions.

4. Downstream transcriptional activation: the case of prospero

From the data described so far, the general model of Ras pathway function in the developing eye
is as follows. An RTK activates Ras and MAPK, leading to changes in the transcriptional profile
of the stimulated cell mediated by the transcription factors PntP2 and Yan (among others). The
same pathway is used repeatedly for all cell types, so specificity is generated by the presence of
other transcription factors in undifferentiated cell. A study by Xu et al (169) set out to test these
assumptions.

They chose as a model the prospero (pros) gene. Pros is a transcription factor required in the
eye for the proper axonal projection of the R7 photoreceptor. Prospero is expressed more
strongly in R7 than the cone cells. This difference might reflect a difference in RTK activation
because R7 is the only photoreceptor in the eye that requires two RTKs (Sev and EGFR) for
differentiation. R7 and the cone cells compose the "R7 equivalence group," so called because
cone cells are readily transformed into ectopic R7s by an increase in Ras pathway activation.
Hyperactivation of Ras in presumptive cone cells increases Pros expression. Conversely, loss of
Sev reduces Pros expression in the presumptive R7. From these data, it appears that the higher
level of Pros expression normally seen in R7 is the result of higher levels of Ras pathway activation. Thus, the pros locus should be a good model for gene activation downstream of RTKs in the eye.

A regulatory region of the pros locus was identified that can recapitulate the endogenous expression pattern when fused upstream of a lacZ reporter. This pros-lacZ expression shows the expected reduction in expression in the presence of a constitutively active Yan, Yan^{ACT}, or EGFR^{DN}. Likewise, broad expression of EGFR^{ACT} expands reporter expression. Two experiments demonstrate that these effects are mediated by PntP2 and Yan. First, both PntP2 and Yan can bind to several ETS Binding Sites (EBSs) within the pros enhancer in vitro. Second, mutation of two EBSs in the pros enhancer obliterate reporter expression in the eye, even in the presence of EGFR^{ACT}. The ability of PntP2 and Yan to bind to the same EBSs in the pros enhancer also provides support for the hypothesis that PntP2 and Yan compete for binding upstream of target genes.

As mentioned above, all eye cells are stimulated by EGFR at some point. So, from where comes the cell-specific expression of pros? In mutants of lozenge (lz), Pros expression is not activated. Lz is a Runt domain transcription factor expressed in undifferentiated cells, R1, 6, 7, cone and pigment cells. Two Lz binding sites were found in the pros enhancer. Lz can bind specifically to these sites in the presence of a necessary co-factor in vitro, and mutation of these sites abolishes in vitro Lz binding and in vivo reporter expression. Furthermore, broad expression of EGFR^{ACT} results in ectopic Pros expression wherever Lz is expressed. Thus, Lz is capable of providing a spatially restricted signal that allows cell specific expression of an RTK target gene.
This study confirms a model of target gene activation that previously had not been tested for a specific endogenous target. It also demonstrates how cell-type specific expression can be achieved with a signal that is reused.

E. The elegance of the Notch pathway

Compared to the RTK/Ras/MAPK pathway, the Notch pathway is surprisingly simple in its molecular composition (Figure 1.5). There are two stimulatory ligands for Notch in Drosophila: Delta and Serrate (49, 126). These ligands have transmembrane domains, and thus are thought to remain tethered to the plasma membrane. However, there is some evidence that the extracellular domain of Delta may be proteolytically liberated from the transmembrane domain, allowing limited diffusion (124). The Notch extracellular domain contains 36 EGF-like repeats (164). Only repeats 11 and 12 are necessary for interaction with Delta and Serrate (126). Upon ligand binding, Notch is cleaved in its transmembrane domain by a Presenilin proteolytic complex (144, 173). This cleavage allows the Notch Intracellular Domain (NICD) to translocate to the nucleus and interact with the DNA-binding transcription factor, Suppressor of Hairless, (Su(H), (52, 143).

In the absence of NICD, Su(H) can act as a transcriptional repressor, but the NICD/Su(H) complex functions as an activator (10). The transcription factor Mastermind (Mam) can also associate with this complex and is required for the Notch pathway’s inhibition of neuroblast fate in the embryo (168, 170). No phenotype has been observed in eye clones of a mam hypomorphic allele, despite the presence of mam transcript anterior to the MF (103). Perhaps Mam functions redundantly with another transcription factor, or is not utilized by the pathway at all in the eye.
Transcription of the *Enhancer of split* (*E(spl)*) locus is a common response to Notch activation (83). *E(spl)* is a complex locus that encodes several basic Helix-Loop-Helix DNA binding transcription factors (37). E(spl) proteins physically associate with the transcriptional co-repressor Groucho to block expression of proneural genes, such as the genes of the *Achaete-Scute Complex* and the eye proneural gene *atonal* during the lateral inhibition step of R8 photoreceptor selection (72, 103, 118). In this way, the Notch pathway restricts neural cell fate.

1. **Notch has multiple roles in ommatidial assembly**

Like EGFR, the Notch receptor is used throughout Drosophila eye development, and its function in and around the MF has been intensely studied, as described above. Less is known about Notch’s function once R8 has been established. Perhaps this is because Notch null eye clones that lack R8 cells never initiate the ommatidial assembly process (6, 103). For this reason, studies of Notch function posterior to the MF have relied upon genetic tricks to avoid interfering with the R8 selection process. Two studies, one which used a temperature sensitive (t.s.) allele of Notch (25), and another which used a combination of t.s. Delta alleles (122), produced remarkably similar results (Table 1.1). In both studies, t.s. inactivation of the pathway immediately posterior to the MF resulted in the massive overproduction of neurons in the larval eye disc and excess cone cells in the larval and pupal discs. These defects manifest themselves in adults as a scar that contains highly disorganized ommatidia. Interestingly, in ommatidia that are approximately seven ommatidial rows posterior to the scarred region (and thus, approximately 14 hrs more mature), inactivation of Notch or Delta results in the loss of R7 as well as one or more cone cells. Similar bi-phasic influences on cell fate were seen for other cell types (Table 1.1).
From these experiments, it is obvious that the Notch pathway, like the EGFR pathway, is used reiteratively throughout the process of ommatidial assembly. Among Notch’s many roles in ommatidial assembly, only some have been studied in detail. The following section will discuss how Notch helps determine the fates of R7, the cone cells and the pigment cells.

2. Notch and R7

The t.s. experiments described above establish that loss of Notch or Delta can lead to an R7-less phenotype. In these eyes, R7 becomes transformed into an R1/R6 cell type because there is an additional nucleus that expresses the R1/R6 marker, BarH1 (151). Conversely, constitutive activation of the Notch pathway leads to ectopic R7 cells and the loss of BarH1 nuclei (31, 53, 151). Thus, it appears that when R1/R6 and then R7 join the ommatidium, one way that they are distinguished is by activation of Notch in the presumptive R7. Notch activation leads to repression of BarH1 in R7, steering it away from the R1/R6 fate. The source of Delta ligand in this case is R1 and R6 (151). Perhaps their slightly earlier integration into the ommatidium explains why they activate Notch in R7, but do not themselves receive sufficient Notch activation to become R7s. Activation of the Ras/MAPK cascade by two RTKs, EGFR and Sevenless, is also essential for assigning R7 cell fate (57, 150). It is unclear if EGFR/Sevenless and Notch work sequentially or in parallel in this process.

Flores et al (51) claim that Notch is not normally activated in the presumptive R7, and that such activation should transform R7 into a cone cell. However, such a conclusion ignores the presence of ectopic R7s in eyes that express NotchACT (53). Subsequent work has shown that the
presumptive R7 is not transformed in Notch\textsuperscript{ACT} eyes. Furthermore, newly recruited R7 cells in wild type eyes express \textit{E(spl)m\delta}, demonstrating that Notch is indeed activated in these cells (31). This point will become important later when the relevance of Notch signaling in specifying R7 is discussed.

3. Notch and cone cells

The role of Notch signaling in cone cell development is explored in more detail by Flores \textit{et al} (51). This study shows that the reduction of Notch signaling results in loss of the cone cell marker, D-Pax2. This outcome can be observed in larval eye discs a number of ways: by looking at \textit{Notch\textsuperscript{iz}} alleles, mild \textit{Delta} alleles, overexpression of the pathway antagonist Hairy, or overexpression of dominant negative Notch or Delta. In this case, the source of ligand is the R cells, where Delta is produced in response to EGFR activation (154). The link between Notch activation and cone cell expression of \textit{D-Pax2} is direct, because Su(H) can bind to a \textit{D-Pax2} control element (the \textit{sparkling} minimal enhancer, or SME) in \textit{vitro}, and cone cell-specific transcriptional control by the SME requires functional Su(H) binding sites.

Likewise, they show that the EGFR pathway is also necessary for cone cell expression of D-Pax2. Temperature sensitive inactivation of EGFR, expression of EGFR\textsuperscript{DN}, expression Yan\textsuperscript{ACT}, and loss of \textit{pointed} function all lead to the loss of D-Pax2 expression in the larval eye disc. Yan and PntP2 both bind to ETS Binding Sites within the SME in \textit{vitro}, and mutation of these sites eliminates cone cell expression of the SME. Finally, Lz is also required for cone cell expression of D-Pax2. This is demonstrated by loss of D-Pax2 expression in \textit{lz} mutant eye discs, by the
ability of Lz protein to bind to the SME in vitro, and by the necessity of Lz binding sites for cone cell expression of the SME.

Altogether, these data appear to illustrate the combinatorial input required for cone cell specification. A model is offered stating that cone cell precursors are exposed to a unique combination of factors: RTK activation, Notch activation, and the presence of Lz. However, the assumption that regulation of D-Pax2 per se is the determining step in cone cell fate is not entirely accurate. In the $spa^{pol}$ mutation of D-Pax2, the entire SME and two early coding exons are deleted (60). In homozygous $spa^{pol}$ mutants, expression of the cone cell marker Cut is completely lost in the larval eye disc, but recovers to above-normal levels in pupal eye. Morphologically, the number of cone cells in pupal eyes are only mildly reduced in $spa^{pol}$ mutants (60). While Lz significantly alters the fate of cone cells, the relatively low penetrance of the $spa^{pol}$ mutant suggests that additional factors may work redundantly with Lz, or that $spa^{pol}$ is not a complete loss of function.

Flores et al conclude that cone cells are specified by the combined activities of EGFR, Notch and Lz. However, this same combination appears to be necessary for R7 cells (31, 151, 169). If both R7 and cone cells require Lz, Notch and Ras, then what other factors distinguish their fates? The most likely explanation is the use of two RTKs by R7. The importance of using two RTKs, and the mechanism that distinguishes between activation of one or two RTKs are discussed below.
4. Notch mediates the choice between pigment cell and apoptotic fates

Another closely studied example of Notch function during ommatidial assembly is the role of Notch in deciding between 2\textsuperscript{\textdegree}3\textsuperscript{o} PC fate or programmed cell death. In the larval eye disc, Notch is expressed ubiquitously posterior to the MF, but later in pupal development, Notch expression becomes restricted to the undifferentiated cells that surround the nearly mature ommatidium (inter-ommatidial cells, or IOCs)\textsuperscript{(88)}. Alleles of Notch that specifically reduce expression in the IOCs (known as facets mutants, or Notch\textsuperscript{\textasciitilde}) lack 1\textsuperscript{o} PCs and have a vast excess of 2\textsuperscript{\textdegree}/3\textsuperscript{o} PCs \textsuperscript{(25)}. This result can be phenocopied by t.s. inactivation of Notch at mid-pupal stages (Table 1.1). Not surprisingly, the overproduction of 2\textsuperscript{\textdegree}/3\textsuperscript{o} PCs results in a corresponding loss of apoptosis in the IOCs of Notch\textsuperscript{\textasciitilde} pupal eye discs \textsuperscript{(174)}. Thus, Notch appears to function upstream of a cell death decision in the pupal retina.

At first it was believed that the loss of 1\textsuperscript{o} PCs in developing Notch\textsuperscript{\textasciitilde} eyes somehow relieved the IOCs from a death signal. To test this hypothesis, 1\textsuperscript{o} PCs and cone cells were precisely laser ablated from a large region of the pupal eye \textsuperscript{(109)}. In fact, the exact opposite result occurred: loss of the cone and 1\textsuperscript{o} PCs results in a reduction in 2\textsuperscript{\textdegree}/3\textsuperscript{o} PCs, and this effect was more severe in the middle of an ablated region than on the edges. From this outcome, it was concluded that the cone cells and 1\textsuperscript{o} PCs actually provide a survival signal to the IOCs, allowing some of them to differentiate as 2\textsuperscript{\textdegree}/3\textsuperscript{o} PCs. Thus, the absence of 1\textsuperscript{o} PCs in Notch\textsuperscript{\textasciitilde} mutants does not actually cause the cell death defect. Other observations suggest that the hypothesized survival signal could be secreted Spitz. For example, Spitz is expressed in the pupal cone cells, t.s. inactivation of EGFR in pupal eyes increases apoptosis, and expression of EGFR\textsuperscript{\textasciitilde} or activated Ras (Ras\textsuperscript{\textasciitilde\textsuperscript{V12}}) can
induce ectopic 2º/3º PCs (109, 174). In this light, perhaps the role of Notch in the IOCs is to antagonize the EGFR pathway.

In this case, loss of Notch activity in the IOCs would lead to hyperactivation of EGFR and suppression of apoptosis. Genetic epistasis shows that the Egfr⁺⁺ ectopic cell death phenotype is epistatic to the Notch⁻⁻ suppressed cell death phenotype (174). Thus, Notch acts upstream or in parallel to Egfr in the IOCs. A possible molecular link between the Notch and EGFR pathways in the pupal retina is the transmembrane immunoglobulin superfamily protein, Irregular chiasma C-roughest (IrreC-rst).

5. IrreC-rst: determinant of death

As in Notch, loss of irreC-rst function results in strong suppression of apoptosis in the pupal retina (167). In the wild-type pupal eye, the IOCs organize themselves into single-file rows between the maturing ommatidia. In irreC-rst mutants, this transition does not occur, with the result that some IOCs never form contacts with the 1º PCs of the ommatidia (129). Presumably, these contacts are important for specifying secondary/tertiary pigment cell vs. apoptotic fates, but no correlation has yet been drawn between position in the IOC rows and fate. The IrreC-rst protein localizes to the plasma membrane in IOCs in the early pupal eye. This localization starts out fairly evenly distributed throughout the plasma membranes of IOCs, but by the time the single-file rows form, IrreC-rst has become concentrated at the junctions between IOCs and the 1º PCs (129). This re-localization depends upon Notch pathway activation and is not an artefact of suppressed apoptosis. Thus, IrreC-rst may be a molecular link between the Notch and EGFR
pathways in the retina. The next step is to demonstrate that the concentration of IrreC-rst to the IOC/1º PC junction ultimately antagonizes the EGFR pathway.

6. Notch vs. Ras

a. Notch downregulates RTK ligand production

In specifying apoptotic fate in the pupal retina, Notch appears to inhibit RTK signaling. Antagonism between Notch and RTK/Ras/MAPK pathways has been observed in a number of developmental contexts in Drosophila. Generally speaking, RTK/Ras/MAPK tends to promote the adoption of a specific fate, such as a myoblast founder cell or a fusion cell at the tip of a developing tracheal branch, while Notch restricts this fate by preventing differentiation and/or promoting a competing cell fate (Table 1.2). While the molecular details of this relationship will vary depending upon the context, some general trends can be noted. First is that Notch tends to inhibit production of RTK ligands, either directly or indirectly. For example, in the developing trachea, Notch activation leads to transcriptional repression of branchless, which encodes and FGF-like ligand (79). In other developing tissues, such as embryonic muscle, the pupal wing and the pupal thorax, Notch inhibits transcription of rhomboid, thereby indirectly blocking production of active, secreted Spitz (26, 32, 36).

b. Notch deactivates MAPK

Based on the tendency of Notch to prevent RTK ligand production, it is not surprising that loss of Notch or Delta function can lead to an increase in activated MAPK, while expression of Notch\textsuperscript{ACT} reduces MAPK activation (26, 79). However, there is also evidence to suggest that Notch can more directly interfere with the activation state of MAPK. In C. elegans, the RTK
LET-23 promotes anchor cell fate in the vulva by activation of MPK-1, a MAPK homolog. This function is opposed by signaling through the Notch homolog, LIN-12 because loss of lin-12 function results in ectopic anchor cells (160). LIN-12 antagonizes the LET-23 pathway by activating lip-1, which encodes a MAPK phosphatase. LIP-1 phosphatase in turn dephosphorylates MAPK to its inactive state (14). This example demonstrates that Notch antagonism of Ras is evolutionarily conserved. Furthermore, this specific mechanism may also be at work in Drosophila because loss of Notch function in the developing eye increases MAPK activation in proneural clusters (113). Efforts are currently underway to determine if this effect on MAPK is mediated by the Drosophila lip-1 homolog, mkph (76).

c. Notch activates yan?

Another molecular link between Notch and RTK/Ras/MAPK may be the transcriptional regulation of yan. Loss of Su(H) or Notch function in the larval eye disc results in loss of yan expression from undifferentiated progenitor cells posterior to the MF (131). An enhancer element upstream of the yan locus was identified that recapitulates the wild type expression pattern posterior to the furrow, and in vitro assays show that Su(H) can bind to multiple sites within this enhancer. However, the effects of genetic perturbation on the yan enhancer's expression pattern prove difficult to predict, and suggest complex regulatory mechanisms (131).

It should be noted that all cells within a Su(H) eye clone begin to differentiate as neurons, and most of these are R8 photoreceptors (103, 104). It may be possible that the loss of Yan in Su(H) eye clones is merely a secondary consequence of these extra R8 cells rather than a primary phenotype. It seems unlikely that production of excess R8s is a result of yan downregulation.
because there is no increase in R8 cells in yan hypomorphic eye discs or in yan null clones (96, 130). Likewise, Egfr null clones presumably have elevated levels of Yan, yet no effect upon the number of R8 cells is seen (8, 43). Thus, loss of Yan in Su(H) eye clones may not indicate direct regulation of the yan locus.

d. Feedback: RTK/Ras/MAPK upregulates Delta

Interestingly, loss of the RTK ligands Spitz, Vein and Breathless results in a local reduction in Delta expression in various developmental contexts (36, 79). Conversely, activation of the RTK/Ras/MAPK pathway increases Delta production (26, 79). In this light, perhaps activation of the Ras pathway is one way in which asymmetry is generated in a group of otherwise equivalent cells. According to the theory of lateral inhibition, upregulation of Delta in a cell increases the likelihood that this cell will differentiate, while at the same time maintaining the undifferentiated state in neighboring cells. Thus, by increasing Delta production, RTK signaling can influence the lateral inhibition mechanism, and the resulting local Notch activation can serve as a negative feedback loop, helping to ensure that the effects of RTK ligand expression are restricted.

An example of this circuitry can be seen in the eye, where EGFR stimulation of presumptive photoreceptors induces production of Delta. Delta in turn acts in parallel to EGFR in the specification of cone cells (154). In this example, Notch most likely prevents presumptive cone cells from becoming photoreceptors. Thus, the mechanism for restricting photoreceptors also serves as part of the specification signal for another essential cell type. The coupling of these
two pathways may be an evolutionarily conserved cassette for regulating cell fate because a similar series of events is used in C. elegans to assign fates in the vulva (160).

III. The Combinatorial Code

In theory, Ras and Notch can provide some of the required information to specify eye cell fates, but the constant use of both pathways indicates that these are generalized signals. Ras activation induces all eye cell types except R8, so it acts as a permissive signal rather than an instructive one. Notch can both induce and prevent multiple fates, so it does not appear to be truly instructive either. Somehow cells already know the appropriate response when stimulated by a particular combination of permissive signals. Based upon these conclusions, researchers have proposed that the exact response of undifferentiated cells to a particular combination of permissive signals changes over time (55, 67, 89, 115). If this is true, then there must be distinct “competence zones” which occur posterior to the MF. It seems likely that these changes in cellular competence are governed by the expression of different transcription factors.

A. The importance of prepatterns

Several transcription factors are expressed broadly posterior to the MF. Early expression patterns that encompass undifferentiated cells will be referred to here as “prepatterns.” Many prepatterned proteins are necessary for cell fate determination. For example, the Glass transcription factor is required in all photoreceptors because many genes need Glass for expression posterior to the MF (45, 112, 114, 152). While Glass is an important determinant of eye cell fates, its non-specific function does not explain how specific cell fates arise. In order to narrow the competence of undifferentiated cells, a given factor must be expressed in a subset of
undifferentiated cells. This division is an incremental step towards generating the more complex patterns necessary to specify multiple cell fates. Thus, of the many prepatterns of cell fate determinants in the eye, the most informative are those that generate asymmetry in the pool of undifferentiated cells. Overlap of prepatterns and inductive signals in the developing eye should explain how all the cell fates of the eye are derived. This section will summarize the available data in an attempt to reconstruct the combinatorial code of signals and prepatterns that specifies each eye cell fate (Tables 1.3 and 1.4).

B. Photoreceptor R8

As described above, R8 founder cells are selected by refinement of an Atonal prepattern. As Atonal expression is gradually lost from cells of the PNC, there is a complementary increase in expression of the homeobox protein, Rough (40). In rough mutants, resolution of the Atonal expression pattern by lateral inhibition is delayed and incomplete, resulting in the production of 2-3 R8 cells per ommatidium (70). Conversely, heat-shock induced overexpression of rough completely prevents the initiation of Atonal expression (40). These data demonstrate that Rough is a negative regulator of Atonal. The failure of lateral inhibition in rough mutants also suggests that rough expression is activated by Notch. Consistent with this hypothesis, inactivation of a Notch allele reduces the Rough expression domain. However, the Atonal and Rough patterns never overlap, so Rough retains its ability to repress atonal in the absence of activated Notch (40). Therefore, Notch does not post-translationally activate Rough. In summary, a Notch-mediated lateral inhibition mechanism is used to refine prepatterns of Atonal and Rough. The result is evenly spaced Atonal-expressing R8s in a field of Rough-positive cells.
C. Photoreceptors R2 and R5

Subsequent production of secreted Spitz by R8 and expression of Rough in cells other than R8 serve as the determinants for photoreceptors R2 and R5. In rough mutant eyes, presumptive R2 and R5 photoreceptors express a marker normally seen in R1,3,4 and 6 (70). These rough mutants also show non-cell autonomous defects in ommatidial assembly, presumably because R2 and R5 are important sources for secreted Spitz (148). Not far behind the MF, strong Rough expression is maintained only in R2 and R5, and weak expression can be seen in R3 and R4 (87). Thus, Rough is the key to establishing R2 and R5 cell fates.

D. Photoreceptors R3 and R4

Unlike R2 and R5, loss of rough does not affect R3 and R4 in a cell autonomous fashion. The nuclear hormone receptor Seven-up (Svp) is necessary to maintain R3 and R4 (110), butsvp is not expressed in undifferentiated cells, so it can’t be a critical determinant for R3 and R4. It is known that signaling through the transmembrane receptor Frizzled lies upstream of Notch pathway signaling between R3 and R4, but these events occur after the R3/R4 fates have already been specified (47). Perhaps the only source of R3/R4 specificity is the presentation of secreted Spitz by R2 and R5.

E. Photoreceptors R1 and R6

Lz is initially expressed posterior to the MF in all undifferentiated nuclei with the exception of those cells that give rise to the five-cell precluster. After the second mitotic wave, newly differentiated cells maintain Lz expression, where it participates in establishing cell type specific transcriptional programs (50, 51). For the presumptive R1 and R6, Lz is necessary for BarH1
expression (35). Genetic mosaic analysis shows that BarH1 and BarH2 are necessary for R1, R6 and 1º P.C. fates (74), therefore Lz acts as a determinant for R1 and R6. Paradoxically, Lz is known to repress expression of svp when misexpressed in R3 and R4 (35), but Lz and svp are both expressed in R1 and R6 (50, 110).

Another determinant for R1 and R6 may be Dachsund (Dac), which is expressed in undifferentiated cells until ommatidial row 15, and briefly maintained in R1, 6, 7 and cone cells (67, 107). Overexpression of Dac can induce ectopic expression of a svp-lacZ enhancer trap. Loss of svp-lacZ expression is seen in dac, eyes absent (eya) double mutant clones, but not in clones of either separately (67). Eya is expressed strongly posterior to the MF (17), so if it acts redundantly with Dac, some other factor is necessary to confine Eya’s activation of svp-lacZ to the appropriate region posterior to the MF. Perhaps Dac activates expression of svp in R1 and R6, despite the presence of Lz.

F. Photoreceptor R7

The R7 photoreceptor fate requires inductive signals from EGFR, Sev, and Notch. As described above, activation of Notch prevents R7 from assuming the R1/R6 fate. EGFR and Sev are both RTKs upstream of Ras, so they are expected to have the same effects. One important function of RTKs in the presumptive R7 is to activate an E3 Ligase complex composed of Seven in absentia (Sina), Phyllodop (Phyl) and Ebi (18, 44, 101, 102, 146). This E3 complex ubiquinates Tramtrack88 (Ttk88), a transcriptional repressor, targeting it for proteolysis. Without Sev, Ttk88 is stabilized and prevents neural differentiation, causing the presumptive R7 to become a cone cell. Ttk88 is expressed in all undifferentiated cells posterior to the MF, so presumably R1 and
R6 need to downregulate Ttk88 as well (95). This conclusion is supported by the observation that in phyI null eye clones, R1, 6 and 7 become cone cells (27, 39). Why is it that R7 needs two RTKs to accomplish a task that R1 and R6 perform with only one? Perhaps the need for Notch pathway activation in R7 has the side effect of reducing RTK/Ras/MAPK activity, possibly by transcriptional activation of a MAPK phosphatase as seen in C. elegans. An alternative explanation is that Notch signaling upregulates Ttk88. Notch is already known to upregulate both Ttk88 and Ttk69 isoforms in the developing PNS in three ways: by activating transcription of ttk, by stimulating translation of ttk message, and by repressing transcription of phyI (65, 119, 123). Thus, the necessity of using Notch to specify the R7 fate may result in high concentrations of Ttk88 in the presumptive R7. As a consequence, two RTKs are necessary to downregulate this anti-neural factor.

R7 also requires Lz to prevent expression of svp, and to upregulate prospero. In the absence of Lz, Svp transforms the presumptive R7 into an “outer” photoreceptor type, i.e., R1-R6 (35). It seems likely that these extra outer photoreceptors are of the R3/R4 fate because the lack of Lz should prevent them from expressing BarH1 (R1/R6 fate), and it seems unlikely that Rough would become activated so late in ommatidial assembly to allow a transformation to R2/R5.

G. Cone cells

As discussed above, Lz is also necessary for the specification of cone cells, and for the expression of cone cell markers like D-Pax2 and Cut. Cone cells are members of the R7 equivalence group, that is, they are readily transformed into R7 cells when the Ras pathway is inappropriately activated (54). Conversely, loss of the Sev RTK transforms R7 into a cone cell.
(150). A critical difference between R7 and cone cells is that the later maintain Ttk88 expression (95). Loss of ttk function is sufficient to transform cone cells to R7 (96). Thus, Ttk88 stability is the critical readout of varying degrees of Ras pathway activation that distinguishes cone cell fate from R7 fate.

The Notch pathway is also required for cone cell specification (25, 122, 154). Part of this function is related to the transcription of differentiation factors like D-Pax2 (51). As noted above, activation of Notch may also affect Ttk88 levels. This possibility seems likely for cone cells because they express Ttk88 at higher levels than undifferentiated cells (158). This observation implies that some factor involved in the specification of cone cells upregulates Ttk88.

**H. Pigment Cells and Apoptosis**

There have been fewer efforts to uncover the mechanisms of pigment cell fate determination. Like all other cell types in the eye, PCs need EGFR, and Notch is specifically required for 1º PC fate (24, 57). There are no published studies of ttk's role in pigment cell specification. Based upon the broad expression of Ttk88 in undifferentiated cells, perhaps Ttk88 and/or another factor prevent presumptive PCs from becoming neurons. Although it is known that Lz is expressed in presumptive pigment cells, there are no descriptions of PC fates in lz mutants (50). Presumably, 1º PCs require Lz to activate expression of D-Pax2 and BarH1, because Lz is necessary to activate these genes in cone cells and R1/R6 cells, respectively (35, 51). In turn, D-Pax2 is also necessary for BarH1 expression in 1º PC. However, D-Pax2 does not help to specify 1º PC fate because it is not expressed in undifferentiated cells (60). D-Pax2 mutants have fewer 2º and 3º
PC fates, but it is not expressed in these cells, so this is most likely a non-cell autonomous effect stemming from the loss of 1º PCs (109).

Another potential determinant for pigment cells is the Homeodomain transcription factor, Homothorax (Hth). Hth is only expressed in the most mature undifferentiated cells in the larval eye disc, so it is ideally positioned to influence cell fate (15). However, there are no descriptions of pigment cell development in hth mutant tissue. Finally, as described above, apoptotic fate results from Notch activation, lack of secreted Spitz ligand, and a prepattern of IrreC-rst protein in the undifferentiated IOCs. In this context, Notch acts as a death signal by its inhibitory effects on the Ras pathway. IrreC-rst may represent the molecular link between the Notch pathway and Ras in the context. Possibly, this connection is mediated by reorganization of IOC cell membranes (129).

Conclusions
A combination of inductive signals and prepatterned expression of cell fate determinants are the basic components used to establish all cell fates in the developing Drosophila eye. Before a specific cell fate is assigned to a cell, it progresses through a number of earlier stages. In the second instar larval eye disc, cells are allocated to the antennal or eye cell fates (90). Anterior to the furrow, Dpp initiates the pre-proneural state (64), then the proneural state follows (82). Eventually, cells end up posterior to the MF where a specific fate is assigned. In general, Ras activation promotes acquisition of a cell fate (55). The specific cell fate that Ras promotes is determined by the timing of the this permissive signal. All undifferentiated cells appear to cycle through a number of fates for which they are competent to become when stimulated. The key to
keeping all cells from differentiating all at once appears to be the strict production of Spitz ligand coupled with the production of an inhibitory signal, Argos (58, 59, 69).

A considerable amount is known about the prepatterns necessary to specify cell fates in the eye. The importance of prepatterns of Atonal, Rough and Lozenge has been well studied. In the case of Lozenge, the cell fates it promotes include all those born after the second mitotic wave (50). Diversity is generated within this prepattern by the use of the Notch pathway. For example, presumptive R1, R6 and R7 are very similar in their developmental potential. What distinguishes them is the activation of Notch in R7 (31, 151). R7 is the only photoreceptor in which Notch activation is necessary for cell fate specification. As noted earlier, the connection between Notch and expression of the neural inhibitor Ttk88 may pose an obstacle in the acquisition of neural fate by R7. Perhaps this explains the need for activation of two RTKs in R7. Notch is also necessary for the specification of cone cells, 1\degree pigment cells and 2\degree/3\degree pigment cells (25). Activation of EGFR and prepatterned expression of Lz are also necessary determinants for all these fates (50, 57), but very little is known about the determinants that specify any one non-neural fate vs. the other. It is interesting that Notch is necessary to specify all the non-neural cell fates in the eye. This requirement seems like another example of restriction of neural cell fate by Notch, evolutionarily adapted for the specific needs of making an eye.
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Table 1.1: Eye phenotypes induced in Notch and Delta t.s. mutants

<table>
<thead>
<tr>
<th>Notch t.s. inactivation</th>
<th>Delta t.s. inactivation</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Near MF*</td>
<td>Near MF*</td>
<td>Many ectopic R cells and cone cells, “scarred” eye in adult at position of MF during non-permissive incubation.</td>
</tr>
<tr>
<td>= ommatidial column 7*</td>
<td>= ommatidial column 7*</td>
<td>Loss of R7, loss of one or more cone cells per ommatidium.</td>
</tr>
<tr>
<td>6-16 hr APF† (8-21 hr)**</td>
<td>11-18 hr APF</td>
<td>Loss of 2° and 3° P.C.s</td>
</tr>
<tr>
<td>20-28 hr APF (27-37 hr)**</td>
<td>34-44 hr APF</td>
<td>One extra 1° P.C. per ommatidium.</td>
</tr>
<tr>
<td>28-36 hr APF (37-48 hr)**</td>
<td>&gt;40 hr APF</td>
<td>Loss of all 1° P.C.s, many ectopic 2° P.C.s. In Delta mutants, 1° P.C.s are unaffected and there is a slight increase in 2° P.C.s.</td>
</tr>
</tbody>
</table>

*These rows describe phenotypes produced by t.s. inactivation in wandering 3° instar larvae.
†APF = After Puparium Formation.
**Pupae from the Notch experiments were grown at 20° C while those from the Delta experiment were grown at 18° C, both were shifted to a non-permissive temperature of 32° C. The numbers in parentheses are the projected ages of the Notch pupae if they had been grown at 18° C, based upon Fig. 8.27 from Ashburner, 1989.
<table>
<thead>
<tr>
<th>Context</th>
<th>Cell fate promoted by RTK/Ras</th>
<th>Notch function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trachea</td>
<td>Fusion cells (tip of tracheal branch)</td>
<td>Restricts fusion cell fate</td>
<td>79</td>
</tr>
<tr>
<td>Muscle</td>
<td>Myoblast founder</td>
<td>Specifies fusion-competent cells</td>
<td>26</td>
</tr>
<tr>
<td>Eye-Antenna</td>
<td>Antennal</td>
<td>Specifies eye fate</td>
<td>90</td>
</tr>
<tr>
<td>Wing</td>
<td>Vein</td>
<td>Specifies vein boundary</td>
<td>36</td>
</tr>
<tr>
<td>Notum bristles</td>
<td>Sensory mother cell (SMC)</td>
<td>Restricts SMC fate</td>
<td>32</td>
</tr>
<tr>
<td>Chordotonal organ</td>
<td>Sensory organ precursor (SOP)</td>
<td>Restricts SOP fate</td>
<td>178</td>
</tr>
<tr>
<td>Eye</td>
<td>Secondary/tertiary pigment cell</td>
<td>Apoptosis</td>
<td>109, 174</td>
</tr>
</tbody>
</table>
Table 1.3: Examples of prepatterned gene expression in the larval third instar eye disc

<table>
<thead>
<tr>
<th>Cell Fate Determinant</th>
<th>Expression pattern in larval third instar eye disc</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>Undifferentiated cells</strong></td>
<td><strong>Specified cells</strong></td>
</tr>
<tr>
<td>Atonal</td>
<td>Anterior and within MF, proneural clusters</td>
<td>Briefly in R8</td>
</tr>
<tr>
<td>Dachshund</td>
<td>From anterior of MF to ommatidial row 15</td>
<td>Briefly in R1, 6, 7, cone cells</td>
</tr>
<tr>
<td>Eya</td>
<td>From anterior of MF to posterior edge of eye disc</td>
<td>Cone and pig. cells</td>
</tr>
<tr>
<td>Glass</td>
<td>All cells posterior to MF</td>
<td>Photoreceptors, pig. cells</td>
</tr>
<tr>
<td>Lozenge</td>
<td>All cells posterior to MF except R2, 3, 4, 5, 8</td>
<td>R1, 6, 7, cone and pig. cells</td>
</tr>
<tr>
<td>Rough</td>
<td>All non-Atonal cells until ≈ommatidial row 3</td>
<td>R2, 5, weakly in R3, 4</td>
</tr>
<tr>
<td>Ttk88</td>
<td>All cells posterior to MF</td>
<td>Cone cells</td>
</tr>
<tr>
<td>Homothorax</td>
<td>Posterior edge of larval eye disc</td>
<td>Not determined</td>
</tr>
<tr>
<td>IrreC-rst</td>
<td>All cells posterior to MF</td>
<td>Briefly in all specified cells</td>
</tr>
</tbody>
</table>
Table 1.4: A combinatorial code for the specification of all eye cell fates

<table>
<thead>
<tr>
<th>Cell fate</th>
<th>Signals</th>
<th>Prepatterened determinants *</th>
<th>Factors necessary for cell type specific differentiation**</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>R8</td>
<td>lack of Notch</td>
<td>Atonal</td>
<td>Atonal, Spalt</td>
<td>81, 111</td>
</tr>
<tr>
<td>R2, 5</td>
<td>EGFR</td>
<td>Rough</td>
<td>Rough</td>
<td>148, 87</td>
</tr>
<tr>
<td>R3, 4</td>
<td>EGFR</td>
<td>Dachshund?</td>
<td>Svp</td>
<td>67, 110</td>
</tr>
<tr>
<td>R1,6</td>
<td>EGFR</td>
<td>Lozenge</td>
<td>Lozenge, Svp, BarH1,2</td>
<td>35, 50, 110</td>
</tr>
<tr>
<td>R7</td>
<td>EGFR, Sev, Notch</td>
<td>Lozenge</td>
<td>Lozenge, Prospero, Spalt</td>
<td>35, 111, 86</td>
</tr>
<tr>
<td>Cone cells</td>
<td>EGFR, Notch</td>
<td>Lozenge, Ttk88</td>
<td>Lozenge, D-Pax2, Cut, Ttk88</td>
<td>50, 51, 95</td>
</tr>
<tr>
<td>1º P.C.</td>
<td>EGFR, Notch</td>
<td>Lozenge?, Ttk88?, Hth?</td>
<td>Lozenge, BarH1, D-Pax2, Hth?</td>
<td>50, 51, 95</td>
</tr>
<tr>
<td>Apoptosis</td>
<td>Notch</td>
<td>IrreC-rst, Hth?</td>
<td>IrreC-rst</td>
<td>129, 15</td>
</tr>
</tbody>
</table>

*A prepatterened determinant is a protein that is expressed in undifferentiated cells when the indicated cell type is specified. Mutation of the determinant must also negatively affect the cell type in question. This list only includes those determinants whose expression pattern is somewhat restricted, and thus can account for the generation of different types of cells in the eye.

**These factors are necessary for the differentiation that is unique to the indicated cell type, but not necessarily for the specification of that cell type.
Figure 1.1: The organization of the Drosophila eye. A. An scanning electron micrograph of an adult eye. The hexagonal arrangement of ommatidia allows these subunits to be precisely packed together in a honeycomb like arrangement. B. The organization of photoreceptors for an ommatidium. The dark circles are the light sensing organelles of the photoreceptors, called rhabdomeres. There is one rhabdomere per photoreceptor and they are arranged in a stereotyped pattern. The identities of the photoreceptors, R1-R7 are indicated. The larger rhabdomeres belong to the outer photoreceptors, R1-R6. The inner photoreceptors, R7 and R8, have smaller rhabdomeres. The rhabdomere for R8 is not visible in this plane of section. Pigment granules from the pigment cells form a hexagonal arrangement around the photoreceptors.
Figure 1.2: The organization of pigment cells and cone cells in the ommatidium. These accessory cells provide structural support for the ommatidium. Lying above the photoreceptors are the cell bodies of the two primary (1°) pigment cells and the four cone cells (C). These cells secrete the material that makes up the lens. On the periphery of the ommatidium are the secondary (2°) and tertiary (3°) pigment cells, and the bristle cells (B).
Figure 1.3: Ommatidial assembly in the larval third instar eye imaginal disc.
The eye imaginal disc is drawn here in cartoon form. The position of the morphogenetic furrow (MF) is indicated by an arrow. The furrow moves in a posterior to anterior direction. The region of the disc anterior to the furrow is undifferentiated, while the region of the disc posterior to the furrow is undergoing the ommatidial assembly process. Anterior to the furrow, broad expression of the proneural gene product, Atonal is seen (green). Within the furrow, the broad expression gives way to the evenly spaced proneural clusters. These proneural clusters gradually resolve themselves into evenly spaced, single cells. Note that new proneural clusters are staggered relative to older, more resolved clusters. After single Atonal-positive R8 cells are established, the photoreceptors are added sequentially, as indicated by the enlarged drawing of photoreceptor nuclei to the right (blue). After R8, the photoreceptors are added in pairs: R2 and R5, R3 and R4, R1 and R6. Lastly, the R7 photoreceptor is added, and then the cone cells (yellow).
Figure 1.4: The RTK/Ras/MAPK pathway. Shown here is the RTK EGFR. Upon binding of to Spitz, EGFR dimerizes and transphosphorylates. This event leads to Drk binding of EGFR, thus allowing Sos to stimulate the exchange of GDP for GTP on Ras. Activated GTP-Ras recruits Raf to the plasma membrane, setting off the MAPK cascade. The activated form of MAPK translocates into the nucleus, where it phosphorylates the ETS DNA binding domain transcription factors, PntP2 and Yan. Phosphorylation of PntP2 increases its ability to activate transcription, while phosphorylation of Yan targets it for proteolysis. PntP2 and Yan are known to bind to the same DNA sequence, the ETS Binding Site. The net result of MAPK phosphorylation of these two proteins is that downstream transcriptional targets that had been repressed by Yan are activated by PntP2.
Figure 1.5: The Drosophila Notch signalling pathway. Upon binding Delta, Notch is cleaved in its transmembrane domain. This cleavage allows the Notch Intracellular Domain (NICD) to translocate to the nucleus and interact with the DNA-binding transcription factor, Su(H). In the absence of NICD, Su(H) can act as a transcriptional repressor, but the NICD/Su(H) complex functions as an activator. The transcription factor Mastermind (Mam) can also associate with this complex. Transcription of the Enhancer of split (E(spl)) locus is a common response to Notch activation. E(spl) proteins physically associate with the transcriptional co-repressor Grocho to block expression of proneural genes, such as the genes of the Achaete-Scute Complex and the eye proneural gene atonal during the lateral inhibition step of R8 photoreceptor selection. In this way, the Notch pathway restricts neural cell fate.
Chapter 2

Genetic Analysis of $\text{Enhancer of } Yan^{ACT} 3-5$

Reveals a Multigenic Phenotype
Abstract

The RTK/Ras/MAPK signaling pathway is an important regulator of cell fate determination in the developing eye (8). One effect of RTK/Ras/MAPK signaling is to downregulate the ETS DNA-binding domain transcription factor, Yan (21, 25). Downregulation of Yan is a general requirement for cellular differentiation in the eye (17). A previously reported genetic screen looked for mutations that dominantly modify a constitutively active form of Yan (Yan\textsuperscript{ACT}) in the developing eye (24). From this screen, two alleles of Enhancer of Yan\textsuperscript{ACT}, 3-5 (EY3-5) were identified as strong enhancers. Described here are efforts to characterize EY3-5 genetically and molecularly. The conclusion is reached that the defining phenotype of EY3-5, i.e., the ability to enhance the Yan\textsuperscript{ACT} phenotype, is a multigenic effect caused by large deletions present in the two founding alleles of EY3-5. Specific mutations were isolated in three genes that can enhance the Yan\textsuperscript{ACT} phenotype. The strongest enhancer of Yan\textsuperscript{ACT} among these three genes, called hidden clones, appears to be necessary for growth and involved in early cell fate decisions in the eye.

Introduction

The Drosophila eye is a complex neural tissue with precise cellular architecture (23). This “neurocrystalline lattice” has inspired many studies aimed at uncovering the genetic and biochemical mechanisms that govern eye development (reviewed in (20, 33). Among the many findings of such research is the realization that the RTK/Ras/MAPK pathway (or Ras pathway) is essential for the specification of every fate in the eye except one (9). Furthermore, Ras signaling also stimulates growth, initiates differentiation and

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prevents apoptosis (7, 15, 31). The great importance of this pathway in a diversity of developmental processes suggests that its function may be molecularly integrated with other networks to accomplish specific goals. Thus, examining Ras pathway function in more detail can lead to insights into all aspects of eye development.

Signaling begins when an extracellular ligand binds to an RTK, thus stimulating the Guanine Nucleotide Exchange Factor, Sos to exchange of GDP for GTP on Ras. GTP-Ras then activates the MAPK cascade, which culminates in the phosphorylation of MAPK (reviewed in (12, 13). Di-phosphorylated MAPK can localize to the nucleus, where it phosphorylates many protein targets, including the ETS DNA-binding domain transcription factors, Pointed P2 (PntP2) and Yan (5, 21). Phosphorylation activates PntP2, a transcriptional activator, but inactivates Yan, a transcriptional repressor (21). PntP2 and Yan can bind to the same DNA sequence; thus when phosphorylated, PntP2 can activate genes that had previously been repressed by Yan (21, 30).

Phosphorylation of Yan results in its nuclear export and degradation. Mutation of the MAPK phosphorylation sites on Yan generates a constitutively active form of Yan, Yan$^{\text{ACT}}$, that cannot be exported from the nucleus or degraded when Ras is activated. Expression of Yan$^{\text{ACT}}$ in the developing eye blocks differentiation and greatly increases apoptosis. These developmental defects give rise to a malformed, or “rough,” eye in the adult fly (25). In an attempt to build upon the molecular framework of the Ras pathway, a genetic screen was conducted to identify mutations that dominantly modify the Yan$^{\text{ACT}}$ rough eye phenotype (24).
From this screen, several genes with established roles in the Ras pathway were identified, such as the genes that encode MAPK, Sos, Star, and PntP2. This screen also uncovered previously unknown connections to the Ras pathway. For example split ends (spen) and eyes absent (eya) were identified as enhancers of the Yan<sup>ACT</sup> phenotype. Spen is a novel nuclear protein that is necessary for the Ras-mediated survival of glial cells in the CNS (6, 24). Eya is a member of the retinal determination network of transcription factors that function in the early stages of eye fate determination (4) and reviewed in (22). Subsequent work has shown that MAPK phosphorylation of Eya upregulates Eya function (11). These findings validate the Yan<sup>ACT</sup> screening approach and offer new directions that push the focus of research towards understanding how Ras signaling is integrated with other molecular networks.

In this screen, alleles of Enhancer of Yan<sup>ACT</sup>, 3-5 (EY3-5) were also identified as a dominant enhancer of the Yan<sup>ACT</sup> phenotype (24). To understand the role of EY3-5 in Drosophila development, and the relationship between EY3-5 and the Ras pathway, a molecular and genetic description of EY3-5 was undertaken. The EY3-5 locus was mapped to a small region containing a dozen genes. Also, a screen to isolate additional alleles of EY3-5 was undertaken. From this screen, alleles of not one, but three genes that can enhance the Yan<sup>ACT</sup> eye phenotype were identified. Of these three, the strongest enhancer is hidden clones (hic). Initial analysis suggests that hic is necessary for growth and may be involved in early cell fate decisions in the eye.
Results

*EY3-5* was identified as a strong dominant enhancer of the rough eye phenotype generated from developmental expression of Yan\textsuperscript{AC} (Figure 2.1). This effect can be seen regardless of the eye-specific enhancer used to express the Yan\textsuperscript{AC} transgene. *EY3-5* can also dominantly modify a dominant rough eye phenotype seen in the gain of function mutant, yan\textsuperscript{s2382} (24). Together, these results suggest that the *EY3-5* dominant enhancement phenotype is not an effect on expression of the Yan\textsuperscript{AC} transgenes.

Mapping of *EY3-5* locus

Among the lines isolated from the Yan\textsuperscript{AC} screen are XZB970 and XKR845. These lines both map to the third chromosome and fail to complement each other for homozygous lethality. Based on this evidence, it was concluded that these lines are alleles of the same gene. This gene was named *EY3-5* and the alleles will be referred to as *EY3-5\textsuperscript{XZB970}* and *EY3-5\textsuperscript{XKR845}* . Both of these alleles were generated by X-ray mutagenesis, and thus may be the results of chromosomal rearrangements. Polytene chromosome banding analysis performed on salivary gland squashes revealed that each allele contains a deletion in chromosomal band region 61A-B (24). It should be pointed out that the relevant phenotype regarding the identification of *EY3-5* is the ability to enhance Yan\textsuperscript{AC}. The lethality associated with the X-ray induced alleles may be due to disruption of a gene near *EY3-5* that is uncovered by the deficiencies. The lethal phenotype simply demonstrates that the two deficiencies lie near each other, and thus their effect on Yan\textsuperscript{AC} is likely to result from the disruption of the same gene. The lethal phenotype of the deficiencies does not indicate that the null phenotype of *EY3-5* is lethal.
To map further the deleted regions, and potentially identify a new allele of *EY3-5*, several lethal mutations that map to band region 61 were obtained and tested for the lethal non-complementation of the *EY3-5* alleles. From these tests, it was found that both alleles of *EY3-5* complement the gene *tracheless* (*trh*). This information provides a defined genomic interval that contains *EY3-5*. The proximal extreme is *trh* at chromosomal band 61C, and the distal extreme is the telomeric region of chromosome arm 3L that is immediately adjacent to chromosomal band 61A (2). Based upon the Drosophila genome sequence, this defines a region of approximately 400 kilobases (kb) (1). Two P-element lines were identified that fail to complement *EY3-5* alleles. Insertion *l(3)rH321* fails to complement both *EY3-5* alleles while insertion *l(3)ry122* fails to complement only *EY3-SxZ7870*. These data confirm the polytene band analysis. Also, the fact that *l(3)ry122* fails to complement only one *EY3-5* deficiency allele suggests that the two deficiencies are not perfectly overlapping. The insertion lines also were tested for the ability to enhance *Yan^ACT*. Insertion *l(3)rH321* strongly enhances *Yan^ACT*, while *l(3)ry122* does not. Therefore, *l(3)rH321* is an allele of *EY3-5*.

To identify the exact site of insertion of these two P-elements, inverse PCR was used to amplify small regions of genomic sequence that flank the P-element insertion site on either end. The inverse PCR products were sequenced and compared to the Drosophila genome. Sequence recovered from one side of the *l(3)ry122* insertion indicates that this insertion lies in the 5' region of *nurfl301* (Figure 2.2). Sequence recovered from both sides of *l(3)rH321* derive from genomic loci that are separated by ≈50 kb. The distal
Inverted Repeat of the P-element is inserted into the 5' untranslated region (UTR) of *rhinoceros (rno)*, while the proximal Inverted Repeat lies in the 5' region of *nur301* (Figure 2.2). This result suggests that a deletion of ≈50 kb lies adjacent to the *l(3)rH321* insertion site. The presence of a deletion in this position was confirmed by the failure to PCR amplify small genomic sequences within the deletion from *l(3)rH321* homozygous genomic DNA (data not shown).

The deletion in *l(3)rH321* also helps to explain the genetic data. The two insertion lines fail to complement each other. On the surface, this result might mean that they both affect the same, single gene. However, as noted above, *l(3)rH321* fails to complement both *EY3-5* deficiency alleles while *l(3)ry122* only fails to complement one of the two. If the insertions only affected one gene, then their complementation pattern vs. all other mutations should be identical. The presence of a deletion that uncovers multiple genes in *l(3)rH321* also explains why *l(3)ry122* does not enhance Yan\(^{ACT}\), while *l(3)rH321* does. Taken together, these data show that *EY3-5* most likely resides within a ≈50 kb region that is deleted in *l(3)rH301*.

**Screen for new alleles of EY3-5**

The three *EY3-5* alleles described are not ideally suited for phenotypic analysis because they affect multiple genes. Therefore a genetic screen was designed to recover alleles that specifically disrupt *EY3-5* (Figure 2.3). The basic approach was to test EMS mutagenized third chromosomes for the failure to complement an *EY3-5* allele. The true loss of function phenotype of *EY3-5* is not known, so the non-complementation
phenotype could take the form of lethality, or it could result in some form of morphological perturbation in the eye and other tissues. Mutagenized chromosomes were simultaneously tested for the ability to dominantly enhance Yan\textsuperscript{ACT} (Figure 2.3). This phenotype could arise from mutations anywhere in the genome, not just the third chromosome, so it was important to retest recovered alleles after establishing a stock.

The results of the EY3-5 allele screen are summarized in Table 2.1. Over 24,000 mutagenized lines were screened, and 93 third chromosome lines were recovered that fail to complement the lethality phenotype of an EY3-5 deficiency allele. Several lines that show a rough eye phenotype were also recovered, but in every case this phenotype was dominant, and thus unlikely to result from mutation of EY3-5. Given the size of the EY3-5 deficiencies used for the complementation test (from \(\approx50\) kb for \(Df(3L)EY3-5^H321\) to \(\approx400\) kb for \(Df(3L)EY3-5^ZK970\)), it seems likely that these 93 lines represent mutations in several different genes. Complementation crosses between the various alleles identified four complementation groups. Each group contains approximately 20 alleles (Table 2.1). Interestingly, several of the lines behave as alleles of more than one complementation group. This suggests that these lines may in fact represent deficiencies, because EMS can induce deficiencies as well as point mutations (2).

Of the 93 lines isolated, 15 can enhance the Yan\textsuperscript{ACT} phenotype (Table 2.2). These 15 lines are alleles of three complementation groups: 1, 2 and 4. Also, 5 of these 15 lines are alleles of more than one group. In order to discern which complementation group is most likely to represent EY3-5, the different groups were compared for strength of
phenotype (Tables 2.2 and 2.3). Of the 15 YanACT-enhancing lines isolated, 73% are alleles of group 1 including the two strongest, AC5 and CK2. Furthermore, half of all group 1 alleles can enhance YanACT. In contrast, 33% of enhancing lines are group 4 alleles, while 27% are group 2 alleles. For either group 2 or 4, only about 20% of their alleles can enhance YanACT. This comparison shows that group 1 alleles have the strongest effect upon the YanACT phenotype.

Interestingly, the strongest enhancers of YanACT are allelic to multiple groups. Both of the two strongest alleles AC5 and CK2 are alleles of both groups 1 and 4, while the strongest EY3-5 phenotype is seen in the deficiency line, Df(3L)EY3-5XZB970, which uncovers groups 1, 2 and 4 (Table 2.2). These data suggest that the EY3-5 phenotype is the result of a multigenic effect on YanACT, perhaps through the combined inactivation of groups 1, 2 and 4. Alternatively, EY3-5 could be a single gene that is not strongly disrupted in any of the mutant lines isolated except the deficiency lines. However, the degree of mutational saturation of the four defined complementation groups (Table 2.1) suggests that EY3-5 is not a mutable single gene. It seems more likely that the EY3-5 phenotype is a multigenic effect.

**Phenotypic analysis of hidden clones mutants**

The strong YanACT enhancement phenotype of Df(3L)EY3-5XZB970 may result from the simultaneous loss of several adjacent genes. From the screen for new EY3-5 alleles, complementation group 1 emerged as a strong interactor with YanACT, suggesting that a large part of the EY3-5 phenotype may derive from loss of group 1 function. Genetic and
phenotypic analysis of group 1 was begun in order to understand its role in eye
development and how this role relates to the Ras pathway. The gene representing group
1 mutants will be referred to as hidden clones (hic), for reasons that will soon be obvious.

As an enhancer of Yan\textsuperscript{ACT}, hic could function as a positive regulator of Ras pathway
function. To test this hypothesis, hic alleles were crossed to a sev-Ras\textsuperscript{N17} transgenic line.
This line expresses a dominant negative form of Ras in the presumptive R7 photoreceptor
in the developing eye, causing the loss of R7 from about 12% of all ommatidia in the
adult eye (Figure 2.5, Table 2.4). This phenotype results because of the requirement of
high levels of Ras activity in the R7 photoreceptor. When sev-Ras\textsuperscript{N17} transgenic flies are
also heterozygous for an allele of hic, the organization of the eye is further disrupted and
the number of ommatidia lacking an R7 increases approximately four-fold (Figure 2.5,
Table 2.4). This result is consistent with a role for hic as a positive regulator of Ras
pathway signaling.

To understand the role of hic in normal development, the loss of function phenotype was
examined. Alleles of hic were first “cleaned” of linked background mutations by
recombination (see Material and Methods). Crosses between cleaned alleles reveal that
loss of hic function results in lethality before the larval third instar stage. Scoring for
lethality in embryos from these same crosses reveals that lethality occurs some time after
hatching (data not shown).
In order to analyze hic function in the adult eye, the larval lethality must be circumvented. This can be done using the Flipase/Flipase Recombination Target (FLP/FRT) system to stimulate recombination during mitosis in the developing eye (Figure 2.6). In this way, homozygous mutant tissue can be generated in the eye of an otherwise hic heterozygous animal. Furthermore, this system is designed such that homozygous mutant tissue can be conveniently distinguished by the lack of red eye pigment (Figure 2.6). Several hic, FRT80B recombinant lines were generated. These lines were crossed to y, w, eyeless-FLP; w+, FRT80B flies. The resulting y, w, eyeless-FLP; hic, FRT80B / w+, FRT80B progeny are expected to show three eye colors, each color corresponding to a different hic genotype: white (homozygous mutant), red (heterozygous), dark red (homozygous wild type). Surprisingly, progeny of the appropriate genotype show no eye color variegation that would indicate that recombination has occurred, and there are no visible eye defects either. In contrast, control animals that are wild type for hic contain numerous clones marked by the absence of red eye pigment, as well as the corresponding dark red twin spots. From this experiment, it appears that hic alleles somehow interfere with the generation of eye clones.

There are many technical reasons that can explain the failure to generate hic eye clones. From the control crosses, it seems clear that the eyeless-FLP and w+, FRT80B elements work as expected, so these can be ruled out as the source of a problem. One possibility is that the hic, FRT80B lines actually lack an FRT insertion. The FRT insertion contains a neo\(^R\) marker that was used to select resistant larvae grown on G418-containing food (see
Materials and Methods). If the selection was not performed correctly, then the presumed \textit{hic}, FRT80B lines might not actually have an FRT element. However, two lines of evidence argue against the G418 selection as a source of error. First, several recombinant group 4, FRT80B lines were made in exactly the same way, and all of these lines generate clones as expected. Second, the expected recombination frequency between band region 61A where \textit{hic} resides and FRT80B is about 50\% (18). A total of 40 different \textit{hic}, FRT80B lines were made that include 9 different \textit{hic} alleles. If one were to randomly select 40 potential recombinants that are already known to carry an allele of \textit{hic}, then the odds of not choosing at least one that bears and FRT80B insertion is $0.5^{40} \approx 9 \times 10^{13}$. However, none of the 40 \textit{hic}, FRT80B lines have ever generated offspring with detectable eye clones when mated to \textit{y}, \textit{w}, eyeless-FLP; \textit{w*}, FRT80B flies, while all positive controls work as expected. These data strongly suggest that \textit{hic} alleles dominantly inhibit FLP/FRT mediated mitotic recombination.

An alternative explanation is that clones of \textit{hic} are cell lethal. In other examples of cell lethal mutations, such as loss of Egfr function, the generation of eye clones usually produces a scar in the eyes of adults where mutant tissue once was (31). Such defects are never seen in \textit{hic} eye clone experiments, perhaps because \textit{hic} mutants are cell lethal at a much earlier stage, so the eye has sufficient time to remove and replace the dying mutant tissue before ommatidial assembly begins. This hypothesis does not explain the lack of red/dark red eye color variegation in the adult eye, but perhaps this result occurs for a different reason unrelated to the absence of homozygous mutant tissue.
To test the cell lethal hypothesis, double mutant lines were made that should simultaneously create clones for hic and for Df(3L)H99. This deficiency uncovers the important programmed cell death pathway genes, hid, reaper and grim. Removal of these three genes is known to efficiently block programmed cell death in the embryo and eye (16, 29, 32). Thus, if clones of hic ever do form, perhaps blocking cell death will allow these clones to survive long enough to have an impact on the adult eye.

Homozygous mutant Df(3L)H99 eye clones in adults show no obvious defects (Figure 2.7A, B). The only abnormality is the presence of extra pigment cells that can be seen by increased spacing between ommatidia (16). However, double mutant hic, Df(3L)H99 eye clones produce an unexpected result. These clones somehow cause the formation of indentations in the eye (Figure 2.7C, D). When indentations form along the edge of the eye, ectopic bristles and cuticular structures form there, which raises the possibility that mutant tissue has lost eye cell fate and become transformed to bristle cells and other cell types.

This transformation phenotype is similar to one seen in eye clones of axin mutants (3). The Axin protein is known to antagonize Wg signaling by binding to Armadillo and facilitating its downregulation (10), thus axin mutant clones result in increased Wg signaling. This ectopic activation of the Wg pathway is thought to direct cells of the eye disc towards non-eye fates that are normally derived from the eye disc, such as the head cuticle. Furthermore, this transformation appears to occur through the inhibition of the retinal determination (RD) genes eyes absent, sine oculis and dachshund (3). It seems
possible that the transformation of eye tissue seen in hic, Df(3L)H99 clones represents a link between hic and the retinal determination genes.

Eye sectioning reveals that hic, Df(3L)H99 double mutant cells can become photoreceptors. These photoreceptors tend to form small, irregularly shaped rhabdomeres (Figure 2.7E). Thus, loss of hic does not prevent a cell from acquiring photoreceptor fate, but it does inhibit the ability of a photoreceptor to differentiate properly. Interestingly, hic, Df(3L)H99 clones do not form contiguous patches of unpigmented tissue. White patches are commonly seen in clonal experiments. This result suggests that after being established, these clones grow and divide very little. Perhaps every double mutant cell in the adult eye is an individual clone.

The ability to generate homozygous mutant tissue shows that recombination does occur in the presence of hic alleles. Therefore, hic does not dominantly suppress FLP/FRT mediated mitotic recombination as previously suspected. Rather, it seems likely that when hic homozygous mutant tissue is generated, severe growth and survival defects prevent these cells from contributing to the adult eye. However, even if the homozygous mutant tissue is eliminated by apoptosis, this still does not explain why eye color variegation is not observed between the remaining heterozygous and homozygous wild type cells, as is seen in control eye clones. One possible explanation of the non-variegation phenotype is that gene dosage of hic strongly influences how these cells are allocated during development. This idea is described in more detail in the discussion section.
Discussion

A screen for dominant modifiers of a Yan\textsuperscript{ACT} rough eye phenotype has identified two new links to the RTK/Ras/MAPK pathway, \textit{spen} and \textit{eya} (6, 11, 24). \textit{EY3-5} is a third Yan\textsuperscript{ACT} interactor with a potential connection to the Ras pathway. A screen was performed to isolate gene-specific alleles of \textit{EY3-5}. This screen successfully isolated several mutant lines capable of enhancing Yan\textsuperscript{ACT}. Surprisingly, not all lines that can enhance Yan\textsuperscript{ACT} are mutated in the same gene. From the screen, three complementation groups were identified that contain some alleles that can enhance Yan\textsuperscript{ACT} (Table 2.2). Furthermore, the strongest enhancers of Yan\textsuperscript{ACT} are alleles of more than one complementation group. This finding implies that more than one gene needs to be inactivated in order to recapitulate the original \textit{EY3-5} phenotype. In fact, none of the EMS-induced alleles recovered can enhance Yan\textsuperscript{ACT} as strongly as \textit{Df(3L)EY3-5\textsuperscript{XZZY70}} (Table 2.2). This deficiency is the only allele that simultaneously uncovers groups 1, 2, and 4. Thus, the full \textit{EY3-5} phenotype may be the result of the combined loss of these three genes.

A combination of genetic and molecular data has allowed the \textit{EY3-5} locus to be mapped to a region of \textasciitilde50 kb (Figure 2.2). This region contains 11 genes of diverse putative functions, including two chromatin remodeling factors, two glycerol kinases, a protein kinase, an exoribonuclease, a serine protease inhibitor, a fusion of Nitrilase and Fragile Histidine Triad proteins, a calcium ion channel, and three novel proteins, one of which is known to bind to the zinc-finger transcription factor, Disconnected. It is easy to imagine roles for some of these proteins in signal transduction. Many signaling pathways include
a protein kinase component. Several pathways could also use a protease inhibitor to control their activation. For example, initiation of the Toll pathway begins with a proteolytic cascade culminating in the production of an activated form of the Spatzle ligand (19, 26, 27). Also, upon binding a ligand, the Notch receptor is cleaved in its transmembrane domain, allowing its C-terminus to activate transcription in the nucleus (28). In the next chapter of this thesis, evidence is presented showing that the mno gene from the EY3-5 region is linked to the Ras pathway and corresponds to complementation group 4. The last chapter of this thesis presents evidence that the nurf301 gene corresponds to complementation group 2 and describes the roles of NURF301 in heat-shock transcription, homeotic gene expression and JAK/STAT signaling. The identity of the hic gene is still unknown, but is presumably one of the other 9 genes uncovered by Df(3L)EY3-5-H321.

Of the genes identified in this screen, hic has the strongest effect on YanACT. Half of hic alleles enhance YanACT, and 11 of the 15 lines isolated that enhance YanACT are hic alleles (Table 2.3). Alleles of hic can also dominantly enhance an eye phenotype caused by expression of a dominant negative allele of Ras in the developing eye. This genetic activity supports the model of hic as a Ras pathway agonist. In order to analyze the phenotype of hic the FLP/FRT system was used to stimulate mitotic recombination in the developing eye, thus circumventing the larval lethality of hic alleles. Surprisingly, this system seemed unable to stimulate recombination in the presence of hic even though careful control of the experiment indicated that it should work.
The assumption was made that recombination does happen, but \textit{hic} mutant clones are eliminated very early by apoptosis. Thus, to observe the effects of \textit{hic} mutants on eye development, cell death was blocked in the eye by making double mutant clones of \textit{hic} and \textit{Df(3L)H99}. By itself, loss of cell death in the eye causes no defects in patterning other than the overproduction of pigment cells (Figure 2.7A, B, (16). Photoreceptors that are double mutant for \textit{hic} and \textit{Df(3L)H99} are individually distributed throughout the eye (Figure 2.7E). The failure of clones to form patches of tissue indicates that \textit{hic} activity is essential for growth. Mutant photoreceptors also have malformed rhabdomeres, suggesting that differentiation is incomplete. Thus, \textit{hic} is also required for proper differentiation. Simultaneous loss of \textit{hic} and apoptosis produces in morphological defects in the retina (Figure 2.7C, D). Indentations were observed in the middle and along the edge of the eye. Frequently, there were ectopic bristles and cuticular protrusions associated with the indentations. The reason for these defects is unclear, but it may be related to early cell fate decisions in the eye, as discussed below.

The most curious phenotype seen in eyes in which \textit{hic} clones have been induced is the absence of eye color variegation. Generation of mitotic eye clones in both wild type and group 4 mutant control crosses both resulted in three distinct eye colors: white, red and dark red. These colors correspond to the three genotypes that exist in the eye after recombination. The absence of white tissue in \textit{hic} homozygous mutant eye clones is explained by an early cell lethal defect. What happens to the two shades of red?
One possible explanation for the absence of variegation is that gene dosage of hic has strong influences on eye cell fate. Perhaps the developing eye-antennal imaginal disc is highly sensitive to hic gene dosage. Thus, after inducing eye clones, the two different genotypes are biased towards different fates. In this scenario, one of the two genotypes is favored to become eye cells, while the other genotype would be directed to another fate, such as antennal cells, macrochaetae, the maxillary palp, or any other tissue that is derived from the eye-antennal disc. This hic dosage sensitivity is only obvious in a context in which there are cells with different numbers of a wild-type hic allele, such as when mitotic recombination is stimulated in the eye. In the absence of genetic mosaicism, such as in normal, non-mosaic hic heterozygotes, this dosage sensitivity is undetectable.

Cells of the eye-antennal disc become committed to either eye or antennal cell fate during the larval second instar stage (14). This allocation event utilizes the Ras and Notch pathways to modulate the activity of the retinal determination gene network (i.e., eyeless, twin of eyeless, eye gone, eyes absent, sine oculis, dachshund). This eye vs. antennal fate decision occurs early enough to explain why both heterozygous and homozygous wild type hic tissue is not intermixed in adult eyes. It may also explain the apparent transformation of eye tissue to macrochaetae and cuticular tissue along the edge of the eye that results when hic, Df(3L)H99 double mutant clones are made. On a molecular level, the ability to sort hereterozygous vs. homozygous wild type cells could be explained by a Notch/Delta lateral inhibition mechanism. For example, cells of one
genotype may produce more Delta ligand. Once this initial bias arises, feedback through
the Notch/Delta network could reinforce the distinction.

Materials and Methods

Inverse PCR

Inverse PCR was performed as described by Spradling et al (Genetics, 1999). For both
the l(3)rH321 and l(3)ry122 insertions, the Plac1 and Plac4 primers were used to inverse
PCR from the 5' end, while Pry1 and Pry4 primers were used for the 3' end.

EMS mutagenesis

EMS-induced mutations were induced by feeding isogenic w118 males 25mM EMS in
10mM Tris, pH 7.5 and 1% sucrose for 12hrs at room temperature. Mutagenized males
were batch-crossed to TM3/TM6B virgins, and F1 male progeny then crossed individually
to one of three deficiency lines that uncover EY3-5: Df(3L)EY3-5XZ8970, Df(3L)EY3-5XK8845,
or Df(3L)EY3-5X331. Each of these deficiencies was balanced over TM3, sev-YanACT. F2
progeny were scored as described in Figure 2.3.

Fly maintenance

Flies were maintained at 18° or 25° C on standard corn meal, molasses and agar medium.

Fly stocks

y, w, eyeless-FLP was obtained from the Bloomington Stock Center. Df(3L)H99,
FRT80B was obtained from H. Steller.
Chromosome cleaning

hic alleles were recombined onto a marker chromosome for the third containing the following markers: h, th, st, cu, sr, e, ca. The markers were then removed by recombination with a wild type chromosome. The closest marker to hic is h, located at band region 66.

hic, FRT80B recombinants

Cleaned hic alleles were crossed to FRT80B flies, then hic/FRT80B virgins were crossed to TM3/TM6B males. The eggs from this cross were collected on fly medium containing 0.3 ml of 25 mg/ml G418 (GIBCO) per 10 ml of medium. The progeny were raised at 25°C, then individual males were testcrossed vs. a different allele of hic. If this cross failed to complement, then a stock was made from the progeny.

Fixing, embedding and sectioning of adult eyes

Heads were fixed in 1% OsO4 (Ted Pella) and 1% glutaraldehyde (Ted Pella) in phosphate buffer, pH 7.2, for 30 minutes on ice, washed in phosphate buffer, then fixed in 2% OsO4 for 2 hours on ice. Fixed eyes went through an ethanol dehydration series (30%, 50%, 70%, 90%, 100%, 100%, 10 minutes each on ice), followed by 3 x 10 minute washed in propylene oxide (Ted Pella) at room temperature. Heads were then incubated overnight in 50% propylene oxide, 50% Durcapan resin (Fluka). Heads were then incubated in resin for 6 hours, then embedded in resin and cured at 65°C for 24 hours. Embedded
eyes were then cut into 1 micron thick sections and mounted on glass slides in DPX (Fluka).

References


Table 2.1: Summary of screen for EY3-5 alleles

<table>
<thead>
<tr>
<th>Category</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>F2 families screened</td>
<td>24,167</td>
</tr>
<tr>
<td>Recessive lethal mutations recovered</td>
<td>93</td>
</tr>
<tr>
<td>Alleles of complementation group 1</td>
<td>22*</td>
</tr>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>20*</td>
</tr>
<tr>
<td></td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>16*</td>
</tr>
<tr>
<td></td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>24*</td>
</tr>
<tr>
<td>Alleles not assigned to a comp. group</td>
<td>19*</td>
</tr>
</tbody>
</table>

*Note that the sum of the number of alleles for each category is 101, which is greater than the total number of lines recovered (93). Some lines fail to complement alleles from two or more groups, and are therefore counted as members of different groups.
Table 2.2: List of lines that enhance the Yan\textsuperscript{ACT} phenotype

<table>
<thead>
<tr>
<th>Line</th>
<th>Fails to complement</th>
<th>Strength of Yan\textsuperscript{ACT} enhancement</th>
</tr>
</thead>
<tbody>
<tr>
<td>AR1</td>
<td>1</td>
<td>average</td>
</tr>
<tr>
<td>BH1</td>
<td>1</td>
<td>average</td>
</tr>
<tr>
<td>CN4</td>
<td>1</td>
<td>average</td>
</tr>
<tr>
<td>DG1</td>
<td>1</td>
<td>average</td>
</tr>
<tr>
<td>ER1</td>
<td>1</td>
<td>average</td>
</tr>
<tr>
<td>FQ1</td>
<td>1</td>
<td>average</td>
</tr>
<tr>
<td>FA1</td>
<td>2</td>
<td>weak</td>
</tr>
<tr>
<td>G1</td>
<td>4</td>
<td>weak</td>
</tr>
<tr>
<td>G4</td>
<td>4</td>
<td>average</td>
</tr>
<tr>
<td>AD2</td>
<td>4</td>
<td>average</td>
</tr>
<tr>
<td>AC5</td>
<td>1, 4</td>
<td>strong</td>
</tr>
<tr>
<td>CK2</td>
<td>1, 4</td>
<td>strong</td>
</tr>
<tr>
<td>CM1</td>
<td>1, 2</td>
<td>average</td>
</tr>
<tr>
<td>CM2</td>
<td>1, 2</td>
<td>average</td>
</tr>
<tr>
<td>EX1</td>
<td>1, 2</td>
<td>average</td>
</tr>
<tr>
<td>\textit{Df(3L)EY3-5}\textit{^R321}</td>
<td>1, 2</td>
<td>strong</td>
</tr>
<tr>
<td>\textit{Df(3L)EY3-5XKR845}</td>
<td>1, 4</td>
<td>strong</td>
</tr>
<tr>
<td>\textit{Df(3L)EY3-5ZIB970}</td>
<td>1, 2, 3, 4</td>
<td>very strong</td>
</tr>
</tbody>
</table>

Listed above are the 15 EMS mutant lines that enhance Yan\textsuperscript{ACT}, as well as the three original deficiency lines. Alleles of group 1 seem to have the greatest effect upon Yan\textsuperscript{ACT} (see Table 3). The strongest enhancers of Yan\textsuperscript{ACT} are those that fail to complement multiple groups, suggesting that the original \textit{EY3-5} phenotype is a multigenic effect, perhaps through the combined loss of groups 1, 2, and 4.
Table 2.3: Comparison of Yan\textsuperscript{ACT} enhancement between different complementation groups

<table>
<thead>
<tr>
<th>Complementation group</th>
<th>Alleles that enhance Total of alleles in group</th>
<th>Alleles that enhance Total of lines that enhance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>11/22 (50%)</td>
<td>11/15 (73%)*</td>
</tr>
<tr>
<td>2</td>
<td>4/20 (20%)</td>
<td>4/15 (27%)*</td>
</tr>
<tr>
<td>3</td>
<td>0/16 (0%)</td>
<td>0/15 (0%)</td>
</tr>
<tr>
<td>4</td>
<td>5/24 (21%)</td>
<td>5/15 (33%)*</td>
</tr>
<tr>
<td>unassigned</td>
<td>0/19 (0%)</td>
<td>0/15 (0%)</td>
</tr>
</tbody>
</table>

The ability of the different complementation groups to enhance the Yan\textsuperscript{ACT} eye phenotype was compared two ways. First, the total percentage of alleles of a given group that can enhance Yan\textsuperscript{ACT} was determined (second column). Second, the percentage of all Yan\textsuperscript{ACT}-enhancing lines that are allelic to a given group was determined (third column). From this analysis, group 1 appears to have the strongest effect on Yan\textsuperscript{ACT}. Half of group 1 alleles can enhance Yan\textsuperscript{ACT}, and of all 15 lines isolated that do enhance Yan\textsuperscript{ACT}, 73\% of them are alleles of group 1. Groups 2 and 4 also have some effect on Yan\textsuperscript{ACT}.

*Note that the percentages in the third column do not add up to 100\%. This is because 5 of the 15 mutant lines that enhance Yan\textsuperscript{ACT} are alleles of more than one complementation group.
Figure 2.1: EY3-5 dominantly enhances the sev-Yan<sup>ACT</sup> phenotype. Top row: SEMs of eyes of the indicated genotypes. Bottom row: sections of adult eyes. Expression of Yan<sup>ACT</sup> leads to loss of cells in the eye, giving a rough surface and disrupted cellular arrangement. Yan<sup>ACT</sup> flies that are also heterozygous for EY3-5 show a much stronger phenotype.
Figure 2.2: Schematic representation of the EY3-5 locus. Rectangles represent genes that have been experimentally discovered or predicted. The annotation for this region was done by the Berkeley Drosophila Genome Project. Regions uncovered by deficiencies are indicated by dashed lines. Breakpoint positions are approximate, except for Df(3L)rH321, for which the precise breakpoint positions are known. The right breakpoint of Df(3L)EY3-5XR845 is inferred by the fact that this deficiency genetically complements l(3)ry122. P-element insertion sites are indicated by triangles. The telomeric region of chromosomal arm 3L is approximately 250 kb to the left. The trh locus resides approximately 150 kb proximally to EY3-5. This drawing is approximately to scale. The identities and functions of the genes are below.

rhinoceros (rno): PHD zinc finger domain. Predicted chromatin remodeling factor.
1: CG1216. Glycerol kinase.
2: Glycerol kinase.
4: BcDNA:GH04978. Protein kinase.
7: CG17142: Calcium ion channel.
8: CG32492: Novel.
nurf301: Nucleosome Remodeling Factor.
Figure 2.3: Screen for alleles of EY3-5. EMS mutagenized males were crossed to a third chromosome balancer stock en masse. Individual F1 males were then test-crossed to an allele of EY3-5. The EY3-5 allele is balanced over TM3, sev-Yan^{ACT}. If the F1 male bears a mutation in EY3-5, then it will not complement the EY3-5 deficiency. This non-complementation could result in lethality. If the EY3-5 allele is not lethal over the deficiency, perhaps an eye defect can be detected. The mutagenized chromosomes can also be tested for enhancement of Yan^{ACT}. 
Figure 2.4: *hic* alleles dominantly enhance the *sev-Yan^{ACT}* phenotype.
Top: Photographs of eyes. Bottom: Sections of adult eyes. The *hic* allele AR1 noticeably enhances the *sev-Yan^{ACT}* phenotype.
Figure 2.5: hic alleles dominantly enhance a dominant-negative Ras phenotype in the eye. Adult eye sections. Left panel: Expression of Ras\(^{N17}\) causes a mild loss of R7 photoreceptors (circled ommatidia). The rhabdomere of an R7 photoreceptor in an unaffected ommatidium is indicated with an arrowhead. Right panel: sev-Ras\(^{N17}\) flies that are also heterozygous for a hic allele show an overall loss of organization as well as fewer R7 cells. In this view, all of the ommatidia except one is missing R7 (arrowhead).

Table 2.4: hic alleles dominantly reduce the number of R7s in sev-Ras\(^{N17}\) flies

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Ommatidia missing the R7 photoreceptor</th>
<th># ommatidia scored</th>
</tr>
</thead>
<tbody>
<tr>
<td>sev-Ras(^{N17}) / +</td>
<td>11.9%</td>
<td>263</td>
</tr>
<tr>
<td>sev-Ras(^{N17}) / hic(^{AR1})</td>
<td>42.8%</td>
<td>283</td>
</tr>
<tr>
<td>sev-Ras(^{N17}) / hic(^{CK2})</td>
<td>45.5%</td>
<td>292</td>
</tr>
</tbody>
</table>

The effect of hic alleles on R7 number was quantified. sev-Ras\(^{N17}\) flies that are also heterozygous for a hic allele show a four-fold increase in the number of ommatidia lacking an R7.
Figure 2.6: FLP/FRT mediated mitotic recombination. This technique allows loss of function analysis of advanced stage developmental processes by avoiding earlier developmental defects. Here the technique is applied to the eye, but in theory it can be used to study the role of any gene in any tissue. In this example, the mutation in question is Df(3L)H99, which is normally homozygous lethal. Chromosomes are indicated above by grey lines. On the left is a cell in mitotic metaphase. The cell is heterozygous for the mutation in question (H99). The two third chromosome homologs are shown paired with their sister chromatids. The centromeres are indicated by circles. The FRT sequences are indicated by triangles and reside proximally to the locus of interest. One the wild type chromosome is a dominant marker, in this case a w+ transgene that cell autonomously rescues the w- mutation present in the background. The FLP recombinase is expressed in the eye using an eyeless-FLP transgene. FLP stimulates recombination between homologous chromosomes specifically at the FRT locus. This results in heterozygosity for the H99 mutation between the two pairs of sister chromatids. Therecombined chromosomes then segregate such that both of the daughter cells are homozygous: one for H99, the other for the wild type allele. The H99 daughter cell can then grow and divide, giving rise to a clone that is marked by the absence of the w+ transgene, and thus lacks red eye pigment.
Figure 2.7: Suppressing cell death reveals hic mutant phenotypes. A. Eye clones of Df(3L)H99 have a normal gross morphology. B. Section of adult eye shows normal photoreceptors within a Df(3L)H99 clone (circled). Suppression of cell death by Df(3L)H99 allows hic mutant clones to generate morphological defects. C. An indentation in the middle of the eye. D. An indentation along the edge of eye. Ectopic bristles and cuticle are often seen here. E. Section of an eye reveals that several hic, Df(3L)H99 double mutant photoreceptors are found in the eye, which are distinguished by their lack of pigment granules (arrowheads). The double mutant photoreceptors make irregularly shaped rhabdomeres.
Chapter 3

The PHD domain protein Rhinoceros Restricts Cell

Fate Specification in the Drosophila Eye and is

Necessary for Proper Developmental Timing
Abstract

The Drosophila eye is a complex tissue composed of neural and non-neural cell types arranged in a highly organized pattern. Inductive signaling between cells in the developing eye is very important for establishing the correct number and identity of cell fates in the adult eye. Here, the role of the rhinoceros (rno) gene in regulating eye cell fates is described. Mutations in rno cause overproduction of photoreceptors and cone cells and strongly inhibit apoptosis. These phenotypes are similar to those seen when EGFR is inappropriately activated by mutation of the argos gene. Tests between argos and rno alleles show a strong genetic interaction. Furthermore, rno mutant eye tissue shows reduced production of Argos ligand. These data suggest a role for rno in the inhibition of EGFR by regulating expression of argos. Alleles of rno also display a general delay in the expression of differentiation markers in photoreceptors and cone cells. The rno gene encodes a nuclear, PHD zinc finger protein, implying that it functions as a transcription factor. Thus, the Rno protein may be necessary for the timely transcription of genes that control the differentiation of the eye, as well as for the production of Argos.

Introduction

The Drosophila eye is a complex neural tissue with precise cellular architecture (45). It is composed of several hundred reiterated subunits called ommatidia, of which each contains eight photoreceptors and a complement of non-neural support cells arranged in an invariant pattern. This "neurocrystalline lattice" has inspired many studies aimed at uncovering how specific cell fates are assigned during eye development (reviewed in (37,
Careful observations have been made of the morphological events that occur eye development (11, 45, 52, 55). Differentiation in the eye begins in the cells at the posterior edge of the eye disc of third instar larvae and gradually proceeds to the anterior edge. This wave of differentiation is called the morphogenetic furrow (MF). As cells exit the MF along the posterior edge distinct eye cell fates are added to a developing ommatidium in a strict sequence. First the R8 photoreceptor is established, then the R2 and R5 photoreceptors, followed by R3 and R4, then R1 and R6, and finally, R7. After these photoreceptors have been specified, the non-neural cell fates are recruited to the ommatidium. First the cone cells appear, followed by the primary, secondary and tertiary pigment cells. At the end of this process, there is an excess of undifferentiated cells that are eliminated by apoptosis.

Analysis of the genetic controls of eye development has revealed that inductive signals are important for the specification of each cell type. The EGFR/Ras/MAPK signaling pathway is particularly important because every eye cell except R8 requires activation of this pathway in order to acquire a specific cell fate (19). In fact, EGFR activation is necessary for the production of a given cell type exactly when that cell type joins the ommatidium. Furthermore, if EGFR is inappropriately activated, then an excess of cellular differentiation results (19, 20). The particular cell fates that are induced depends upon when EGFR is activated. These data show that EGFR is a general, permissive signal for differentiation that specifies multiple cell fates.
EGFR is also necessary for all eye cells to escape programmed cell death. Loss of Egfr function in the third instar or later stage eye disc results in apoptosis for all cell types (14, 57). In addition, undifferentiated cells in the pupal retina that lack access to the EGFR ligand Spitz die by apoptosis instead of becoming photoreceptors (3). Therefore, activation of EGFR is a necessary survival signal in the developing eye.

Activation of EGFR in the developing eye triggers feedback mechanisms that restrict EGFR activation and efficiency in neighboring cells. Upon binding to Spitz, EGFR activates Ras which in turn triggers the MAPK cascade (reviewed in (24, 31)). This signal culminates in activated MAPK translocating into the nucleus and phosphorylating several protein targets, including PntP2 and Yan (10, 42). This phosphorylation of PntP2 allows it to activate transcription of specific target genes, while phosphorylation of Yan prevents this repressor from blocking expression of these same target genes (42, 46). One common transcriptional response to EGFR activation is expression of argos (22). Argos is a secreted, EGF domain protein that can bind directly to EGFR (50). This binding inhibits dimerization of EGFR monomers, thus blocking activation (26). The Argos protein can diffuse several cell diameters to inhibit EGFR (20). In argos mutants, EGFR is inappropriately activated, leading to the overproduction of all eye cell types and the inhibition of cell death (9, 20).

Described here is a previously uncharacterized gene, rhinoceros (rno). Mutations in rno were isolated as part of a screen to recover alleles of EY3-5 (Chapter 2). The ability of rno to contribute to the multigenic EY3-5 phenotype initially suggested that it might
function as an agonist of RTK/Ras/MAPK signaling. However, detailed phenotypic analyses have demonstrated that rno exhibits the characteristics of an inhibitor of EGFR signaling. Specifically, loss of rno function in the eye results in the overproduction of photoreceptors and cone cells and strongly inhibits apoptosis. Evidence is provided that these effects occur due to a lack of Argos ligand. Loss of rno function also leads to a general delay in the differentiation of the eye. The Rno protein is a putative chromatin remodelling factor, suggesting that the rno mutant phenotypes may ultimately be the result of inefficient activation of an eye-specific transcriptional program.

Results

In a screen to isolate EMS-induced alleles of Enhancer of Yan\textsuperscript{ACT}, 3-5, (EY3-5) EMS mutagenized chromosomes were screened for failure to complement deficiency alleles of EY3-5 (see Chapter 2). From this screen, 24 alleles of complementation group IV (referred to here as rhinoceros \{rno\}) were recovered. These alleles fail to complement the lethal phenotype of EY3-5 deficiency alleles, and 5 of the 24 alleles enhance Yan\textsuperscript{ACT} (Table 2.2, 2.3). Thus, mutation of the rno gene may contribute to the dominant enhancement of Yan\textsuperscript{ACT} seen in EY3-5 deficiency alleles. Disruption of the hic and nurf301 genes also appears to contribute to the Yan\textsuperscript{ACT} enhancement seen in EY3-5 deficiency alleles (Chapter 2).

Phenotypic analysis of rno during eye development

To assess how loss of rno affects eye development, the FLP/FRT recombination system was used to generate mutant eye clones of rno (see Materials and Methods). The outward
appearance of \textit{rmo} eye clones in adult eyes is normal, except for some mild irregularity in the arrangement of ommatidia. However, examination of these clones in fixed and sectioned eyes reveals the presence of extra outer photoreceptors (Figure 3.1A-D). Extra outer photoreceptors were seen in sections of all six alleles examined (\textit{rmo}^{G4}, \textit{rmo}^{A02}, \textit{rmo}^{CF2}, \textit{rmo}^{CM3}, \textit{rmo}^{CT1}, \textit{rmo}^{CV1}). Typically, there is one extra outer photoreceptor per ommatidium, although occasionally an extra R7 or more than one extra photoreceptors are observed. The percentage of affected ommatidia in a mutant eye clone varied from 35\% to 45\% (Table 3.1). Rarely, an affected ommatidium is entirely composed of wild type cells (Figure 3.1E). This phenomenon has been observed in a total of eight ommatidia and for three different \textit{rmo} alleles. The occurrence of such ommatidia is a clear demonstration that the \textit{rmo} phenotype can occur in a non-cell autonomous manner.

To determine the identity of these ectopic photoreceptors, larval and pupal retinas containing \textit{rmo} eye clones were stained with markers for specific subtypes of photoreceptors. No extra nuclei were seen with anti-Rough staining, implying that the extra photoreceptor is not of the R2/R5 subtype (data not shown). Extra nuclei were observed with anti-Spalt, which shows that the ectopic photoreceptors in adults are most likely to be of the R3/R4 subtype (Figure 3.2A, B). This result is supported by the presence of an ectopic nucleus that appears between R3 and R4 in pupal \textit{rmo} eye clones that have been labeled with \textit{seven-up-lacZ} (Figure 3.2C). Therefore, the extra photoreceptors are of the R3/R4 subtype.
The *rno* phenotype is reminiscent of the *argos* mutant phenotype in which there is also a non-cell autonomous production of extra photoreceptors (20, 33). In *argos* mutants, cone cells are also overproduced (9, 20). To look for ectopic cone cells, eye discs from third instar larvae were stained with anti-Cut, which specifically labels cone cell nuclei in the eye. Instead of an increase, there is an absence of Cut-positive nuclei within *rno* mutant eye clones (Figure 3.3A). However, the presence of a few Cut-positive nuclei along the posterior edge of *rno* mutant clones suggests that in older tissue, cone cells form normally. To test this hypothesis, pupal retinas bearing *rno* eye clones were stained with anti-Cut. Wild type control clones show a normal expression pattern of four cone cell nuclei per ommatidium (Figure 3.3B). In *rno* mutant eye clones, there are many ommatidia that contain five cone cell nuclei (Figure 3.3C). This result shows that like *argos*, *rno* mutants cause the overproduction of multiple cell types. It also shows that there is a delay in the development of cone cells in *rno* mutant eye tissue.

Mutants of *argos* have a profound effect upon the cell determination events in the pupal retina. By allowing EGFR to become ubiquitously activated, essentially all the undifferentiated cells of the pupal retina are able to escape apoptosis and differentiate as 2° and 3° pigment cells (20). From detailed studies in the developing eye, it is known that apoptosis peaks in the pupae at around 50 hours after puparium formation (APF) at 20° C (56). The effects of *rno* on apoptosis were tested by staining pupal eyes that contain *rno* clones with the cell death marker, acridine orange. While wild type control clones show extensive acridine orange staining at 50 hours APF, staining in *rno* pupal eye clones is
almost completely absent (Figure 3.4). Thus, loss of rno and argos during eye
development have many of the same effects.

**Molecular cloning of the rno gene**

Alleles of rno were isolated for their non-complementation of Df(3L)EY3-5\textsuperscript{XZB970}. This
deficiency uncovers \( \approx 400 \) kb from band region 61 AB (Chapter 2). Within this region is
a smaller deficiency, Df(3L)EY3-5\textsuperscript{H321}, that complements rno and uncovers \( \approx 50 \) kb.
Therefore, the rno gene lies within Df(3L)EY3-5\textsuperscript{XZB970} but outside of Df(3L)EY3-5\textsuperscript{H321}. In
order to map rno to a smaller region, additional deficiency alleles of rno were generated
by imprecise P-element excision of the \( l(3)rH321 \) insertion. The breakpoints of these
deficiencies were mapped using a PCR-based technique (see Appendix). Briefly,
genomic DNA from embryos homozygous for a deficiency was used as a template to
amplify small, \( \approx 500 \) bp sequences found throughout the chromosomal region in question.
Failure to amplify a particular sequence tagged site, or STS, indicates that the deficiency
being tested is deleted at the position of the STS. In this way, the breakpoints of several
deficiencies in the region were mapped.

Two deficiencies were mapped that allow the identification of the rno gene. As
mentioned above, Df(3L)EY3-5\textsuperscript{H321} uncovers \( \approx 50 \) kb and complements rno.
Df(3L)rH321A26 fails to complement rno and has the same proximal breakpoint as
Df(3L)EY3-5\textsuperscript{H321}. However, its distal breakpoint deletes an additional 5 kb (Figure
3.5A). This breakpoint occurs in the middle of the coding region for the predicted gene,
CG7036 (2), referred to here as rmo. This identification was confirmed by comparing the
sequence of this gene in rno mutant alleles to wild type sequence (Table 3.2). Eight rno alleles contain nonsense mutations that truncate the predicted protein. None of these sequence substitutions are seen in the wild type stock from which the rno alleles were derived.

Small regions of the rno coding region were PCR amplified from genomic DNA and used to probe cDNA libraries. Among the several clones identified were a 7.6 kb 3' cDNA with a poly-adenylated tail, and a 2.7 kb 5' piece that contains many in-frame stop codons upstream of the presumed initial methionine codon. This left a small region between the two clones that had not been recovered from the cDNA libraries. Instead, a 1.8 kb piece of the rno coding region was RT-PCRed. The resulting full-length rno cDNA is 10,821 bp and encodes a protein of 3,241 amino acids. The full-length rno cDNA was entirely sequenced and compared to the Drosophila genome sequence (2). Several nucleotide polymorphisms between the cloned rno cDNA sequenc and genome sequence were found, but none of these differences are predicted to alter the amino acid composition of the Rno protein.

Comparison of the Rno sequence vs. Pfam (6) reveals the presence of a PHD class zinc finger motif near the amino terminus. The PHD domain is followed by a zinc finger-like Cysteine Rich Region (CRR) (34). A predicted Rno ortholog from the genome of the mosquito, Anopheles gambiae (23), shows that the N-terminal domain is highly conserved (62% identical and 72% similar, Figure 3.6). Outside of the conserved N-terminal domain are other short and widely spaced regions of conservation that are
typically rich in basic amino acids. Otherwise, both Drosophila and Anopheles Rno have frequent stretches of low complexity sequence, such as polyserine or polyglutamine. The positions of the repetitive regions do not seem to be well conserved between the two species. The conserved N-terminal domain can also be found in the predicted human protein, KIAA0215, but this protein lacks the long, repetitive tails found in Drosophila and Anopheles Rno.

PHD fingers have been found in over 400 eukaryotic proteins and they most commonly occur in members of chromatin remodelling complexes (1, 49). Biochemically, they have been shown to act as protein-protein interaction domains by binding to other PHD domains, or to structurally unrelated motifs (15, 41). The CRR domain is likewise capable of protein-protein interactions (34). Thus, the PHD domain suggests a role for Rno as part of a chromatin remodeling complex. Its involvement in such a complex could be mediated by protein binding activity in the PHD and CRR domains.

**Transgenic overexpression of rno**

The *rno* cDNA was subcloned into the pUAST expression vector and six transgenic UAS-rno lines were established. A *sevenless-Gal4* line was crossed to all six UAS-rno transgenic line to drive expression of *rno* in the developing R1, 3, 4, 6, 7 and cone cells (8). In all six cases, a mild blistering of the eyes was seen in *sev-Gal4; UAS-rno* flies. Sectioning of these eyes reveals a normal complement of photoreceptors, but a strong reduction in the number of pigment cells (Figure 3.7). This phenotype can result from a block of the second mitotic wave (12). Larval third instar eye discs overexpressing *rno*
were tested for defects in the progression of S-phase or mitosis, but no defects were observed (see below, Figure 3.14). Alternatively, the loss of pigment cells could be the result of an increase in apoptosis in the eye, which would complement the rno loss of function phenotype (Figure 3.4). Furthermore, this phenotype appears to be non-cell autonomous because the sevenless enhancer is not believed to be active in the pigment cells (5). This further supports that rno can function through a non-cell autonomous mechanism.

Characterization of Rno expression

To learn more about the context in which Rno functions, an antibodies were raised to the C-terminal 600 amino acids. Staining of blastoderm stage embryos with anti-Rno immunoserum reveals strong nuclear staining (Figure 3.8A). Expression of Rno is ubiquitous throughout embryonic development (data not shown). Cultured Drosophila S2 cells that express a rno transgene also show nuclear localization (Figure 3.8B, C).

Anti-Rno staining was also observed in the developing third instar eye disc. To simultaneously test the staining specificity, clones of rno were stained with anti-Rno. These mutant clones express a Rno protein that is truncated well before the C-terminal region that was used to raise anti-Rno antiserum (Table 3.2). Rno is expressed throughout the eye disc, both anterior and posterior to the morphogenetic furrow (Figure 3.10B). Upon closer examination, nuclear staining can be seen in the peripodial epithelium as well as in the R cells of the disc proper. The peripodial staining also includes a perinuclear component, but this perinuclear staining is not strongly reduced in
*rno* mutant clones (Figure 3.9C), which suggests that this staining may be non-specific. Anti-Rno staining in the R cells is significantly reduced in *rno* clones, although not completely lost. This result suggests that at least part of the nuclear staining is specific, but may overlap with non-specific staining. Alternatively, Rno protein may be very stable. Perhaps the protein that was available when the clones were made has been maintained and inherited by the clonal lineage. Finally, Rno expression is seen in the basally located, undifferentiated cells posterior to the furrow (data not shown). Expression in the undifferentiated cells means that Rno is in a position to influence early cell fate specification events.

**rno is necessary for proper expression of Argos in the eye**

The similarities between *rno* and *argos* phenotypes suggest that these genes regulate the same process in eye development, i.e. the recruitment of cells to the ommatidium. To test this hypothesis genetically, alleles of *rno* were tested for the ability to modify a rough eye phenotype seen in viable *argos* mutants. From this test, it was observed that *rno* alleles can very strongly enhance the *argos* eye phenotype (Figure 3.10A). This result was observed for three alleles of *rno*. These data reinforce the link between *rno* and *argos* function. However, it is not clear if these two proteins function in one pathway or in parallel pathways.

As a nuclear protein with similarity to chromatin remodeling proteins, one possible explanation for the overproduction of cone and R cells in *rno* mutant tissue is that Rno activates transcription of the *argos* locus. This hypothesis was tested by staining *rno*
mutant clones anti-Argos antibody (18, 51). Wild type eye tissue shows a punctate expression pattern for Argos, which is suggestive of localization of Argos in vesicles. Anti-Argos staining is lost in larval third instar eye clones of argos\textsuperscript{wt1}, which demonstrates the specificity of this antibody (data not shown). In rno mutant eye clones, anti-Argos staining is also strongly reduced (Figure 3.10B). This result is consistent with the idea that Rno is necessary for proper transcription of the argos locus.

To test the effects of rno on argos transcription more directly, the requirement of rno to express a reporter from an argos enhancer trap line was tested (20). In rno mutant clones, expression of β-gal from an argos enhancer trap line was assayed by anti-β-gal staining. Clones of rno did not show a loss of β-gal staining (data not shown). However, this experiment is flawed because rno and argos are on the same chromosome arm and consequently clones of rno and the argos enhancer trap are made simultaneously. This changes the dosage of the enhancer trap relative to the surrounding heterozygous tissue. Also, enhancer trap clones result in a loss of argos activity because the enhancer trap lines are hypomorphic alleles of argos. Loss of argos activity results in the hyperactivation of EGFR (20, 50), and it is known that EGFR activation results in increased transcription of the argos locus (22). Therefore, loss of argos activity is predicted to result in increased transcription of the argos locus via the EGFR to argos negative feedback loop. For the above two reasons, it is difficult to determine how loss of rno function effects argos transcription in rno, argos double mutant clones.
To get away from the requirement to generate loss of rno function clonally, rno mutant embryos were assayed for expression from the argos enhancer trap. No difference was seen in anti-βgal staining in rno vs. wild type embryos (data not shown). This experiment is not perfect either because anti-Rno staining of early embros shows that there is a strong maternal contribution of Rno protein. Thus, this experiment needs to be repeated in a rno germ line clone to be certain of the result. Another drawback is that it is not clear that rno and argos have the same relationship in the embryo as they do in the eye. Mutant rno animals die late in embryogenesis or as early first instar larvae, but the primary defect in these animals is unknown (see Appendix B).

rno is necessary for the production of the Notch ligand, Delta

The non-cell autonomous mechanism in which extra outer photoreceptors are produced in rno eye clones is also reminiscent of loss of Delta function (43). Furthermore, Delta expression in photoreceptors is known to be necessary for the specification of cone cells (17, 53). This might explain the delay in cone cell markers seen in rno eye clones (Figure 3.3). In wild type tissue, Delta is expressed in endocytic vesicles near the apical tips of the photoreceptors (32) (Figure 3.11A). In rno mutant eye clones, this staining is strongly reduced (Figure 3.11B). If this loss of Delta expression is significant, one would expect that signaling through the Notch receptor to be reduced. A convenient readout of Notch pathway activity is expression of the E(spl) proteins (25). Expression of E(spl)m7 was assayed in rno mutant clones. E(spl)m7 is expressed broadly posterior to the morphogenetic furrow (Figure 3.12A). In rno mutant eye clones, expression of E(spl)m7 is mildly reduced (Figure 3.12B). Thus, production of Delta ligand is another possible
means by which the photoreceptor and cone cell phenotypes could arise. Delta expression is not completely lost, and E(spl) expression is only mildly reduced, thus cone cell fate could still be specified, but after a longer timeperiod.

**rno mutants display a general delay in expression of developmental markers.**

In the course of examining the *rno* eye phenotype, the expression of several developmental markers was assayed in *rno* eye clones. From this series of experiments, it became clear that there is a delay in the rate of differentiation in *rno* eye clones (Table 3.3). There were three effects on developmental marker expression seen in *rno* eye clones. First, there are proteins whose expression is unaffected by *rno*. This includes the proteins Notch, Rough and Yan. While expression of these markers is upregulated near or in the furrow, their increased expression is by itself not a sign of differentiation. The second effect observed is that several markers of photoreceptor differentiation are delayed. Within this category, very early, general neural markers such as ELAV and 22C10 are only mildly affected (Figure 3.13), while other markers that are expressed in more mature R cells, such as *seven-up-lacZ* and Chaoptin, are more severely affected. This indicates that differentiation is initiated without much delay, but more specific, later differentiation events are significantly delayed. Expression of these markers is not lost entirely, though. Expression of *seven-up-lacZ* can be seen in the pupal eye. Also, loss of *seven-up* and *chaoptin* function have very distinct phenotypes, i.e., transformation of outer photoreceptors into ectopic R7 cells and loss of rhabdomeres, respectively (35, 54). Neither of these phenotypes is seen in adult *rno* mutant eye clones, thus the abrogation of expression for these genes is not absolute. The third effect observed is that several

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markers of cone cell differentiation are delayed. Cone cells seem to be particularly sensitive to the effects of \textit{rno} eye clones because no cone cell marker tested is expressed in the larval eye disc. However, as shown above, pupal \textit{rno} eye clones have an excess of cone cells, and strong expression of the nuclear protein, Cut. Altogether, these data show that \textit{rno} is necessary for the proper timing of development in the eye.

\textbf{Cell cycle progression occurs normally in \textit{rno} eye clones}

In the developing eye, there is a coordinated cell cycle posterior to the morphogenetic furrow in which all undifferentiated cells participate. Upon completion of this "second mitotic wave," cells become postmitotic and differentiate into mature cell types (55). One possible explanation for the developmental delay is that \textit{rno} is necessary for the timely progression through the cell cycle. To test this hypothesis, larval \textit{rno} mutant eye clones were assayed for BrdU incorporation to measure the onset and completion of S-phase in the second mitotic wave. No effect was observed in eye discs bearing \textit{rno} eye clones (Figure 3.14A, B). Likewise, overexpression of \textit{rno} posterior to the morphogenetic furrow did not interfere with S-phase (Figure 3.14C, D). To test for an effect on onset or completion of mitosis, larval \textit{rno} eye clones were labeled with the mitosis-specific chromatin marker, anti-phospho-Histone-H3. No change in the pattern of mitotic nuclei was observed in \textit{rno} eye clones, nor did overexpression of \textit{rno} posterior to the MF cause an obvious change in the second mitotic wave (data not shown). From these experiments, it is concluded that the developmental delay seen in larval \textit{rno} eye clones does not arise from a defect in cell cycle progression.
Discussion

Loss of rno function causes very specific phenotypes in the adult eye. There are extra outer photoreceptors and extra cone cells (Figures 3.1-3). There may also be an excess of pigment cells owing to the lack of apoptosis in rno mutant tissue during the pupal stages (Figure 3.4). These phenotypes are very similar to those seen in argos mutants. Furthermore, rno and argos mutants interact very strongly genetically and rno is necessary for production of Argos ligand in the larval eye disc (Figure 3.10). Thus it seems likely that rno acts upstream of argos in a pathway to prevent inappropriate differentiation during the ommatidial assembly process. Given that rno encodes a putative chromatin remodeling factor, the level of control may be transcriptional. Attempts to support this hypothesis have so far been unsuccessful. In order to answer this question definitively, argos transcription in rno mutant tissue must be assayed directly.

Another possible source of the main rno phenotypes, i.e., extra photoreceptors and cone cells, may be the partial loss of Delta expression in rno eye clones (Figure 3.11). As noted above, Delta loss of function can result in extra outer photoreceptors and the loss of cone cells (17, 43). These events are likely to occur in a sequence, as it has recently been shown that the source of Delta signal for presumptive cone cells is the photoreceptors (53). This hypothesis is not inconsistent with the excess cone cells seen in rno mutant pupal tissue because Delta expression is not lost completely, nor is expression of a Notch pathway readout, E(spl)m7 (Figure 3.12). A likely scenario is that assignment of cone
cell fate is a much slower event in rno mutant tissue. When cone cell fates are eventually specified, the lack of Argos ligand results in their overrecruitment. In this way, the rno mutant phenotype in the adult eye may result from a combination of defects, none of which are absolute, producing a relatively mild phenotype.

The effects of rno loss of function on Delta and Argos expression seem to be the hallmarks of a much more general phenotype. Expression of a whole cadre of developmental markers is slowed, but apparently none are lost (Table 3.3). In many cases, a marker that is absent in the larval eye disc has been observed later in the pupal retina. For other markers, like Chaoptin, eventual expression can be inferred simply by the absence of the expected mutant phenotype (54). The global nature of the rno phenotype suggests a general role in eye specific expression that begins with the differentiation of photoreceptors, similar perhaps to the general role of the Glass transcription factor in photoreceptor differentiation (38). This idea is supported by the similarity of Rno to chromatin remodelling factors, and by the ubiquitous, nuclear expression of Rno in differentiating photoreceptors.

It is curious that such broad effects on the expression of differentiation markers should have such relatively mild effects. Part of the answer to this puzzle is that rno mutant tissue never seems to completely eliminate expression. The marker in question is either expressed at a reduced level and/or its expression is fully restored at a later stage. Nonetheless, the bias in thinking about developmental processes is that timing is essential. Frequently a secreted signal or a transcription factor is used repeatedly
throughout many stages of a developing organism. This is certainly true for the Drosophila eye, especially with regard to the Notch and Ras pathways which are used reiteratively throughout eye development (Chapter 1). Two important ligands for these pathways are strongly affected by rno loss of function. By all rights, chaos should ensue.

Maybe timing is not so essential if the development of every cell type is delayed in unison. For example, Delta is necessary for the specification of cone cells, and in rno mutant eye clones, Delta expression is severely reduced. Presumably, a consequence of low Delta levels is the delay in the onset of cone cell marker expression. Existing models for the specification of cone cells predict that such a defect would result in the loss of cone cells (17, 40, 53), but of course this doesn't happen in rno mutants. The critical difference may be that the cells which normally induce cone cells are themselves delayed, thus the developmental clock for a whole region of tissue is set back. If it is true that in rno mutants, all cells are delayed equally, it raises the question of why the resulting mutant phenotype is the overproduction of cell fates. Perhaps once the differentiation program of the Drosophila eye is set in motion, the specification of cell types is not as dependent upon timely gene expression as are the brakes that keep the process from running out of control.
Materials and Methods

For deletion mapping by PCR, see Appendix A

Chromosome cleaning

*rno* alleles were recombined onto a marker chromosome for the third containing the following markers: *h, th, st, cu, sr, e, ca.* The markers were then removed by recombination with a wild type chromosome. The closest marker to *rno* is *h,* located at band region 66. Seven alleles were cleaned in all: *rno*<sup>G4</sup>, *rno*<sup>Ad2</sup>, *rno*<sup>CF3</sup>, *rno*<sup>Cm3</sup>, *rno*<sup>CT1</sup>, *rno*<sup>CV1</sup> and *rno*<sup>F51</sup> (see Table 3.2 for the mutation associated with each allele). All experiments described in Chapter 3 that use an EMS allele of *rno* were performed with a cleaned allele. For five of these alleles, the mutagenic sequence change has been identified (see Table 3.2).

Generation of *rno* eye clones

*rno, FRT80B* recombinant chromosomes were made as described in Chapter 2. For adult eye clones, *rno, FRT80B/TM6B* males were crossed to *ey-FLP; P[w+]70C, FRT80B* (Bloomington). For pupal eye clones, *rno, FRT80B/TM6B* males were crossed to *to ey-FLP, GMR-lacZ, RpS17, P[w+]70C, FRT80B/TM6B* (Bloomington). For larval eye disc clones *rno, FRT80B/TM6B* was crossed to *eyFLP; P[Ubi-GFP]61E-F, FRT80B* (Bloomington). For pupal clones, white prepupae were marked and aged 40-50 hours at 20° C before dissection. For larval clones, larvae were grown at 20° C until they reached
the wandering third instar stage. The Tb marker on TM6B allowed the unambiguous genotyping of pupae and larvae.

**argos enhancer trap experiment**

In order to measure transcriptional activity from the argos locus in rno eye clones, the argos<sup>W11</sup> enhancer trap line (Bloomington) (20), was recombined onto a rno, FRT80B chromosome. Next, rno, argos<sup>W11</sup>, FRT80B/TM6B males were crossed to eyFLP; P[Ubi-GFP]<sup>61E-F</sup>, FRT80B virgins. By following the Tb marker on TM6B, third instar larvae of the genotype eyFLP/+; rno, argos<sup>W11</sup>, FRT80B/ P[Ubi-GFP]<sup>61E-F</sup>, FRT80B were selected and eye imaginal discs were dissected and fixed (see below for details). An anti-βgal antibody was used to visualize transcription from the enhancer trap insertion (see below for antibody details). This experiment was repeated using rno<sup>Cm3</sup> and rno<sup>CT1</sup> with the same results.

**Larval and pupal eye disc staining**

Dissected larval or pupal eye discs were fixed at room temp for 10 minutes in 4% paraformaldehyde in PBS plus 0.1% Triton-X100 (PBT) to keep discs from sticking. Discs were then washed in PBT and blocked in PBT plus 1% normal goat serum (PNT) for 1 hour at room temp. After blocking, primary antibodies were added in PNT and incubated overnight at 4º C. Discs were then washed 3X briefly and once for 15 minutes. Secondary antibody was added in PNT and incubated at room temp for 2-3 hours. Discs were then washed in PBT 3X briefly and once for 15 minutes. For HRP secondaries, discs were incubated in PBT with 0.5 mg/ml diaminobenzidene, 0.04% NiCl, and 0.001%
H₂O₂ until dark brown staining appeared. Stained discs were washed 6 times in PBT, then mounted in 90% EM-grade glycerol in PBS. For fluorescent secondaries, discs were mounted immediately in 1mg/ml p-phenylene diamine in 90% glycerol in PBS. The following primary antibodies and concentrations were used: mouse anti-Cut (G.M. Rubin, 1:100) (53), rabbit anti-Spalt (R. Bario, 1:200) (36), Rat anti-ELAV (G.M. Rubin, 1:100) (47), mouse anti-Notch (R. Fehon, 1:5,000) (16, 32), mouse anti-Yan (G.M. Rubin, 1:50) (46), mouse anti-Rough (G.M. Rubin, 1:100) (13, 29), mouse 22C10 (Developmental Studies Hybridoma Bank, 1:1000) (30), mouse anti-E(spl) m7 (S. Bray, 1:10), mouse anti-Delta (S. Artavanis-Tsakonas, 1:5,000) (44), mouse anti-Argos (DSHB, 1:10) (18, 51), mouse anti-Chaoptin (DSHB, 1:200) (21, 54), rabbit anti-Tramtrack 88 (A. Travers, 1:250) (39), mouse anti-Prospero (R. Carthew 1:1,000) (27), rabbit anti-βgal (Cappel, 1:20,000), rabbit anti-phospho-Histone-H3 (Upstate, 1:40) (4). Secondary antibodies conjugated to HRP, CY2 or CY3 (Jackson Immunoresearch) were used at concentrations of 1:1,000-2,000. Fluorescent microscopy was performed on a Zeiss LSM510 confocal microscope. For acridine orange staining, pupal eye discs of exactly 50 hours were dissected and immediately incubated in 1 mM acridine orange (Sigma) for 5 minutes, then immediately mounted in PBS and photographed.

**BrdU incorporation into eye imaginal discs**

In order to test BrdU incorporation into *rho* eye clones, eye imaginal discs from third instar larvae were dissected then incubated in Schneider's insect medium (Sigma) with 1 mg/ml BrdU (Roche) for one hour at room temperature. Labeled discs were washed twice in PBT then fixed in 4% paraformaldehyde for 30 minutes. Fixed discs were
washed 3 times in PBT, then the discs were incubated in 2N HCl in PBT for 30 minutes. Discs were then washed 3 times in PBT, then washed twice more for 10 minutes each. Discs were blocked in PNT for one hour, then incubated in mouse anti-BrdU (Becton Dickson, 1:100) (7). Antibody hybridized discs were treated as described above. This analysis was performed on alleles rno$^{CM3}$ and rno$^{CTI}$ with the same results.

**Embryo anti-Rno staining**

Wild type embryos were collected for two hours, the dechorionated in 50% bleach and fixed with vigorous shaking in 5ml 4% paraformaldehyde and 5ml heptane for 20 minutes at room temp. Fixed embryos were devitellinized by shaking in 5 ml methanol and 5 ml heptane for one minute. Devitellinized embryos were washed 3X in methanol, then 3X in PBS plus 0.1% Triton-X100 (PBT), then blocked for one hour at room temp in PBT plus 1% normal goat serum (PNT). Anti-Rno was then added at 1:1000 and incubated overnight at 4°C. Embryos were then wased in PBT 3X briefly then once for 15 minutes. Secondary antibody (1:1000) and DAPI (final concentration of 0.1 μg/ml) was added in PNT and incubated at room temp for 2-3 hours. Embryos were then washed briefly 3X in PBT plus once for 15 minutes. Embryos were then mounted in 1mg/ml p-phenylene diamine in 90% glycerol in PBS.

**Generation of excision lines**

The $l(3)rH321$ insertion in band region 61A was chosen as the line to be excised (see Figure 3.5). This insertion is dominantly marked by a $r_y^+$ transgene. This line was crossed to $Sb$, $P(\Delta2-3)99B$. $l(3)rH321/Sb$, $P(\Delta2-3)99B$ males were crossed to $TM2$,
ry/TM6B, ry virgins and rosy-eyed males and virgins were recovered in the next generation and used to establish a stock. A total of 90 lines were established, one third of which are alleles of rno.

**Sequencing of rno mutant alleles**

rno alleles were balanced over TM6B, Ubi-GFP (Bloomington), then non-flourescing first instar larvae were collected and mashed in TE plus 25 mM NaCl. These smashesites were digested in 200 µg/ml of proteinase K for 30 minutes at 37° C, then heat inactivated at 95° C for 2 minutes. The rno locus was PCR amplified in pieces of 500 to 1000 bp. The PCR primers were also used to sequence using the Big Dye Terminator kit from ABI. Sequence was collected on a ABI 373 laser scanner using ABI 373XL Data Collection software. Gel images were tracked and extracted using ABI Sequencing Analysis 3.4.1 software, and editing and sequence comparison was done using Sequencher 4.1 (Gene Codes Corp).

**Screening of libraries for rno cDNA**

Probes of the PHD region of rno, and the 3' end of rno were made by PCR using the following primer pairs. For the PHD region, primers ZFS (5'TGTTCGATGGGAATTAAAGCCGGAC3') and ZFA (5'CTTCTTACCGGCCTGACTGTG3') were used to generate a 386 bp fragment by PCR from a genomic template, while for the 3'end of the rno coding region, primers BOS (5'TGCGTAGCTTTGATGCGAATTTAG3') and BOA (5'TTGGATCGAGTTGCTATTGCAGGAG3') were used to generate a 1,206 bp fragment. Probes were then made and used to screen filter lifts of λ phage plaques from
the LD (poly dT primed, 0-22 hour embryonic mRNA, Berkeley Drosophila Genome Project (48)) and ERP (random primed embryonic mRNA, BDGP (48)) libraries. Four 3’ probe-hybridizing clones were isolated from the LD library, while two PHD-hybridizing clones were isolated from the ERP library. The cDNAs were recovered from these plaques by excision of pBluescript plus insert from the λZAP arms (Stratagene). End sequencing revealed that the 5’-most, 2.6 kb clone contains several in frame stop codons upstream of the predicted reading frame, indicating that the 5’ end of the coding region had been recovered. The longest 3’ clone was 7.6 kb and contained a poly-A tail. To join the 5’ and 3’ clones, a 1.8 kb fragment was RT-PCRred from total RNA. A full length clone was constructed by subcloning a 2.1 kb Not I/Mlu I 5’ fragment, a 1.8 Mlu I/Bgl II RT-PCR fragment, and a 7.4 Bgl II/Xho I 3’ fragment into the Not I and Xho I sites of pBluescript (Stratagene).

Overexpression of rno

The full length rno cDNA was subcloned into the Not I/Xho I sites of the pUAST transformation vector (8) by ligating the rno cDNA as two pieces. The 5’ piece was an 8.8 kb Not I/Sph I fragment and the 3’ piece was a 2.1 kb Sph I/Xho I fragment. In order to add a Myc epitope to the N-terminus of Rno, a PCR primer was designed to encode a Not I site followed by a Kozac consensus, an ATG, the 11 codons of the myc epitope, and 18 nucleotides of homology to the 5’ end of rno excluding the initial methionine (MV48 5’ CAGTGCAGCCGACAAACATGGAGCAAAAAGCTCATTTTCTGAAGAGGACTTT GAGGTCACAAAGAGGTAAGCGC 3’). PCR of the 5’ end of rno using the full length clone as template was performed using oligos MV48 and MV22 (5’
GATCGCTGTGTAGAGACGCA 3'). The approximately 800 b.p. product was digested with Not I and Sac II and the resulting 275 b.p. fragment was subcloned into Not I/Sac II digested pUAST-rno. The resulting clone, pUAST-myc-rno lacks the 5' UTR of rno and encodes a Myc tag fused to the N-terminus of Rno. Sequencing of the PCR-amplified region and the region derived from the oligo MV48 confirmed the expected sequence of this clone. Qiagen-maxipreped plasmid was then injected to make six transgenic lines by standard techniques (28). To overexpress rno, UAS-myc-rno lines were crossed to sev-Gal4 (Bloomington) and GMR-Gal4 (M. Freeman, line 15) (19).

Generation of anti-Rno antiserum

In frame fusions of rno coding sequence were made with the GST coding sequence in the bacterial expression vector, pGEX-4T-1,2 or 3 (Stratagene). The shortest fusion made joined 234 a.a. to the C-terminus of GST, while the longest added 401 a.a. In this way, almost all of the Rno protein sequence was fused to GST in small pieces. The various fusions were then tested for high level expression when expressed in the E. coli protein production strain BL21. Dense cultures were induced with 0.1 mM IPTG for 1 hour, then 1.5 ml samples were spun down and lysed in 2x SDS protein loading buffer, boiled for 5 minutes, spun briefly and loaded into a gradient gel for SDS-PAGE. Four fusions showed sufficient induction of protein at the predicted size: IR1 (a.a. 700-1018), IR46 (a.a. 602-836), MV1 (a.a. 2607-2853), and MV2 (2815-3215). For these four fusions, larger scale cultures were grown to high density at 37° C, then cooled to 15° C with agitation. Once at 15° C, the cultures were induced with 0.2 mM IPTG for 4 hours. Harvested bacteria was resuspended in TE and 100 mM NaCl and Lysozyme
added to 0.5 mg/ml. The bacteria were incubated on ice for 30 minutes before adding DTT to 5 mM and Triton-X100 to 1% and rocking at 4°C for 15 minutes. A series of 3-6 freeze/thaw lyses followed, then the preps were french pressed at 20,000 lbs/psi. The resulting lysate was centrifuged and incubated with glutathione conjugated agarose beads at 4°C for 1 hour. The beads were washed several times with cold PBS plus 5 mM DTT, then protein was eluted with 0.3% glutathione in 50 mM Tris, pH 8.0. Protein concentration was estimated by comparison with known standards by SDS-PAGE and Coomassie staining. Three mice were inoculated for each of the four GST-Rno antigens prepared. Test bleeds were assayed for immunoreactivity vs. embryos and transfected S2 cells. After three test bleeds the IR1 and IR46 injected mice were discontinued. After seven test bleeds, the MV2 injected mice were discontinued. The MV1 injected mice were injected a total of eleven times, then exsanguinated.

References


Table 3.1: Strength of \textit{rno} phenotypes in different alleles.

<table>
<thead>
<tr>
<th>\textit{rno} allele</th>
<th># eyes examined</th>
<th># ommatidia affected</th>
<th># ommatidia scored</th>
<th>% ommatidia affected</th>
</tr>
</thead>
<tbody>
<tr>
<td>CF2</td>
<td>5</td>
<td>264</td>
<td>582</td>
<td>45.4%</td>
</tr>
<tr>
<td>CM3</td>
<td>4</td>
<td>269</td>
<td>775</td>
<td>34.7%</td>
</tr>
<tr>
<td>CT1</td>
<td>6</td>
<td>393</td>
<td>985</td>
<td>40.0%</td>
</tr>
</tbody>
</table>

Ommatidia composed of \textit{rno} mutant tissue were scored for the presence of extra photoreceptors. Large eye clones were generated using a \textit{Minute} on the \textit{rno} wild type chromosome (see Materials and Methods). Greater than 90% of all ommatidia scored were entirely composed of \textit{rno} mutant cells. Ommatidia were scored if they had at least one mutant R cell.
Table 3.2: List of mutations in *rno* alleles

<table>
<thead>
<tr>
<th>Allele</th>
<th>Codon substitution</th>
<th>Amino acid substitution</th>
<th>Predicted protein length, # a.a.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>Not applicable</td>
<td>Not applicable</td>
<td>3,241</td>
</tr>
<tr>
<td>N1</td>
<td>CGA&gt;UGA</td>
<td>R319Stop</td>
<td>318</td>
</tr>
<tr>
<td>BL1</td>
<td>CAG&gt;UAG</td>
<td>Q2425Stop</td>
<td>2424</td>
</tr>
<tr>
<td>CF2</td>
<td>UGG&gt;UGA</td>
<td>W292Stop</td>
<td>291</td>
</tr>
<tr>
<td>CM3</td>
<td>CAG&gt;UAG</td>
<td>Q617Stop</td>
<td>616</td>
</tr>
<tr>
<td>CT1</td>
<td>CGA&gt;UGA</td>
<td>R319Stop</td>
<td>318</td>
</tr>
<tr>
<td>CV1</td>
<td>CGA&gt;UGA</td>
<td>R1136Stop</td>
<td>1135</td>
</tr>
<tr>
<td>ED1</td>
<td>CAG&gt;UAG</td>
<td>Q1878Stop</td>
<td>1877</td>
</tr>
<tr>
<td>FS1</td>
<td>CAG&gt;UAG</td>
<td>Q719Stop</td>
<td>718</td>
</tr>
</tbody>
</table>
### Table 3.3: Expression of developmental markers in \textit{rno} eye clones

<table>
<thead>
<tr>
<th>Expression test</th>
<th>Normal pattern</th>
<th>\textit{rno} larval clones</th>
<th>\textit{rno} pupal clones</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 anti-Notch</td>
<td>ubiquitous</td>
<td>normal</td>
<td>N.D.</td>
</tr>
<tr>
<td>2 anti-Yan</td>
<td>undifferentiated cells</td>
<td>normal</td>
<td>N.D.</td>
</tr>
<tr>
<td>3 anti-Rough</td>
<td>R2 and R5</td>
<td>normal</td>
<td>N.D.</td>
</tr>
<tr>
<td>4 22C10</td>
<td>all R cells</td>
<td>slight delay</td>
<td>presumed normal*</td>
</tr>
<tr>
<td>5 anti-ELAV</td>
<td>all R cells</td>
<td>slight delay</td>
<td>presumed normal*</td>
</tr>
<tr>
<td>6 anti-E(spl!) m7</td>
<td>broadly posterior to MF</td>
<td>mild reduction</td>
<td>N.D.</td>
</tr>
<tr>
<td>7 anti-Delta</td>
<td>all R cells</td>
<td>strong reduction</td>
<td>N.D.</td>
</tr>
<tr>
<td>8 anti-Argos</td>
<td>all R cells</td>
<td>strong reduction</td>
<td>N.D.</td>
</tr>
<tr>
<td>9 anti-Chaoptin</td>
<td>all R cells</td>
<td>lost</td>
<td>presumed normal*</td>
</tr>
<tr>
<td>10 seven-up enh trap</td>
<td>R1,3,4,6, cone cells</td>
<td>lost</td>
<td>normal</td>
</tr>
<tr>
<td>11 anti-Spalt</td>
<td>R3,4, cone cells</td>
<td>normal in R 3,4, lost in cone cells</td>
<td>normal</td>
</tr>
<tr>
<td>12 anti-Cut</td>
<td>cone cells</td>
<td>lost</td>
<td>normal</td>
</tr>
<tr>
<td>13 anti-Tramtrack 88</td>
<td>cone cells</td>
<td>lost</td>
<td>N.D.</td>
</tr>
<tr>
<td>14 anti-Prospero</td>
<td>R7, cone cells</td>
<td>lost in cone cells</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

Expression phenotypes fall into three general categories. Expression tests 1-3 are unaffected in \textit{rno} mutant tissue. Tests 4-10 show a reduction of expression in the photoreceptors. Tests 10-14 show loss of expression in cone cells. *Presumed normal means that expression for the given marker in \textit{rno} pupal eye clones has not been tested, but is expected to be normal. In the case of 22C10 and anti-ELAV, this is because normal expression is recovered after approximately two ommatidial rows in \textit{rno} larval eye clones. In the case of Chaoptin, expression must have been recovered sometime during pupal stages because this protein is essential for the formation of rhabdomeres, and \textit{rno} eye clones in adults have normal rhabdomeres.
Figure 3.1: Eye clones of rno alleles produce extra outer photoreceptors. A. The wild type arrangement of photoreceptor rhabdomeres. The outer photoreceptors, R1-6 have larger rhabdomeres that are arranged on the outside. The inner photoreceptors, R7 and R8 have smaller rhabdomeres located in the middle (R8 not shown). B. A wild type ommatidium. C. An affected ommatidium from a rno mutant eye clone. There are seven large, outer rhabdomeres, thus there is an extra outer photoreceptor. D. A large rno mutant patch shows that about half of the ommatidia are affected (circled). E. This ommatidium has the rno mutant phenotype of seven outer photoreceptors. The presence of dark pigment granules associated with each rhabdomere (arrowhead) indicates that this ommatidium is entirely composed of wild type cells. Thus, the rno mutant phenotype can occur in a non-cell autonomous manner.
Figure 3.2: The extra outer photoreceptors are of the R3/4 subtype. 

A. A larval third instar eye disc with *rno* clones. The nuclei of R3, R4 and the cone cells are stained with anti-Spalt. The dashed box indicates the area of detail shown in B. The morphogenetic furrow is indicated by the arrow. 

B. An ectopic nucleus between R3 and R4 stains strongly for anti-Spalt. This indicates that the extra photoreceptors seen in adults correspond to the R3/R4 subtype. 

C. A pupal retina containing *rno* clones and a *svp-lacZ* reporter transgene. The β-galactosidase produced is localized to the nucleus. This disc has been stained for anti-β-gal. Reporter expression is strongest in R3 and R4. On the left is circled an unaffected ommatidium. On the right is an ommatidium with the *rno* mutant phenotype. Between R3 and R4 is an ectopic nucleus that contains intermediate anti-β-gal staining. This result supports the conclusion that the extra photoreceptors in adults are of the R3/R4 subtype.
Figure 3.3: *rno* eye clones contain ectopic cone cells. A. *rno* eye clones in third instar larval eye disc. GFP (top) dominantly marks wild type tissue. Anti-Cut (middle) stains the nuclei of cone cells. The merged image (bottom) shows that Cut staining is largely absent from *rno* eye clones, except for some nuclei along the posterior edge of the clones (arrowheads). The presence of Cut-positive nuclei near the more mature, posterior edge of the clone suggests that cone cells may be added later. B. A 40 hour old pupal retina in which a control wild type clone has been generated. Each ommatidium contains four Cut-positive cone cell nuclei (one example is circled). C. In pupal retinas that contain *rno* eye clones, ommatidia with five Cut expressing nuclei are often seen (one example is circled).
Figure 3.4: rno mutants show a drastic reduction in cell death. Large wild type control clones or rno mutant clones were made using a Minute on the wild type chromosome. This results in pupal retinas that are almost completely composed of homozygous mutant tissue. Pupae of the appropriate genotypes were aged to exactly 50 hours APF, upon which the eyes were dissected and stained with acridine orange to label cell death. Wild type eyes show extensive cell death (top) while cell death in rno mutant eyes is suppressed (bottom). This result was observed for alleles rno$^{CM3}$ and rno$^{CT1}$.
Figure 3.5: Deletion mapping of the rno locus. A. Region surrounding the rno locus. New deficiency alleles of rno were generated by P-element excision of the insertion l(3)rH321. Df(3L)EY3-5rH321 is not an allele of rno, whereas Df(3L)rH321Δ26 fails to complement rno. PCR-based deletion mapping of Df(3L)rH321Δ26 shows that the proximal breakpoint (right) is the same as in Df(3L)EY3-5rH321 whereas the distal breakpoint uncovers an additional 5 kb of the rno gene. B. The genomic arrangement of Df(3L)EY3-5rH321. It is not clear why this deficiency can complement rno alleles. Df(3L)EY3-5rH321 uncovers the 5' region of rno, and might be expected to severely reduce transcription of rno. However, none of the coding region is deleted. Perhaps transcription of rno occurs in this mutant by control elements present in the l(3)rH321 insertion, or by the fusion of the nurf301 promoter region to the proximal side of the P-element insertion (marked by asterisk, compare A to B).
**Figure 6: Rno is a PHD zinc finger protein.** Schematic drawing of Rno protein is shown. Within the first several hundred amino acids of Rno, there is a PHD zinc finger, followed by a zinc finger-like Cysteine Rich Region (yellow boxes). The positions of nonsense mutations found in *rno* alleles are indicated by arrows (see Table 2). Comparison of Rno to a predicted ortholog in the mosquito, *Anopheles gambiae*, and a predicted homolog in humans shows that the N-terminal domain is highly conserved (62% identical and 72% similar between D. m. and A. g. Rno, 46% identical and 61% similar between D. m. and human KIAA0215). Other conserved regions are small and dispersed (indicated by small boxes). Most of these sequences are rich in basic amino acids. The two purple boxes represent a direct repeat that is not found in any other predicted protein. Outside of the conserved regions, the sequence is highly repetitive, containing long stretches of poly-serine or poly-glutamine. The positions of these low-complexity regions are not strictly conserved relative to the other conserved domains, and thus are not indicated in this drawing.
Figure 3.7: Overexpression of rno in the developing eye results in a loss of pigment cells. Pictured above are sections of adult eyes. A severe loss of pigment cells can be noted by the gaps in the black lattice surrounding each ommatidium. This is a non-cell autonomous mechanism, because the sevenless enhancer used to drive expression of Gal4 is not expressed in pigment cells (Basler et al, 1989).
Figure 3.8: Rno is expressed in the nucleus. A. Late blastoderm stage embryo stained with anti-Rno shows strong nuclear expression. B. Culture Drosophila S2 cell transfected with Ubiquitin-Gal4, UAS-rno stained with anti-Rno also shows nuclear localization. C. Image from B merged with phase contrast image.
Figure 3.9: Rno is expressed broadly throughout the third instar eye disc. Eye clones of rno mutants were stained with anti-Rno to demonstrate the specificity of the staining. A. Wild type cells are dominantly marked with GFP. Top: a view of the whole eye disc. The magnified region shown in the lower panels is indicated by the dashed box. The edges of a prominent clone are outlined. B. Anti-Rno staining is seen broadly throughout the eye disc starting anterior to the morphogenetic furrow and extending all the way to the posterior edge of the eye. The furrow is indicated by an arrow. Anti-Rno stains the nuclei of the R cells (bottom). Punctate staining can be seen in nuclei, as well as more diffuse nuclear staining. Staining within the outlined clone is reduced relative to the wild type tissue. C. Merged images.
Figure 3.10: *rno* interacts genetically with *argos* and is necessary for Argos expression. **A.** Left: a viable combination of *argos* alleles results in flies with severe blistering along the posterior edge of the eye. This results from a confluence of excess cone cells. Right: alleles of *rno* can dominantly enhance this phenotype, producing a smaller, rougher eye. This result has been seen with *rno*<sup>CM3</sup>, *rno*<sup>CT1</sup> and *rno*<sup>Cvi</sup>. **B.** A *rno* eye clone stained with anti-Argos (outlined). Wild type cells are dominantly marked by GFP (left). Anti-Argos staining is drastically reduced in *rno* eye clones (outlined).
**Figure 3.11: rno is necessary for production of Delta**

A. The wild type expression pattern of anti-Delta antibody. Staining is seen in vesicles in the apical part of the R cells. The morphogenetic furrow is indicated by an arrow. B. Staining of anti-Delta in rno mutant eye clones. Left: Wild type cells are dominantly marked by GFP. A prominent rno clone is outlined. Middle, Right: anti-Delta staining is strongly reduced, but not abolished in rno eye clones. This result has been seen for \( rno^{CM3} \), \( rno^{CT1} \) and \( rno^{CV1} \).
Figure 3.12: Expression of E(spl)m7 is reduced in rno eye clones. Left: GFP dominantly marks wild type cells. A prominent rno clone is outlined. Middle, Right: anti-E(spl)m7 staining is reduced in rno mutant clones. This Result has been observed for rno^{CM1}, rno^{CT1} and rno^{CV1}. This result is consistent with a reduction in Notch receptor activation that would accompany the reduction in Delta expression in rno clones. The morphogenetic furrow is indicated by an arrow.
Figure 3.13: The onset of differentiation is delayed in rno eye clones. Expression of an early marker for photoreceptor differentiation is slightly delayed. Left: wild type cells are dominantly marked by expression of GFP. Middle, Right: Expression of ELAV is delayed in the rno mutant clone by two ommatidial rows, corresponding to roughly four hours. The white arrowheads in the middle and right correspond to wild type photoreceptors while the blue arrowheads are rno mutant photoreceptors. This result has been observed for rno^{CM3} and rno^{CT1}. 
Figure 3.14: The post-furrow S-phase is insensitive to rno gene dosage. In larval eye discs that have been labeled for BrdU incorporation, the coordinated S-phase that occurs posterior to the MF is apparent (A). In eye discs that bear clones homozygous mutant for an allele of rno, there is no obvious perturbation of this pattern (B). This experiment was performed for alleles rno<sup>CM3</sup> (18 eye discs scored) and rno<sup>CTI</sup> (16 eye discs scored). Likewise, overexpression of rno posterior to the MF using a GMR-Gal4 driver had no effect on the onset or completion of S-phase (C,D). Expression of Rno protein in GMR-Gal4; UAS-myc-rno eye discs was confirmed by anti-Myc antibody staining (not shown).
Chapter 4
Biological functions of the ISWI chromatin remodeling complex NURF

This chapter was produced in collaboration with Paul Badenhorst and Carl Wu of the NIH. My specific contributions include the genetic screen that produced all of the EMS alleles of nurf301, the identification of l(3)ry122 as a nurf301 allele (nurf301') and the identification of the macrophage hyperplasia phenotype (also independently discovered by Paul Badenhorst).
Biological functions of the ISWI chromatin remodeling complex NURF

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The nucleosome remodeling factor (NURF) is one of several ISWI-containing protein complexes that catalyze ATP-dependent nucleosome sliding and facilitate transcription of chromatin in vitro. To establish the physiological requirements of NURF, and to distinguish NURF genetically from other ISWI-containing complexes, we isolated mutations in the gene encoding the large NURF subunit, nurf301. We confirm that NURF is required for transcription activation in vivo. In animals lacking nurf301, heat-shock transcription factor binding to and transcription of the hsp70 and hsp26 genes are impaired. Additionally, we show that NURF is required for homeotic gene expression. Consistent with this, nurf301 mutants recapitulate the phenotypes of Enhancer of bithorax, a positive regulator of the Bithorax-Complex previously localized to the same genetic interval. Finally, mutants in NURF subunits exhibit neoplastic transformation of larval blood cells that causes malnautic tumors to form.

[Keywords: NURF, chromatin remodeling, ISWI, Drosophila, JAK/STAT pathway]

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The establishment of the body plan of an animal requires the correct initiation and maintenance of expression of a number of developmental regulators. In insects, segmental identity is conferred by region-specific expression of overlapping sets of homeotic selector genes. Systematic genetic screens for changes in segmental fate have been used to identify a wide class of positive-acting transregulatory factors required for homeotic gene function, the trithorax-group (trxG; Kenison 1995). Members of the trxG define novel chromatin-modifying activities. One class comprises the ATP-dependent chromatin remodeling factors. These are multisubunit protein complexes that utilize the energy of ATP hydrolysis to alter gene architecture by perturbing the dynamic properties of nucleosomes, the basic units of chromatin. They can be divided into broad families depending on the catalytic subunit utilized. Brahma, one of the defining members of the trxG, and its yeast homolog SWI2/SNF2 provide the catalytic subunit of the first group of SWI/SNF chromatin remodelers identified (Tamkun et al. 1992).

Other categories of ATP-dependent chromatin remodeling complexes have been discovered using biochemical approaches. In Drosophila, the ISWI ATPase forms the catalytic core of three protein complexes that have been purified from nuclear extracts: ACF [ATP-utilizing chromatin assembly and remodeling factor; Ito et al. 1997], CHRAC [chromatin accessibility complex; Varga-Weisz et al. 1997] and NURF [nucleosome remodeling factor; Tsukiyama et al. 1995]. All catalyze energy-dependent nucleosome sliding in vitro (Corona et al. 1999; Hamiche et al. 1999; Langst et al. 1999), but with distinct results by the different complexes. ACF and CHRAC can assemble and slide nucleosomes to establish regular ordered arrays [Ito et al. 1997; Varga-Weisz et al. 1997]. In contrast, NURF-induced sliding disrupts nucleosomal periodicity (Tsukiyama and Wu 1995; Hamiche et al. 1999). NURF acts in concert with transcription factors to mobilize nucleosomes at promoters, facilitating transcription activation in vitro (Tsukiyama and Wu 1995; Mizuguchi et al. 1997). These properties suggest that NURF should be required for transcription in vivo.

NURF is a complex of four protein subunits. In addition to NURF140—the ISWI ATPase (Tsukiyama et al. 1995)—NURF contains NURF55, a WD-40 repeat protein found in other protein complexes involved in histone metabolism (Martinez-Balbas et al. 1998; NURF58, an inorganic pyrophosphatase (Gdula et al. 1998); and a large subunit NURF301 (Xiao et al. 2001). NURF301 is distantly related to the ACF1/WCRF1 subunits of the ACf and WCRF chromatin remodeling and assembly complexes [Ito et al. 1999]. NURF301 contains the PHD fingers, bromodomain, WSTF/Acf1/ebp146 (WAC), and WSTF/Acf1/KIAA0314/ZK783.4 (WAKZ) motifs found in these proteins, but also possesses an N-terminal HMG1/Y-like domain. Reconstitution experiments reveal that both NURF301 and ISWI contribute to the

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chromatin remodeling activities of NURF. In addition, 
nur301 provides an interface through which NURF 
can interact with a number of transcription factors, in 
particular the GAGA transcription factor [Xiao et al. 
2001].

We sought to characterize the physiological function 
of NURF. Clues to this have been provided by mutations 
that affect the catalytic ISWI subunit. Analysis of iswi 
mutant animals indicates that ISWI is required for 
homeotic gene activation and chromatin condensation 
in vivo (Deuring et al. 2000). However, as ISWI is present 
in NURF and at least two other Drosophila chromatin 
remodeling complexes—ACF (Ito et al., 1997) and CH 
RAC (Varga-Weisz et al. 1997)—functions could not be 
ascrived to a specific remodeling complex. Given the 
importance of NURF301 for the in vitro activities of NURF, 
we focused our studies on NURF301 and isolated mutations 
in this largest NURF subunit. Here we show that 
nur301 is required for transcription activation in vivo. 
In nur301 mutants homeotic gene expression is 
impaired. Moreover, we demonstrate that nur301 corre 
sponds to an uncharacterized member of the trxG, 
Enhancer of bithorax [Ebx]]]. NURF301 is required for induction 
of the heat-shock genes and has additional functions during larval blood cell development.

Results

Isolation and characterization of nur301 mutants

The gene encoding the large NURF subunit, nur301, 
was mapped by chromosomal in situ hybridization to the 
cytological interval 61A. To isolate nur301 mutants, we 
first screened P-element lines that had been localized to 
this region for those that contained inserts within or 
immediately upstream of nur301. We identified one 
homeozygous lethal P-element line [E3]py122 we call 
nur3011), in which a P-element had inserted in the un 
translated leader sequence of nur301 (summarized in 
Fig. 1A). Subsequently, we recovered a series of EMS 
induced mutations that failed to complement nur3011. 
The three alleles presented [nur3012, nur3013, 
and nur3014] encode truncated NURF301 proteins due to 
the introduction of stop codons at amino acids 546, 750, 
and 1536, respectively. Homozygous nur301 mutants 
heteroallelic combinations were lethal at late-third 
larval instar or early pupal stages. We also identified 
three homozygous viable EP insertion lines [Rorth 1996] 
in which P-elements had inserted upstream of the 
nur301 transcription start site. These were used as substrates for imprecise excision to recover lethal excision 
lines that fail to complement nur3011. The deficiency

Figure 1. Characterization of nur301 mutants. [A] The structure of the nur301 
locus, the position of P-element insertion 
lines, and the location of EMS-induced lesions are shown. nur3012 
and nur3013 encode glutamine to stop codon changes at 
the amino acids 546 and 750, respectively. 
nur3014 contains a splice-donor site muta 
tion that blocks splicing of the fourth 
intronic. The aberrant transcript introduces 
four additional amino acids [GKLF] and an 
in-frame stop codon after proline residue 
1531. [B,C] In situ hybridization using a 
1.3-kb probe spanning the 3' end of 
nur301 (nucleotides 6800–8000) shows 
that ubiquitous expression of nur301 is 
lost in homozygous mutant nur3012 lar 
vae (cf. wild-type and mutant third instar discs). [D] Dot-blot analysis of third larval 
instar total RNA (in triplicate) shows that nur301 RNA is reduced in nur301 
mutants relative to wild-type. In contrast, 
iswi and CG7020, a putative gene located 
upstream of nur301 (a fragment from the 
EST clone LP07661 was used as a probe), 
are unaffected. rp49 provides a loading 
control. [E] Protein levels of the NURF 
subunits, NURF38, NURF55, and ISWI, 
and the ACFI subunit of ACF are un 
changed in nur3012 and nur3014 mut 
ants, relative to α-TUBULIN as shown by Western analysis of extracts from third instar larvae.

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Df(3)L3643 was characterized by PCR and shown to remove the ATG of nurf301 and at least two flanking genes downstream of nurf301.

We showed by in situ hybridization (Fig. 1B,C) and Northern analysis (Fig. 1D) that the normal expression of nurf301 is reduced in the P-element insertion line nurf301. In this mutant, transcription of iswi or a predicted gene upstream of nurf301 (CG7020) is unaffected (Fig. 1D). We confirmed that lethality of this line is caused by the P-element insertion as precise excision of the P-element restores viability. In all the nurf301 mutants, the protein levels of the three other NURF subunits (ISWI, NURF55, and NURF38) and the ACF1 subunit of ACF and CHRAC are unchanged (data shown for nurf301 and nurf301 in Fig. 1E). This indicates that the nurf301 mutations specifically compromise NURF activity without significantly affecting the other SWI-containing complexes ACF or CHRAC.

Heat-shock gene activation is impaired in nurf301 mutant animals

NURF had originally been purified from nuclear extracts as a factor required to disrupt chromatin assembly in vitro on promoters of the Drosophila heat-shock gene hsp70. Using in vitro assays it was shown that NURF cooperates with the GAGA factor to mobilize nucleosomes on the promoter of the heat-shock genes, establishing a nucleosome-free domain over the promoter, thus exposing sites for the heat-shock transcription factor [HSF, Tsukiyama et al. 1994; Tsukiyama and Wu 1995].

We examined if expression of the heat shock genes is affected in nurf301 mutant animals. RNA dot-blot analysis shows that heat-shock-induced transcription of hsp26 and hsp70 is impaired in nurf301 larvae (Fig. 2A). Impaired transcription leads to reduced heat-shock protein accumulation. Although HSF70 is detected in wild-type siblings after 10 min heat-shock, no protein is expressed in mutant animals (Fig. 2B). After 20 min of heat-shock, HSF70 is highly expressed in wild-type larvae. In contrast, nurf301 mutant animals only express low levels of HSF70 (Fig. 2B).

Consistent with these findings, binding of the heat shock transcription factor HSF is impaired in nurf301 mutants. In wild-type animals, immunofluorescence of salivary gland polytene chromosomes shows that, with the exception of the hsp83 locus at 63B, HSF does not generally bind to polytene chromosomes in the absence of heat stress (Fig. 2C). HSF-binding to hsp83 has been shown to be qualitatively different from the other heat-shock loci (Shopland and Lis 1996). The hsp83 locus does not appear to be GAGA factor-dependent as no (CACT) elements are present in this locus [Tsukiyama et al. 1994]. Instead, HSF-binding is proposed to be facilitated by other transacting factors [Shopland and Lis 1996].

Within several minutes of heat-shock, the bulk of HSF forms trimers competent for specific DNA-binding, and binds to the heat-shock loci and a number of other sites [Westwood et al. 1991]. Prominent are the 87A and 87C loci (Fig. 2C, bracketed) which carry two and three copies, respectively, of the hsp70 gene. In nurf301 mutant animals, hsp83 accumulates HSF as in nonshocked conditions. After 2 or 5 min heat-shock, no binding of HSF to hsp70 loci is detectable (Fig. 2C). HSF-binding to the hsp70 loci can only be detected after 10 min of heat-shock, and HSF staining is reduced compared to wild-type animals (Fig. 2C). Impaired HSF-binding is accompanied by reduced accumulation of RNA polymerase II at the hsp70 loci (data not shown).

nurf301 is required for homeotic gene expression

Deuring et al. [2000] had shown that ISWI, the catalytic subunit of NURF, is required for expression of the homeotic gene engrailed [en]. However, ISWI is also a component of two other chromatin remodeling complexes, ACF [Luo et al. 1997] and CHRAC [Varga-Weisz 1997]. To resolve which ISWI-containing complex is required for homeotic gene expression we examined expression of Ultrabithorax (Ubx) and engrailed (en) in nurf301 mutant animals. We found that when both copies of nurf301 are mutated, in homozygous mutant nurf301 larvae, expression of the UBX protein becomes undetectable. The normal expression of UBX in the haltere and third leg discs of wild-type third instar larva is absent in nurf301 mutant animals (Fig. 3A,B). We also showed that expression of the homeotic gene en requires nurf301. The normal expression of EN in the posterior compartment of imaginal discs is abolished in nurf301 mutants (Fig. 3C,D). Semiquantitative RT–PCR analysis confirms that Ubx and en transcript levels are reduced in nurf301 mutant animals. These results confirms that the defects in homeotic transcription seen in iswi mutants are caused by abrogated NURF function.

nurf301 recapitulates E(bx) phenotypes

A positive regulator of the Bithorax-Complex, E(bx), had previously been localized genetically to 61A, the same cytological interval as nurf301 [Bhosker and Babu 1987; Lewis cited in Lindsley and Zimm 1992]. However, unlike numerous regulators of the BX-C, E(bx) has not been cloned. As NURF is required for expression of Ubx, we tested whether nurf301 corresponds to E(bx). Both alleles of E(bx) were no longer extant, so we examined if the mutations we had isolated in nurf301 recapitulated the published morphological properties of E(bx) mutants.

We found that nurf301 mutants, like E(bx), increase the severity of bithorax (bx) mutant phenotypes. bx is a DNA regulatory element required for correct expression of Ubx in regions that give rise to the third (T3), but not second thoracic segment (T2) of the adult fly [Lewis 1978; Bender et al. 1983]. This expression distinguishes T3 from T2 identity. Loss or reduction of UBX levels in bx mutant animals [Ubx<sup>45</sup>]<sup>bx<sup>-</sup></sup> and Ubx<sup>45</sup>/bx<sup>+</sup> mutant combinations [shown in Fig. 4A,B] causes a homeotic transformation of the third thoracic segment to the
Figure 2. *nurfs01* is required for heat-shock gene induction. (A) A quantitation of RNA dot-blot shows delay in hsp26 and hsp70 transcription in *nurfs01* mutant animals. *hsp26* and *hsp70* transcript levels are normalized to *ep49*. Points and bars represent the mean and standard deviation of three independent determinations. (B) Western blotting shows that HSP70 protein synthesis in homozygous mutant animals is reduced relative to the wild-type. (C) HSF was localized to the *hsp70* loci [brackets] and *hsp83* locus (arrowheads) on polytene chromosomes with antibodies against HSF, following 0-, 2-, 5-, 10-, and 15-min heat-shock. HSF-binding to the major heat-shock loci is impaired in *nurfs01* mutants.

anterior second thoracic segment. Thus, the third thoracic segment, which is normally vestigial and naked, is transformed into the second thoracic segment, increasing its size and causing sensory bristles to develop (Fig. 4A). Moreover, the haltere ([T3]) is transformed toward wing fate ([T2]), manifested by increases in size and the development of bristles. The strength of these transformations is increased when one copy of E(bx) also is removed (Bhosekar and Babu 1987). Mutation of one copy of *nurfs01* similarly enhances bx phenotypes. With one copy of either the *nurfs01*, *nurfs01* or a deficiency that removes *nurfs01*—Df[3L]3643—the strength of the
nur301. Like ISWI [Deuring et al. 2000], NURF301 is required for oocyte development.

**Aberrant male X chromosome morphology in nur301 mutant animals**

The defects in homeotic transcription seen in nur301 mutant animals effectively duplicate those reported for animals lacking the catalytic ISWI subunit [Deuring et al. 2000]. However, ISWI is also required to maintain higher order chromosome structure. In iswi mutants the male X chromosome is grossly disrupted relative to autosomes as revealed by polytene chromosome preparations [Fig. 5B; Deuring et al. 2000]. We found that in nur301 mutants the male X chromosome is similarly affected. The male X chromosome, identified by anti-MSL2 staining, is reduced in length and breadth, as seen in iswi mutant animals [cf. wild-type and mutant animals in Fig. 5A,D-F]. The male X chromosome in homozygous mutant nur301 mutant animals is highly aberrant [Fig. 5D]. Disorganized male X chromosome morphology also was observed in nur301/nur301 and hemizygous nur301/Df(3L)3643 mutant animals [Fig. SE,F]. Our results demonstrate that the chromosome condensation defect caused by perturbed ISWI function is mediated through the NURF complex. Although the effects on male X chromosome structure suggest that NURF can influence global chromosome structure, we found that NURF function was not required for heterochromatic gene silencing. Reduction in NURF301 levels has no effect on position effect variegation [data not shown]. This is consistent with results reported by Cryderman et al. [1999] who showed that iswi is not a modifier of PEV.

**Mutations in NURF mutants affect hematopoietic development**

During the course of this analysis we noticed that nur301 mutant animals display a high incidence of melanotic tumors. Melanotic tumors have previously been reported in a number of mutant backgrounds and are generally caused by neoplastic transformation of the larval blood cells. The circulating cells (hemocytes) of the larval blood or hemolymph provide one tier of the innate immune system of insects by encapsulating or engulfing pathogens (for review, see Jemmler and Hoffman 2001, Luo and Deinolf 2001, Tzou et al. 2002). A number of mutations have been shown to trigger the overproliferation and premature differentiation of hemocytes. Tumors form when these cells aggregate, or invade and encapsulate normal larval tissues [Gatelf 1978, Rizki and Rizki 1979].

We observed melanotic tumors both in EMS-induced nur301 mutants that truncate NURF301, the P-element induced mutation that reduces nur301 transcript levels, and allelic combinations of these mutants [Fig. 6A]. Tumor penetrance is extremely high (100% for nur301e at 25°C). Consistent with tumor development, circulating hemocyte cell number was increased dramatically in hemolymph isolated from nur301 mutant animals [Fig.
Figure 4. *nurp301* corresponds to *E(bx)*. In the presence of one copy of either *nurp301*¹, *nurp301*², or the *nurp301* deficiency Df(3L)3643, the strength of the anterior transformation seen in *bithorax* mutant combinations is increased. Transformation is scored both by increases in the size and bristle number on the metathoracic segment and by the severity of the haltere (T3) to wing (T2) transformation. A shows bx· bx· Ubx· Ubx· Ubx· bx combinations and B shows bx· bx· Ubx· Ubx· bx combinations. Mean haltere and T3 bristle numbers ± standard deviation for 20 flies of each genotype are indicated. Arrowheads denote the metathorax (T3). Photographs of nota are composites of three images taken at different focal planes of the animals.

6B). A large percentage of animals lacking ISWI, the catalytic subunit of NURF, also displayed melanotic tumors confirming that disrupted NURF function induces tumor formation [data not shown]. In iswi mutant animals the number of circulating hemocytes is also increased [Fig. 6B]. In both *nurp301* and *iswi* mutant hemolymph, small aggregates of hemocytes are often observed. All hemocyte cell types are present, from small round cells (prohemocytes) to crystal cells and lamellacytes.

In *Drosophila*, larval blood cell transformation and
melanotic tumor formation can be induced by inappropriate activation of either of two distinct signaling cascades: the TOLL or the JAK/STAT pathway. Inappropriate activation and nuclear-localization of the Drosophila NF-κB homolog DORSAL, caused either by constitutive activation of the TOLL receptor or removal of the inhibitor, the Drosophila IκB CACTUS, leads to melanotic tumors in third instar larvae [Qui et al. 1998]. In the second pathway, gain-of-function mutations in HOPSCOTCH (HOP), the Drosophila Janus Kinase (JAK), induce melanotic tumors [Harratt and Denuit 1993; Harrison et al. 1995; Hou et al. 1996]. HOP gain-of-function mutants cause tumor development by triggering constitutive activation and DNA-binding by the Drosophila STAT transcription factor, STAT92E.

To resolve whether the melanotic tumors seen in the nurf301 mutants were caused by misregulation of either the TOLL or HOP/STAT92E pathways, we determined if nurf301 mutants enhance tumor phenotypes seen in constitutively active TOLL or HOP mutant lines. We found that tumor incidence in animals carrying one copy of a gain-of-function HOP mutation—hop<sup>tron-1</sup>—is increased by simultaneous reduction in NURF301 levels [Fig. 7A]. In contrast, removal of one copy of NURF301 fails to enhance the TOLL gain-of-function allele Tt<sup>1{1984}</sup> (data not shown). The results suggest that NURF acts as a negative regulator within the Drosophila JAK/STAT signaling pathway.

Molecular signatures of both JAK and TOLL activation have been defined. It is known that HOP gain-of-function mutants induce expression of a complement-like protein TEF1 [Lagueux et al. 2000]. Overactivation of the TOLL pathway also induces TEF1 synthesis but primarily induces expression of antimicrobial peptides, including DROSOMYCIN (DRS) and DIPTERICIN (DPT). We found that loss of nurf301 induces tep1 but fails to induce drs or dpt (Fig. 7B), demonstrating that NURF301 principally affects the HOP/STAT92E pathway. We tested whether nurf301 interacts genetically with other known components of the HOP/STAT92E pathway. Certain mutations in unpaired (upd, also known as outstretched), which encodes a ligand for the HOP receptor, display a characteristic wings-out phenotype, due to decreased activation of HOP and consequently decreased STAT92E function. When NURF301 levels are simultaneously decreased in these mutant backgrounds, animals are mostly restored to the wild-type [Fig. 7D]. These genetic interactions confirm that NURF301 acts as a negative regulator of the HOP/STAT92E pathway, at a point downstream of HOP. Hence, disruption of NURF could affect either STAT92E or the targets of STAT92E. We found that in nurf301 mutants, levels of the STAT92E transcription factor are not elevated [Fig. 7C], suggesting that NURF acts to repress the activity of STAT92E or the expression of some STAT92E target genes.

Discussion
Here we provide a physiological correlate for the demonstrated biochemical properties of the nucleosome re-
NURF is required for transcription in vivo

Our results demonstrate that NURF is also required in vivo for transcription of the heat shock and homeotic genes. This agrees with the demonstrated biochemical properties of NURF. NURF can catalyze nucleosome sliding in vitro, in this manner disrupting chromatin structure [Hamitche et al. 1999]. Nucleosome sliding on promoters offers a simple way to clear chromatin from promoter elements and has been shown to potentiate the binding of transcription factors and the recruitment of transcription initiation machinery in vitro [Mizuguchi et al. 1997; Kang et al. 2002]. Deployment of NURF at target promoters should be critical for gene activation.

An important question is how NURF is recruited to target sites in vivo. Four genes we show to be dependent on nur301 for expression — bag, en, hasp26, and hasp70—all contain multiple binding sites for the GAGA factor, which is genetically required for their correct expression [Soeller et al. 1993; Farkas et al. 1994; Bhat et al. 1996]. On the Drosophila hasp70 and hasp26 promoters (GA.CG), cognate elements to which the GAGA factor binds are required for HSF-binding. When these sequences are deleted, HSF-binding to transgenes in polyethylene chromosome is impaired [Shopland et al. 1995; Shopland and Lis 1996], consistent with the defects we see in nur301 mutant animals. It is therefore compelling that recent biochemical studies show that NURF and the GAGA factor bind to each other in crude extracts, and that purified NURF and GAGA factor interact directly in vitro [Xiao et al. 2001]. The principal interacting domains map to an N-terminal region of NURF301 and a stretch flanking the Zn finger DNA-binding motif of GAGA factor [Xiao et al. 2001]. These data suggest that NURF is recruited by the GAGA factor through specific, direct interactions with the NURF301 subunit, to catalyze local sliding of nucleosomes at bx, en, hasp26, and hasp70 promoters, increasing accessibility to sequence-specific transcription factors and RNA polymerase II. Curiously, though, reduction of nur301 levels fails to enhance phenotypes of mutations in Trithorax-like, the

Figure 6. Melanotic tumors in NURF mutant animals. Melanotic tumors occur in homozygous mutant nur301 third instar larvae. A) Tumors are observed in all combinations of nur301 mutant alleles. Genotypes displayed are nur301/nur301 [a], nur301/nur301 [b], nur301/nur301 [c], nur301/nur301 [d], and nur301/nur301 [e]. B) The circulating hemocyte cell number in hemolymph isolated from nur301 and isw1 mutant third instar larvae is increased considerably with respect to wild-type animals.

Modeling factor [NURF]. Our results show that the largest subunit of NURF, NURF301, is required for transcription in vivo. These data confirm an earlier report of Tamkun and co-workers [Deuring et al. 2000] that showed that ISWI, the catalytic subunit of NURF, is required for transcription activation in vivo. However, because ISWI is also a subunit of the ACF [Ito et al. 1999] and CHRAC [Varga-Walcz et al. 1997] chromatin remodeling complexes, it had not previously been possible to assign iswi phenotypes to a specific remodeling factor. As NURF301 is not present in ACF and CHRAC, by analyzing nur301 mutations we have been able to resolve specific functions of NURF. We show that for the tested phenotypes, mutations in nur301 and iswi are indistinguishable. We also demonstrate that NURF functions during larval blood cell development. In the absence of either the NURF301 or ISWI subunits, circulating hemocytes undergo a neoplastic transformation that results in the over.proliferation of blood cell types. Melanotic tumors are formed when these cells invade and encapsulate normal larval tissues. Our data suggest this is caused by misregulation of the Drosophila JAK/STAT pathway.

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Figure 7. NURF is a negative regulator of the JAK/STAT signaling pathway. (A) Reduction of nurf301 levels enhances gain-of-function hop701 mutant phenotypes. Vials of flies were raised at either 25°C or 28°C. The percentage of adult female flies of each genotype in a vial that display tumors was scored as tumor incidence. Data are the mean and standard deviation of at least six vials of flies. (B) nurf301 mutant animals express elevated levels of tep1 but neither the antimicrobial peptide gene drosomycin (drs) nor dpt (dpt) is induced. (C) Levels of the STAT92E transcription factor are not elevated in nurf301 mutant animals. (D) Reduction of NURF301 levels suppresses the phenotypes of mutations in a ligand for the JAK receptor UNPAIRED (unp; stretched). (E) Models for how NURF may down-regulate the JAK/STAT92E signaling pathway. NURF could either repress targets of STAT92E or be required for expression of a negative regulator of STAT92E activation. In either case, in the absence of NURF, STAT92E target genes will be transcribed, mimicking STAT92E activation.

gene that encodes the GAGA factor [P. Badenhorst, unpublished].

Conceptual models of the function of chromatin remodeling machines reinforce the view that these complexes are required exclusively during gene activation to expose or “open-up” chromatin. However, nucleosome sliding could equally be harnessed to repress genes. Phenotypic analysis of the orthologous yeast ISW2 complex suggests that ISWI-containing complexes may function both to activate and repress genes. The yeast ISW complexes appear to be recruited to sites within the genome through direct interaction with DNA-binding proteins to activate and repress genes by repositioning nucleosomes (Goldmark et al. 2000, Kent et al. 2001). Drosophila ISWI has been shown to be associated with transcriptionally silent regions of chromatin in salivary gland nuclei, suggesting that it may be involved in repression in this tissue (Deuring et al. 2000). Our analysis of NURF function
during larval blood cell development suggests that NURF can repress targets of the JAK/STAT signaling pathway. We show that in mut mutants the expression of the complement-like protein TEP1 is induced. It remains to be established whether teph is a direct target of NURF.

**NURF and tumorigenesis**

Misregulation of TEP1 is seen in mutants that overactivate the Drosophila JAK/STAT [HOP/STAT92E] signaling cascade and induce the formation of melanotic tumors. Increased signaling through the HOP/STAT92E pathway leads to the overproliferation and aberrant differentiation of larval blood cells that subsequently invade and encapsulate normal host tissue [Henratty et al. 1993; Harrison et al. 1995]. We have found that animals lacking NURF301, or the catalytic ISWI subunit, exhibit an identical neoplastic transformation of larval blood cells. In the absence of NURF, the proliferation and differentiation of hemocytes, and the accumulation of lamellocytes, is triggered.

Our data suggest that NURF normally represses targets of the HOP/STAT92E pathway. Genetic epistasis places nrf301 downstream of HOP. We show that loss of NURF resembles gain-of-function mutations in hop, and that targets of the HOP/STAT92E cascade are upregulated in nrf301 mutants. Normally, HOP activation leads to the expression and posttranslational modification of STAT92E [Chen et al. 2002]. We found that although nrf301 mutants activate the HOP/STAT92E pathway, the levels of the STAT92E transcription factor are unchanged in NURF mutant animals. We suggest that NURF acts downstream of STAT92E. In resting cells, in the absence of HOAl/STAT92E signaling, NURF could normally repress STAT92E target genes. When NURF is removed, repression is no longer maintained, and targets are transcribed mimicking the effects of HOP activation. However, STAT activity is also influenced by a number of inhibitory, STAT-binding proteins. Among these are the suppressor of cytokine signaling (SOCS, Callius and Mathey-Prevot 2002) and protein inhibitor of activated STAT (PIAS) family of inhibitors [Bet et al. 2001; Haire et al. 2001]. It is possible that NURF is required for expression of one such inhibitor, and that loss of NURF activates the HOP/STAT92E cascade by removing a STAT inhibitor.

The involvement of NURF in larval blood cell development agrees well with recent literature implicating a number of chromatin-modifying or chromatin-associated complexes in hemocyte development and melanotic tumor formation. Mutations in *modolo*, which encodes an interacting partner of the coactivator CBP, cause melanotic tumors [Bantignies et al. 2001]. Of particular significance, screens for mutations that cause hematopoietic defects identified *domino* (dom), which encodes a member of the SW2/SNF2 family of DNA-dependent ATPase that is distantly related to ISWI [Rufi et al. 2001]. Mutations in *domino* cause overproliferation of hemocytes, like NURF mutants. However, unlike NURF mutants, hemocytes fail to enter the hemolymph and remain trapped in enlarged lymph glands that become melanized. It will be interesting to assess the relative contributions of the ISWI and DOM complexes in the regulatory hierarchy of larval blood development.

**NURF maintains normal sex chromosome morphology**

A striking feature of male animals that lack either NURF301 or the catalytic subunit ISWI is the distorted, bloated morphology of the male X chromosome [Deuring et al. 2000; this study]. This implicates NURF in the maintenance of normal X chromosome morphology. In flies, X chromosome dosage compensation is achieved by up-regulating transcription from the male X chromosome. One characteristic of the male X chromosome is the specific acetylation of histone H4 at Lys 16 [H4-K16], which is believed to favor a looser chromatin structure that allows increased transcription. These patterns of acetylation are established by the male-specific expression of components of the MSL complex that are tethered on the male X chromosome and subsequently recruit the histone acetyl transferase MOF [for review, see Park and Kuroda 2001].

Genetic studies demonstrate that H4-K16 acetylation antagonizes ISWI function on the X chromosome [Corona et al. 2002]. Biochemical characterization of the ISWI-containing ACF and CHRAC complexes has revealed that they can assemble and slide nucleosomes to establish regular ordered arrays [Ito et al. 1997; Varga-Weisz et al. 1997]. Regular nucleosome arrays are presumed to provide better substrates for chromatin compaction and, thus, it was speculated that ACF and CHRAC might be the complexes that help compact the male X chromosome [Corona et al. 2002]. However, here we show that NURF is the ISWI complex required for normal male X chromosome morphology. Unlike ACF or CHRAC, NURF disrupts regular, ordered arrays of nucleosomes [Tsukiyama et al. 1995]. While it is possible that NURF is required for global aspects of higher order chromosome morphology that are needed to maintain normal male X chromosome structure, other local or transcription-based mechanisms could also account for the nrf301 and iswi phenotypes. The dosage compensation machinery is recruited to the male X chromosome at specific, high affinity sites or entry points and subsequently spreads into flanking chromatin [Kageyama et al. 2001]. NURF may regulate chromatin accessibility at one or a number of these initiation sites. In the absence of NURF, entry of the dosage compensation machinery at such sites may be changed. Alternatively, NURF may control transcription of components of the sex-determination and dosage compensation pathway. Irrespective, the observed antagonistic relationship between ISWI function and H4-K16 acetylation [Corona et al. 2002] suggests that the action of NURF on the X chromosome is correspondingly influenced by H4 lysine acetylation. This influence on NURF could be direct, as suggested by effects of acetylated H4 tail peptide on ISWI ATPase activity in vitro [Corona et al. 2002].
Physiological activities of the ISWI chromatin remodeling machines

ISWI is the catalytic subunit of at least three protein complexes that have demonstrated in vitro chromatin remodeling activity—NURF, ACf, and CHRC. Here we have shown that mutation of a NURF-specific component reproduces the published properties of mutations in iswi. Both iswi and nurf301 are required for homeotic gene expression, proper larval blood cell development, and normal male X chromosome morphology. The specific in vivo functions of the ACf and CHRC complexes remains to be established.

Clues to the function of ACf and CHRC may be derived from studies of the human Williams Syndrome Transcription Factor-ISWI complex [WSTF-ISWI]. The in vitro activities of the WSTF-ISWI complex are essentially identical to ACf. WSTF–ISWI is targeted to pericentromeric heterochromatin during replication and is believed to allow heterochromatin reassembly in the wake of the replication fork [Bozhkov et al. 2002]. It is tempting to speculate that ACf and CHRC may have similar functions in Drosophila and could be implicated in the establishment of repressive chromatin structures after replication. As mutations that selectively compromise individual remodeling complexes become available, whole genome expression analysis will allow the relative contributions of specific complexes to gene activation and silencing events in vivo to be dissected.

Materials and methods

Genetics and fly strains

nurfl01 corresponds to l(3)ry122 [Berg and Spradling 1991] and was mapped to the nurf301 untranslated leader sequence, 382 bp upstream of the ATG. The homozygous viable EP insertions EP(3)837, EP(3)849, and EP(3)543 (Burth 1996) were mapped 880, 720, and 840 bp, respectively, upstream of the ATG of nurf301. The deficiency Df(3L)st643 was generated by imprecise P-element excision from EP(3)3643. EMS-induced mutations in nurf301 were generated by feeding isogenized w118 males 25mg/mL EMS in 10mM Tris, at pH 7.5 and 1% sucrose for 12 h at room temperature. Mutagenized males were back crossed to TM3/TM6B virgins, and F1 male progeny then crossed individually to one of two deficiency lines that uncover nurf301, Df(3L)XX8970 or Df(3L)IrH321. F2 progeny were scored for lethal noncomplementation of the deficiency. Alleles of nurf301 were identified by lethal noncomplementation of the P-element allele, nurf301.

EMS-induced nucleotide changes were determined by amplifying overlapping DNA fragments covering nurf301 using nurf301-specific primers and an Expand Long Range PCR kit (Boehringer, Mannheim) and DNA isolated from homozygous mutant animals as template. DNAs were sequenced and compared with similarly isolated w118 sequence. Altered nurf301 splicing in nurf301 mutants was demonstrated by isolating RNA from nurf301 homozygous mutant larvae using an RNAeasy column (Qiagen) and performing RT-PCR using primers flanking the altered splice-site. First-strand cDNA synthesis was performed using Ready-to-go beads (Amersham). For biochemical experiments, nurf301 mutations were kept over a GFP-marked balancer chromosome [w, TM3, P[w+y>Act-GFP]]

IMR2, Ser1]. This allowed large numbers of homozygous nurf301 mutant larvae to be selected by the lack of the GFP marker. Animals for expression analysis were harvested at mid-third larval instar—well prior to the lethal phase. Varying telomere transgene inserts are as described in Cryderman et al. [1999]. All other fly strains are described in Flybase [http://flybase.bio.indiana.edu]

Microscopy

Adult cuticles were prepared for microscopy by passage through isopropanol and mounted in Euparal (Asco Laboratories). Mutant and wild-type larval imaginal discs were fixed and immunohistochemistry performed in parallel as described [Dominguez and Hafen 1997]. RNA in situ hybridization was performed as described [Dominguez and Hafen 1997]. For polyclonal chromosome analysis, animals were maintained at 18°C until treatment. For heat-shock experiments, salivary glands were dissected from late-third instar larvae, incubated in prewarmed 36°C TBI buffer [Bonser 1981] for the indicated times and chromosomes prepared and stained as described [Gorman et al. 1995]. Rabbit anti-HSF antibody was used at a dilution of 1:1000. For X chromosome staining, polytene chromosomes were prepared as described [Deuring et al. 2000] and stained with rabbit anti-MSL2 antibodies (diluted 1:200). DNA was stained using DAPI and is revealed in purple in the merge.

Expression analysis

For heat-shock gene expression analysis, mutant and wild-type larvae were separated, equilibrated for 12 h, then immersed in 1.5 mL microfuge tubes in a 36.5°C water-bath for the listed times. Samples were snap frozen in liquid nitrogen and stored at -70°C until processing. Total RNA was prepared as described [Andres and Thummel 1994] followed by further purification over an RNAeasy column (Qiagen). Dot blots were performed as described [Sambrook and Russell 2001]. Probes against rp49, hsp26, and hsp70 are as described in Marchler and Wu [2001]. Probes against dipterin, drosomycin, and sptl were PCR fragments amplified from genomic DNA using the following primer sets: dtrs [5'-ATGATCGACCAACTGATCTTGGCTC-3' and 5'-TACGATCTTACCACCCGATCATACTTG-3'], dpt [5'-TTCCTCAATTTGAGAACTGATGCTG-3' and 5'-GAAGTTCGCTCAAATGCTTGGTAA-3'], sptl [5'-CTATGTCATACCCGAGAAATGATAAGAC-3' and 5'-CGAGGCTTCTAGATATTTATATATTCCGACAT-3']. Hybridization was in SUREhyb hybridization buffer (Ambion) using DNA probes labeled using a STRIPcage kit (Ambion). For analysis of Ubx and en mRNA levels, total RNA was isolated as described above, subjected to DNase I cleavage (Amplification grade DNase I, Invitrogen), and transcript abundance assayed using an ACCESS RT-PCR kit (Promega). Primer sets and cycle conditions used were: rp49 [5'-TCTGGAACAGCAGGGCTTGGGCTTG-3' and 5'-TACGATCTTACCACCCGATCATACTTG-3'], Ubx [5'-TCTGATTCATCAAAATCTTTATTTCTGCGACAT-3' and 5'-AACATATTTGTTCAGATTTCTTCCGACAT-3'], and en [5'-CTCGAGATAATATGGACCGCCGCCGGGCTCAGAGG-3' and 5'-CAACGACGAGAACCCGCTCCACGCAACGCGGTT-3'], 25 cycles and 25 cycles and 25 cycles.

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References


Chapter 5

Conclusions and Future Directions
The multigenic source of the EY3-5 phenotype has in turn produced a multigenic effect on the research described here. First, a strong enhancer of YanACT, hidden clones, displays an eye phenotype that suggests an early role in cell fate determination in the eye-antennal imaginal disc. Second, mutation of the putative chromatin remodeling factor, Rno, results in a general delay in the process of ommatidial assembly with a net phenotype that suggests a failure to properly restrict the assignment of cell fates. Lastly, in vivo mutagenesis of a known component of the nucleosome remodeling factor (NURF) has provided genetic evidence for the physiological function of this complex. This analysis has helped to distinguish the roles of NURF from other complexes with similar biochemical activities. Altogether, this work is a survey of the genetic functions contained within a defined genomic interval. While the original goal of carefully charting an unknown course down the stream of Ras signaling has not strictly been met, much has been achieved nonetheless.

Of these three branches of research, further study of Rno has the greatest potential for uncovering the mechanisms of signal transduction during cell fate decisions in the eye. Mutant rno eye tissue shows defects in controlling the number of photoreceptors and cone cells (Chapter 3, Figures 3.1, 3.3). There are also defects in apoptosis (Chapter 3, Figure 3.4), suggesting a corresponding increase in the pigment cells. These phenotypes are reminiscent of argos mutants (10), and additional evidence suggests that rno and argos act in the same pathway. First, alleles of rno show a strong, dominant enhancement of an argos mutant eye phenotype. Second, rno mutant eye clones fail to produce the Argos ligand (Chapter 3, Figure 3.9). These data strongly suggest that the primary cause of the
\textit{rno} phenotype in the adult eye is the loss of Argos. The lack of this inhibitory ligand for EGFR leads to the inappropriate activation of Ras signaling, ultimately resulting in the overproduction of multiple cell types.

In addition to this specific role, Rno is more globally required for the timely expression of developmental markers. In larval \textit{rno} eye clones, photoreceptors show a slight delay in the expression of general neural markers, such as ELAV, and a longer delay in the expression of some markers associated with development of specific subtypes of photoreceptors, such as \textit{seven-up-lacZ}. From these results, there seems to be a continuous requirement for \textit{rno} in developing photoreceptors. First Rno is necessary for the onset of differentiation, and later it is needed for activation of subtype-specific gene expression. The additional need for \textit{rno} for the timely differentiation of cone cells could potentially occur two ways. First, the slowed development of photoreceptors could inhibit their ability to recruit cone cells into the ommatidium, perhaps through a failure to express high levels of Delta, because Delta ligand is a requirement for cone cell specification (20). Alternatively, the affect of \textit{rno} mutants on cone cells could be a cell autonomous effect in the same way that \textit{rno} affects differentiation in photoreceptors. This second possibility seems more likely because there is no non-cell autonomous rescue of cone cell differentiation along the edges of \textit{rno} mutant clones (Chapter 3, Figure 3.3). The later recovery of cone cells in \textit{rno} mutant tissue is probably too extensive to be the result of such a rescue event. Put briefly, Rno is required cell autonomously for the differentiation of both photoreceptors and cone cells.
In light of this general role, perhaps the failure to produce Argos in \textit{rno} mutant tissue is just another symptom of slowed development of the eye. Production of Argos is one of many signs of differentiation of the eye (10). Given the importance of the Ras pathway in initiating differentiation in the eye, perhaps a slowed transcriptional response to Ras activation is the cause of the developmental delay seen in \textit{rno} mutant eye tissue. Another possibility is that the reduced production of Delta in \textit{rno} mutant tissue slows eye development. Activation of Notch is important for upregulation of Atonal expression in proneural clusters, as well as subsequent paternal inhibition to restrict R8 cell fate (2, 3). The partial loss of Delta ligand might impair the efficiency of one or both of these processes, thus setting back the developmental clock at an early stage. As noted earlier, it is unclear why the net result of delayed eye development is extra cell types and not a loss of cell types. Regardless of why this is true, loss of Argos is a likely cause for the overproduction of cell types, and loss of Delta could potentially contribute to the ectopic outer photoreceptor phenotype.

The \textit{rno} gene encodes a predicted protein of 3,241 a.a. protein that includes a PHD-class zinc finger followed by a Cysteine Rich Region (CRR) near the N-terminus. The PHD finger coordinates two zinc ions with its characteristic Cys4-His-Cys3 motif (17). This domain is frequently found in known chromatin remodeling factors and over 400 different proteins have been identified that contain at least one PHD domain (1). The most frequent biochemical property associated with them is the ability to bind to another protein. For example, the human protein MLL1, a homolog of Drosophila Trithorax, contains four PHD domains. One domain mediates homodimerization, while another of
the PHD domains binds to the RNA Recognition Motif protein, Cyclophilin33 (9). In a variation of this theme, the PHD domain of p300/CREB Binding Protein (CBP) is essential for this protein's histone acetyltransferase activity, perhaps because the PHD serves as a general binding site for substrates (12). In all known examples of CRR domains, they are positioned immediately following a PHD domain (14). Their cysteine and histidine rich content also implies a function in coordinating zinc ions, but this has not been formally demonstrated. In the one functional study published, the CRR domain of the human protein AF10 has been shown to bind to itself, and can do so independently of the PHD domain (14). Thus, the majority of the data suggests that the N-terminal region of Rno functions as a protein binding region, most likely as a component of a chromatin remodeling complex.

However, there is another intriguing possibility for Rno function based upon the similarity of PHD domain structure to the RING finger. Both the PHD and RING fingers are known to bind two zinc ions in an overlapping structure (4, 17) (Figure 5.1). In recent years, RING finger proteins have been recognized as required components of multisubunit E3 ubiquitin ligase complexes (11, 13). A multipolypeptide crystal structure of several components of an E3 ligase that includes the RING finger protein Rbx1 suggests that the role of the RING finger component is to recruit the requisite E2 ubiquitin conjugating enzyme to the complex (21).

In the past year, research has shown that some PHD domains perform a function similar to RING fingers. Two studies focus on how the Karposi’s Sarcoma Associated
Herpesvirus proteins, K3/MIR1 and K5/MIR2 can downregulate host defense molecules such as MHC Class I (7, 15). Both K3 and K5 are PHD domain, transmembrane proteins that localize to the plasma membrane. Expression of these proteins leads to ubiquitination of the cytosolic tail of MHC Class I, and subsequent endocytosis of the protein (7). MHC Class I containing endosomes eventually fuse with lysosomal vesicles (15), thus allowing immune evasion of the virus. K5 also shows in vitro E3 ligase activity that is dependent upon the zinc coordinating amino acids in the PHD finger (7). Another study demonstrates that MEKK1, a MAPKKK upstream of the MAPKs ERK and JNK, can act as an E3 ligase towards ERK. This E3 ligase activity is dependent upon a PHD domain located in region of MEKK1 known to bind ERK. Thus, RING protein-like E3 ligase activity is an emerging new function for PHD domains.

There is also data linking Rno the ubiquitination machinery. Alleles of rno interact very strongly with a rough eye phenotype caused by expression of a dominant negative form of Ebi (Ebi\textsuperscript{DN}). Ebi is an F-box protein known to target many proteins for degradation (8, 16, 20). The only problem with this model is that this interaction works the wrong way. Instead of enhancing the Ebi\textsuperscript{DN} phenotype as would be expected for a component of the ubiquitination pathway, rno mutants suppress Ebi\textsuperscript{DN} (Figure 5.2). Furthermore, a reduction in ubiquitination might be expected to globally stabilize proteins, not globally reduce protein expression as seen in rno mutants. Still, the strength of the interaction between rno and Ebi\textsuperscript{DN} is compelling. Perhaps there is a role for Rno in the ubiquitination pathway.
More recent evidence has expanded our understanding of how ubiquitination regulates the transcriptional machinery (6). The ability of E3 ligases to downregulate transcriptional regulatory complexes is well documented. In Drosophila, proteolytic downregulation of Ttk88 and Su(H) containing complexes by Ebi-containing E3 ligase complexes are just two examples (5, 8, 20). Interestingly, among several transcriptional activators that are regulated by proteolytic degradation, the domains that serve as the target for ubiquitination overlap with the activation domains (19). Furthermore, ubiquitin can serve as a post-translational activator of transcription factor function. In the case of VP16, an E3 ligase complex is necessary for its ability to activate transcription. This requirement for the E3 ligase is bypassed if VP16 is expressed as a translational fusion protein with ubiquitin (18). These findings suggest a logical mechanism of transcriptional activation: ubiquitination allows a burst of transcription, and is immediately followed by downregulation.

The idea of ubiquitin as a positive regulator of transcriptional activation allows for the rno mutant phenotype to be explained as a loss of E3 ligase activity. By virtue of its PHD domain, Rno could function as a member of an E3 ligase complex. This complex covalently links ubiquitin to target transcriptional activators, thus upregulating their activity and allowing a brief, timely burst of transcription. In the absence of this E3 ligase activity, transactivation is markedly reduced and the whole developmental program is slowed. Given the prevalent role of ubiquitination in the regulation of transcription (6), and the abundance of PHD domains in chromatin remodeling complexes (1), the potential frequency of such interactions is enormous.
References


Figure 5.1: Overlapping Zn$^{2+}$ coordination of PHD and RING fingers. Based upon Barlow et al., 1994 and Pascual et al., 2000.
Figure 5.2: The nmo suppresses a dominant-negative ebi phenotype. Middle: ebiDN is expressed in all cells posterior to the morphogenetic furrow using the GMR enhancer element. Left: alleles of nmo dominantly suppress this phenotype. This result has been observed for nmo^C4, nmo^A2, nmo^C1, and nmo^CV.
Appendix A

A PCR-based Technique for Mapping

the Breakpoints of Deficiency Chromosomes
Abstract

The molecular mapping of deficiency chromosome breakpoints is a common step in the process of identifying a mutated genetic locus. Traditionally, deficiency chromosome breakpoints are mapped by the genomic Southern technique. While effective, this technique often requires that multiple genomic probes and restriction enzymes be tested before an informative result is obtained. Here is described a PCR-based technique that allows the rapid and straightforward determination of deficiency breakpoints. The speed of PCR relative to genomic Southern and the elimination of a considerable amount of guesswork means that this technique is a quicker alternative for the mapping of deficiency breakpoints.

Introduction

This technique takes advantage of the fact that PCR is a sensitive, reliable and fast way to detect small amounts of a specific DNA sequence. In this application, primers are designed to amplify small pieces of genomic sequence of approximately 400-700 base pairs (b.p.). The genomic loci that are amplified in this way are here referred to as “Sequence Tagged Sites”, or “STSs.” Genomic DNA is isolated from animals that are homozygous for a given deficiency. This DNA is then used as a template for the amplification of several STSs. The successful amplification of a given STS from a genomic template indicates that the STS is not deleted from the deficiency chromosome. However, the failure of an STS to be amplified indicates that this sequence is in fact deleted from the deficiency chromosome. A demonstration of how this technique is predicted to work is shown in the schematic drawing below.
Deficiency chromosome

STS 1  STS 2  STS 3

distal.............. | Deleted region | proximal

PCR product. STS present

No PCR product. STS deleted

PCR product. STS present

In the above example, the PCR primers designed to amplify STS 2 failed to generate a product, thus this region is deleted in the deficiency chromosome. However, both STS 1 and STS 3 are amplified, so these sequences are present in the deficiency chromosome. From this data, it is concluded that the distal breakpoint lies between STS 1 and STS 2, while the proximal breakpoint lies between STS 2 and STS 3. In order to obtain higher mapping resolution, primers to new STSs can be designed between STS 1 and STS 2 for the distal breakpoint, and between STS 2 and STS 3 for the proximal breakpoint. Of course, it is necessary to test any designed primer pair first to make sure that they can robustly and reproducibly amplify the STS in question, and positive controls for a given primer pair should be included for any mapping experiment.

Practical considerations

An important question is how far to space STSs when starting this process.

Once the technique is working, one can rapidly refine the position of a breakpoint from relatively large regions to smaller ones, and the rate-limiting factor is how fast new primers are designed, synthesized and delivered. Since distance between the STSs is not an issue, the main concern is to find at least one STS that gives a negative result and at least one STS that gives a positive result. A good place to locate a “positive” STS is near a gene that has mutant alleles that genetically
complement the deletion. Likewise, a good place to locate a “negative” STS would be near a mutated gene that fails to complement the deficiency. If the deletions were generated by P-element mediated excision, then another good place for a “negative” STS would be very near the P-element excision site. The identification of “positive” and “negative” STSs give a framework for designing new STSs and also provides negative and positive controls for PCRs when one is testing new primer pairs.

The next thing to consider is how to isolate homozygous mutant genomic DNA. If the deletion is homozygous viable to adulthood, then ample amounts of DNA can be recovered from one adult fly by standard methods. If this is not the case, then pupae, larvae or embryos must be correctly genotyped. If the deletions are on the 3rd chromosome, then the TM6B balancer dominantly marked with Tubby (Tb) can be used to unambiguously genotype pupae and 3rd instar larvae. Earlier than 3rd instar, Tb larvae are not as easy to distinguish. Balancers marked with a gfp transgene can also be used to pick larvae, but using a gfp transgene to genotype embryos can be difficult because many of the promoters used to drive GFP expression, such as actin, ubiquitin and armadillo, allow maternal contribution of GFP.

In the case of rhinoceros (rno), all of the available deficiencies are embryonic lethal and the available, transgenically-marked balancers either have weak expression or have maternal contribution of the reporter protein. For this PCR technique to work, it is critical to separate homozygous mutant DNA from wild type siblings, so instead of trying to genotype the embryos, PCR was performed on single embryos. For a given deletion
line, 20 embryos were collected from a breeding stock of heterozygotes and each embryo was placed into a separate tube. Thus, one expects that on average 5 of the 20 embryos are homozygous lethal. A single embryo contains enough genomic DNA to perform at least 10 PCR reactions, so the same collection of embryos can be used to test several STSs. The downside of this blind approach is that the number of PCR reactions increases dramatically. Instead of doing one PCR reaction for a single STS using known homozygous mutant DNA, 20 PCRs are performed for a single STS in order to ensure that at least one mutant embryo is among those embryos selected. However, by performing PCRs in a multiwell plate format and using a multichannel pipettor to mix reagents and load gels, the amount of work is minimized. Here is an example of an experiment performed while mapping the *mo* locus:

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From the 10 single embryos used in this experiment, STS 1 and STS 3 are amplified from the genomic DNA of all 10. Assuming that there at least one mutant embryo was collected, then it appears that these STSs are not deleted from the deficiency chromosome being tested. However, for embryos 3, 5, 6 and 9, STS 2 is not amplified. The conclusion from this data is that those embryos are homozygous for the deficiency chromosome, and that STS 2 is deleted by this deficiency. The fainter, lower band seen
in all lanes are unincorporated primers. The presence of such bands can serve as a loading control for the agarose gel.

**Mapping deletion breakpoints by PCR, detailed protocol**

**Designing primers**

For the mapping of the mo locus, primers were designed by direct observation of the genomic sequence. To ensure robust amplification of STSs, primers were designed to amplify short genomic regions of 400-700 b.p. In order to decrease the amplification of non-specific sequences, the primer length was set at 24 nucleotides (nt.). The following general rules were applied in choosing the primer sequences:

1. 10-12 nt. must be Gs and Cs.
2. Sequence repeats longer than a trinucleotide were avoided (e.g., AAA is acceptable, but not AAAAA).
3. The last 2 nt on the 3' end are G or C.
4. Potentially self-annealing sequences longer b.p. in length were avoided, especially if the duplex would include the 3' end.

Of course, these rules can be difficult to follow in some cases. Several primer pairs were designed that work well, but don’t fit all of the above criteria. Also, primer pairs that were designed for other purposes and are shorter (18-21 nt.) have worked well in this assay.

**Collecting Embryos and Preparing Genomic DNA**

1. Collect embryos from a population of breeding heterozygotes of the deficiency of
interest from 4 hours to overnight.

2) Dechorionate embryos in 50% bleach for 3 minutes, then rinse with water.

3) Place dechorionated embryos in a 10 cm petri dish in 1XPBS to keep the embryos from drying out.

4) Dilute Proteinase K to a final concentration of 200μg/ml in Proteinase K Buffer (see solutions section at end of protocol). Place 10 μl aliquots into the necessary number of PCR tubes (1 tube per embryo). Use an empty tip holder to hold the PCR tubes, then place the tubes on ice.

5) Using a dissecting microscope, pick fertilized embryos that are at least 3 hours old (gastrulating) or later. A microscope that illuminates from below gives the best view of the embryonic morphology. Try to pick the earliest stages possible in case the homozygous mutant embryos develop more slowly that the wild-type siblings. After choosing an embryo, grasp it gently using a #5 watchmaker’s forceps and place it in the mouth of a PCR tube. Using a tip, move the embryo closer to the Proteinase K solution in the bottom, but not actually in the solution (once in the solution, an embryo is very hard to crush because it becomes slippery). Crush the embryo against the wall of the tube several times, then mix the “smashate” with the Proteinase K solution. If an embryo is accidentally pushed into the solution before crushing, it will be difficult to pin against the tube wall, so it is probably easier to throw that tube out and try again.

6) Once you have 20 embryos picked and squashed, put the tubes in a PCR machine and run the following program:

   step1: 37°C, 30 min (digestion)
step2: 95°C, 2 min (heat inactivation of protease)
step3: end

7) Add 90 µl of ddH2O to each tube and pippette up and down. The smashates can be stored at ~20° C indefinitely.

PCR

1) Make the following PCR mix, scaled according to the number of PCRs you need to do. For example, to test 4 different STSs on a collection of 20 embryos requires 80 PCRs. To be on the safe side, make enough PCR mix for 85-90 PCRs, depending upon the accuracy of the multichannel pippettor being. The primer and DNA volumes are calculated below, but they should not be added until later steps.

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<tr>
<td>10X PCR buffer + MgCl2</td>
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<td>10X dNTPs (2mM each)</td>
<td>2.5</td>
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<td>Promega Taq (5U/µl)</td>
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<tr>
<td>ddH2O</td>
<td>9.25</td>
<td>832.5</td>
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<td>primer 2 (20µM)</td>
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2) Divide the PCR mix equally into 4 tubes (i.e., one for each primer pair to be tested, so 225+225+22.5+832.5=1305. 1305 µl/4 tubes=326.25 µl/tube), then add the appropriate amount of each primer, which is 1/4 the amount shown above (22.5/4=5.6 µl) per primer.

3) Arrange PCR tube stripettes in a tip holder and label the tubes. It's easiest to use a system of labeling that is reliable but doesn't require labeling each tube individually.

4) Pippette 15µl aliquots of PCR mix into the appropriate tubes.
5) Set the multichannel pippettor to 10 μl. Pippette the gDNA up and down to mix, then aliquot. There is no need to mix the gDNA and PCR mix.

6) Snap tops onto the strippettes. Avoid spraying the tube contents as the cap snaps on.

7) Run the following PCR program:
   
   step1: 95°C, 3 min
   step2: 95°C, 15 sec
   step3: 55°C, 15 sec
   step4: 72°C, 30 sec
   step5: return to step 2, 34 times
   step6: end

Running gel

1) While PCRs are running, dissolve agarose in TAE to a final concentration of 1.5%. After dissolving the agarose, add 1 μl of 10 mg/ml of Ethidium Bromide per 100ml of gel volume, swirl to mix, then pour a gel with the necessary number of wells. It will save a huge amount of time if the gel combs are spaced such that the gel can be loaded with a multichannel pippettor. Owl Scientific makes 42-well combs that can be loaded in a staggered, or “2X”, pattern with a multichannel pippettor.

2) Remove caps from completed PCRs carefully to avoid splattering the reactions.

3) Pour some 6X Agarose Gel Loading Buffer without Bromophenol Blue (see Solutions at end of protocol) in a reagent reservoir. Set the multichannel pippettor to 3-5 μl and aliquot the dye into the PCR tubes. The same tips can be used to aliquot to all the PCR tubes by touching the tips against the wall of the PCR tubes well above the level
of the liquid in the tubes. Left over Loading Buffer can be poured into a 50 ml Falcon tube and stored at 4°C.

4) Load a DNA ladder into lanes that will not be used. The ladder should contain Bromophenol Blue so you can follow the migration of the gel.

5) Set the multichannel pippettor to 20-25 µl. Using the tips, push the Loading Buffer into the PCR reaction and mix. Suck up the PCR reaction and load.

6) Run gel.

**Solutions**

**Proteinase K Buffer**

10 mM Tris-HCl, pH 8.0

1 mM EDTA

25 mM NaCl

store at room temp

**6X Agarose Gel Loading Buffer***

0.25% Xylene Cyanol FF

30% Glycerol

store at 4°C

*For this PCR assay, it is best to leave out the Bromophenol Blue dye because its migration is similar to that of the amplified PCR products, which can cause it to mask the presence of bands in a gel. The migration of the bands can be followed if the DNA ladder lane contains a buffer with Bromophenol Blue.
Appendix B

Description of phenotypes in rno mutants
Abstract

Mutations in the *rhinoceros* (*rno*) gene have been found to result in the overproduction of multiple cell types in the developing eye (Chapter 3). This phenotype is believed to result from the failure of *rno* mutant tissue to produce the Argos protein, a secreted inhibitor of the Epidermal Growth Factor Receptor (EGFR). To obtain a more complete understanding of the biological functions of *rno*, a broader phenotypic analysis of *rno* mutants was undertaken.

Complementation analysis of *rno* alleles

Alleles of *rno* were recovered in a screen for lethal mutations that fail to complement two deficiencies from cytological band region, 61A-B (Chapter 2). Alleles of *rno* were distinguished from other mutations recovered in this screen by their failure to complement the lethality of the founding *rno* allele, AD2 (Chapter 2). To determine the period of lethality of *rno* alleles, 19 alleles were crossed to each other (Table B.1). Notably, allele W2 allows survival until adulthood when combined with almost any other *rno* allele, although the number of these adult escapers is lower than expected (Table B.1). The resulting adults frequently have wings with a held out, dichaete phenotype. It was determined that *rno*^{CT1}/*rno*^{W2} females raised at 25^\circ\text{C} are sterile, and have ovaries filled with primarily mature eggs, while lacking the usually complement of earlier stage egg chambers. In contrast, *rno*^{CM3}/*rno*^{W2} females are fertile, but the effect of this maternal genotype on the resulting embryos has not been examined. Interestingly, *rno*^{W2/+}
heterozygotes have a dominant phenotype in which a bit of excess wing vein tissue extends from the posterior cross vein. This phenotype appears to be slightly suppressed in rno$^{w2}$/rno escapers. Otherwise, these escapers appear to be normal by gross inspection. A few other allelic combinations also produce adult escapers, such as rno$^{N1}$/rno$^{BD-03}$ and rno$^{AD2}$/rno$^{CP5}$. These flies have obvious eye, antenna and leg defects similar to those seen in pharate adult mutants, which are described below.

By scoring the dominant Tubby (Tb) marker on the TM6B balancer chromosome, it was determined that several allelic combinations of rno survive until pupal stages. Many other allelic combinations survive until the wandering third instar larva stage. Certain alleles seem more capable of allowing survival to late developmental stages, in particular rno$^{N1}$, rno$^{BL1}$ and rno$^{ED1}$. The relative weakness of rno$^{BL1}$ and rno$^{ED1}$ can be explained by the fact that the molecular lesions associated with these alleles are nonsense mutations that occur fairly late in the coding sequence (a.a. positions 2425 and 1878, respectively, see Table 3.2), thus there may be residual biochemical activity from the corresponding truncated proteins. Interestingly, rno$^{N1}$ is a reisolate of a strong allele, rno$^{CTI}$. These alleles introduce a stop codon at a.a. position 319. The ability of rno$^{N1}$ to allow development to late stages, while rno$^{CTI}$ does not (Table B.1, also see below) implies that there may be a suppressor of the rno phenotype in the background of rno$^{N1}$.

**Phenotypes in rno mutant pharate adults**

For many of the allelic combinations that are pupal lethal, mutant animals die at the pharate adult stage. Pharate adults were removed from their pupal cases and examined
for gross morphological defects. The eyes of these pharate are slightly rough with obvious disruptions in the ommatidial organization. In the antennae of pharate mutants, the arista segment is transformed into a leg-like structure, a phenotype known as aristapedia that is seen in mutants such as spineless (4-6), tango (6), extradenticle (15), distal-less (4), homothorax (4), and lawc (19). This phenotype is also seen when rno mutant clones are generated in the eye-antennal imaginal disc (Figure B.1, see chapter 3 for details on how rno clones were generated). The legs of pharate mutants are frequently twisted and the distal-most segments are deleted from the tarsal region, including the pretarsal claw attached to segment T5 (Figure B.2). Loss of tarsal segments has been observed in mutants such as aristaless (2, 17), distalless (2, 3), spineless (5, 6), tango (6) and bric-a-brac(8). Interestingly, it has been observed that interfering with EGFR signaling in the presumptive tarsal region, either by overexpression of the inhibitory ligand Argos or by loss of the stimulatory ligand Vein, leads to deletion of the distal-most tarsal segments (7). The similarity between the tarsal phenotypes of rno and vein (7) seems to contradict the proposed role of Rno as an antagonist of the EGFR signaling pathway in the developing eye (see Chapter 3).

In many allelic combinations of rno, pharate adults are formed that appear to have failed in the fusion of the wing discs and abdominal histoblast nests during metamorphosis. In these animals, gaps can be seen along the dorsal midline of the thorax and abdomen (Figure B.3). Occasionally, only one lateral half of the thorax is visible. These phenotypes are very similar to those seen in pharate adult mutants of hemipterous (hep) (1). The hep gene encodes a MAPKK that is known to activate DJNK, thus this
thoracic closure defect is suggestive of a role for Rno in the DJNK signaling pathway. Another phenotype seen in \textit{rmo} pharate mutants is the loss of normal bristle polarity (Figure B.3, B.4). While most of the microchaetae and macrochaetae on the notum and scutellum have normal polarity, the two posterior scutellar macrochaetae point anteriorly instead of posteriorly. Closer examination of the notum and scutellum reveals that the small bristles are have lost normal polarization, a phenotype that results in swirling patterns. It is known that cell polarity in multiple tissues is regulated by the Frizzled transmembrane protein. Evidence also suggests that signaling through the DJNK pathway is one way in which Frizzled controls cell polarity \cite{11, 12, 14}. Thus, both the thoracic closure and cell polarity defects in \textit{rmo} pharate mutants suggest a connection between Rno and the DJNK signaling pathway. Finally, in \textit{rno} pharates the prothoracic region that is derived from the humeral imaginal disc bulges out relative to the surrounding epidermis (Figure B.5). There are do not appear to be any reported examples of this phenotype and it is not clear how this defect arises.

\textbf{Characterization of embryonic lethal and 1st instar larval lethal phenotypes}

From the complementation matrix, it is clear that several allelic combinations are lethal before the third instar stage. This is particularly true for \textit{rmo}^{CF2}, \textit{rmo}^{CM3} and \textit{rmo}^{CT1}. Not surprisingly, these three alleles contain the nonsense mutations that most severely truncate the Rno protein (Table 3.2, Figure 3.6). Seven \textit{rno} alleles have been "cleaned" of linked background mutations by recombination (see Chapter 3 for details). The removal of these mutations allows the phenotypic analysis of animals that are homozygous for a single allele of \textit{rno}, rather than transheterozygous combinations of
alleles. It should be pointed out that while the vast majority of linked mutations should have been removed by the recombination cleaning protocol, the presence of closely linked secondary mutations is a formal possibility and could potentially confound this analysis. Thus, the true test of a phenotype is its reproducibility between different alleles.

To determine the extent of embryonic lethality, cleaned rno alleles were outcrossed to the wild type, then rno/+ heterozygous males and virgin females were mated to each other. The resulting eggs were collected and the percentage that hatched was determined (Table B.2). From this experiment, it was found that from 14-85% of homozygous mutants do not hatch, depending upon the allele. The strongest phenotype was seen in rnoCvi. The truncated protein encoded by this allele retains the conserved N-terminus, but lacks the majority of the nonconserved C-terminus. The fact that the embryonic lethality seen in rnoCvi is stronger than likely protein null alleles like rnoCf2 and rnoCti (Table 3.2, Figure 3.6) suggests that the truncated protein encoded by rnoCvi may have antimorphic or neomorphic properties. This possibility is further supported by the observation that adult eye tissue that is homozygous mutant for rnoCvi has a different phenotype from the other alleles that have been tested. This phenotype is characterized by the loss of cellular organization (Figure B.6). However, those ommatidia that manage to remain organized have several extra rhabdomeres. Thus, by the criterion of number of ectopic photoreceptors per affected ommatidia, rnoCvi appears to be a stronger allele than rnoCf2 or rnoCti.
Egg collections from breeding populations of rno+ heterozygotes were collected and aged for 48 hours. After 48 hours, all heterozygous and homozygous wild type animals are presumed to have hatched. The unhatched eggs were collected, dechorionated and their cuticles prepared and mounted. Examination of these cuticles did not show any striking differences relative to control, wild type cuticles. This analysis was performed for the cleaned alleles rno$^{G4}$ and rno$^{A02}$ (data not shown). In situ hybridization of rno transcript in embryos revealed that the message is maternally deposited and becomes strongly enriched in the CNS by the latest stages of embryogenesis (Figure B.7). The zygotic expression of rno in the CNS suggests that defects in homozygous mutant embryos might occur in the CNS. To identify defects in the CNS of rno homozygotes, overnight egg collections were taken from breeding rno+ heterozygotes, fixed and stained with various antibodies that allow visualization of the neural architecture.

Antibodies that were used include anti-ELAV (16), which labels all neural nuclei, anti-FasII (9), which labels three main fascicles of longitudinal axons in the nerve cord, BP102, which labels all CNS axons (18), anti-Engrailed/Invected, which labels nuclei along the posterior edge of each segment of the CNS (13), and 22C10, which labels a subset of fascicles in the CNS (10). Particular attention was paid to late stage embryos in which the consequences of developmental defects are expected to be most obvious. In the case of egg collections stained with anti-FasII or BP102, filleted nerve cords were mounted to maximize visualization. Despite many attempts, no obvious or reproducible effects in the embryonic neural structure were found (data not shown).
Material and methods

RNA in situ hybridization

A digoxygenin labeled DNA probe vs. the mnp coding sequence was made using the first
2.6 kb of the mnp cDNA as a template (approximately 0.1 µg per labeling reaction),
random hexanucleotides (Boehringer Mannheim), digoxygenin labeling mix (Boehringer
Mannheim) and Klenow enzyme (New England Biolabs) at a total volume of 20 µl. The
probe was incubated at room temperature overnight, then precipitated and resuspended in
50 µl hybridization buffer (see below). Overnight egg collections were taken from a
population of Canton S. The eggs were dechorionated then fixed in 50% formaldehyde
(4%) in PBS/50% heptane for 30 minutes, then devitellinized in 50% methanol/50%
heptane and washed in methanol several times. Embryos were washed once in 50%
methanol/50% PBS with 0.1% Triton X-100 (PBT), and washed 3 times in 100% PBT.
Embryos were digested in PBT with 50 µg/ml Proteinase K for 5 minutes, then washed
twice in PBT with 2 mg/ml glycine and twice in PBT. Embryos were fixed again in PBT
with 5% formaldehyde for 20 minutes, then washed five times in PBT. Embryos were
then rinsed in 50% PBT/50% hybridization solution, then rinsed in 100% hybridization
solution before being prehybridized at 48°C for 1 hour. The probe in hybridization
solution was boiled then place on ice. The hybridization solution was removed from the
embryos and replaced with just enough probe to cover the embryos. The embryos were
then incubated at 48°C overnight. After hybridization, the probe was removed and the
embryos washed in 50% hybridization solution/50% PBT for 15 minutes at 48 °C,
washed in PBT 4 times, then washed twice in PBT for 15 minutes at 48°C. The embryos
were then incubated in PBT with Alkaline Phosphatase (AP)-conjugated anti-
digoxigenin Fab fragments (Boehringer Mannheim) for 2 hours at room temperature, then washed 4 times in PBT, the last wash lasting 15 minutes. The embryos were rinsed twice in AP buffer (see below), then incubated in AP buffer with 0.375 mg/ml nitro blue tetrazolium and 0.175 mg/ml 5-bromo-4-chloro-3-indolyl phosphate for several hours until blue staining appeared. Stained embryos were rinsed 5 times in PBT, then mounted on slides in glycerol.

**Hybridization solution**

50% formamide, 5 X SSC, 100 µg/ml salmon sperm DNA, 100 µg/ml tRNA, 50 µg/ml heparin, 0.1% Tween-20.

**AP buffer**

100 mM Tris base, pH 9.5, 100 mM NaCl, 50 mM MgCl₂, 0.1% Tween-20.

**Immunohistochemistry**

Egg collections were dechorionated, fixed in 50% PBS with paraformaldehyde (4%)/50% heptane, then devitellinized in 50% methanol/50% heptane. Devitellinized embryos were washed 3 times in methanol, 3 times in PBT, then blocked in PNT (PBT with 1% normal goat serum) for 1 hour at room temperature.Blocked embryos were incubated with the one of the following antibodies at the indicated concentration: rat anti-ELAV (1:50, G. M. Rubin), mouse anti-FasII (1:5, C.S. Goodman), mouse anti-BP102 (1:10, Developmental Studies Hybridoma Bank[DSHB]), mouse 22C10 (1:1000, DSHB), mouse anti-Engrailed/Invected (1:10, DSHB). Embryos were incubated in antibodies
overnight at 4°C, then washed 4 times in PBT at room temperature, the final wash lasting for 15 minutes. Hybridized embryos were incubated with the appropriate secondary antibody at 1:2,000 in PNT (conjugated to Horse Radish Peroxidase [HRP], CY2 or CY3, Jackson Immunoresearch) for 2 hours at room temperature, then washed 4 times with PBT, the final wash lasting for 15 minutes. Flourescently stained embryos were mounted in 90% glycerol in PBS with 0.5% N-propyl gallate. For HRP secondaries, embryos were incubated in PBT with 0.5 mg/ml diaminobenzidene, 0.04% NiCl, and 0.001% H2O2 until dark brown staining appeared. Stained embryos were then washed 6 times in PBT, then mounted in 90% EM-grade glycerol in PBS.

References


Table B1: Complementation matrix of *rno* alleles.

<table>
<thead>
<tr>
<th></th>
<th>G1</th>
<th>G4</th>
<th>I4</th>
<th>N1</th>
<th>O3</th>
<th>W2</th>
<th>Y1</th>
<th>AD2</th>
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Table B1 is continued on the next page.
Table B1: Complementation matrix of *rno* alleles, continued.

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<td>FQ2</td>
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<td></td>
<td>0/62</td>
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</table>

Flies heterozygous for the indicated alleles of *rno* balanced over *TM6B, Hu, Tb* were crossed to each other. After two weeks at 25°C, the progeny were scored. For a given cross, the fraction in the corresponding cell indicates the number of unbalanced adults over the total number of adults produced from that cross. The *TM6B* balancer is homozygous lethal, so if the two alleles being tested produced a 100% viable combination, then 1/3 of the total number of flies would be homozygous mutants for *rno*. The presence of the dominant, *Tb* marker on the balancer also allowed the genotyping of third instar larvae and pupae. Crosses that produced *rno* homozygous mutant third instar larvae, but not pupae are noted with "T.L." for third instar lethal. Crosses that produced *mo* homozygous mutant pupae, but not adults are noted with "P.L." for pupal lethal. The P.L. class includes crosses in which the homozygotes died as pharate adults. Blank cells indicate that data for the corresponding crosses were not collected, or that the data are represented elsewhere in the table. Most crosses versus allele AD2 were performed separately and the presence of homozygous mutant third instar larvae or pupae was not noted.
Table B.2: Degree of embryonic lethality among rno alleles

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<th>Total eggs</th>
<th>% unhatched eggs (lethality)</th>
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<td>6.3% (25.2%)</td>
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<td>3.6% (14.4%)</td>
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<td>186</td>
<td>10.8% (43.2%)</td>
</tr>
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<td>184</td>
<td>4.9% (19.6%)</td>
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<td>189</td>
<td>11.6% (46.4%)</td>
</tr>
<tr>
<td>( rno^{CVI} \times rno^{CVI} ) + +</td>
<td>38</td>
<td>178</td>
<td>21.3% (85.2%)</td>
</tr>
<tr>
<td>( rno^{FSI} \times rno^{FSI} ) + +</td>
<td>10</td>
<td>166</td>
<td>6.0% (24%)</td>
</tr>
</tbody>
</table>

Eggs were collected for 2.5 hours at 25° C, then 200 eggs from each cross were lined up on an agar plate. Eggs were then aged 41 hours at 25° C before scoring. Of the 200 eggs lined up from each cross, a number of unfertilized eggs were accidentally included. These eggs have been excluded from the total number. The number of fertilized, but unhatched eggs was counted. If an allele were 100% lethal, then one would expect to see that 25% of the eggs had not hatched. Thus in the final column, the % of unhatched eggs is given along with the corresponding % of lethality in parentheses.
**Figure B.1: Loss of rno causes aristapedia.** The wild type arista is a feather-like structure at the distal-most part of the antenna (above left). In several rno allelic combinations, pharate adults are seen in which the arista takes on a leg-like appearance (aristapedia, above center). The same phenotype can be seen in antenna that bear rno homozygous mutant clones (above right).
**Figure B.2: Loss of *rno* causes deletion of distal leg segments.** A wild type leg consists of 5 tarsal segments, T1-T5 (above left). The joints between these segments are indicated by arrows. The pretarsal claw is indicated by an arrowhead. In *rno* mutant pharates, legs frequently lack the distal-most tarsal segments (above right). In this case, T4 and T5 are missing, as well as the claw.
Figure B.3: Loss of \textit{rno} causes a failure in dorsal closure during metamorphosis. Mutant pharates frequently showed defects in the closure of the thoracic and abdominal epidermis along the dorsal midline (indicated by dashed line). Also note the polarity defects in the two posterior scutellar bristles (arrows).
Figure B.4: Loss of \textit{rno} disrupts bristle polarity in the scutellum. In both pictures, anterior is to the left. In the wild type (above left), scutellar macrochaetae as well as the small bristles point towards the posterior. Note the posterior macrochaetae (arrow). In pharate \textit{rno} mutants, (above right) the posterior macrochaetae point anteriorly (arrow) and the orientation of the small bristles is disrupted such that swirling patterns are seen. This macrochaetae polarity defect can also be seen in Figure B.3.
Figure B.5: Loss of rno results in a bulging prothorax. In the wild type, the prothorax is a round piece of epidermis located just posterior to the eye that is recognizable by the humeral bristles that project from it. Normally, this tissue is flat relative to the surrounding tissue (not shown). In rmo pharate mutants, this tissue protrudes above the surrounding epidermis. Above left shows a dorsal view and above right shows a lateral view. Anterior is to the left.
Figure B.6: Homozygosity for the \textit{rno}^{CVI} allele produces a dramatic eye phenotype.
In large clones of homozygous \textit{rno}^{CVI} tissue, the adult retina becomes highly disorganized (above). These eyes are characterized by rhabdomeres that are not oriented along the apical/basal axis of the retina, and by the presence of vacuoles. On the edges of large \textit{rno}^{CVI} clones, or in small clones, more organized ommatidia can be seen that contain many more rhabdomeres than are seen in clones of other alleles. On the top right is an ommatidium from a \textit{rno}^{CF2} clone that has 8 rhabdomeres, one more than normally seen in this cross-sectional level. On the bottom right is a clonally-derived \textit{rno}^{CVI} ommatidium from the edge of the same clone pictured to the left. There are 12 separate rhabdomeres seen in this \textit{rno}^{CVI} clone.
Figure B.7: In situ hybridization reveals the expression pattern of the *rno* transcript. There is maternal deposition of the *rno* transcript, as seen in unfertilized eggs. By embryonic stage 10, distribution is still fairly broad. By stage 13, *rno* transcript is strongly enriched in the CNS and remains so until the last embryonic stage, stage 17. Thus, zygotic transcription of *rno* is greatest in the CNS.
Appendix C

Genetic interaction tests of rno alleles
Table C.1: Tests for dominant modification of adult *rno* eye clone phenotype

<table>
<thead>
<tr>
<th>Background</th>
<th><em>rno</em>&lt;sup&gt;G4&lt;/sup&gt;</th>
<th><em>rno</em>&lt;sup&gt;AD2&lt;/sup&gt;</th>
<th><em>rno</em>&lt;sup&gt;CM3&lt;/sup&gt;</th>
<th><em>rno</em>&lt;sup&gt;CVI&lt;/sup&gt;</th>
<th><em>rno</em>&lt;sup&gt;CVI&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>yan&lt;sup&gt;+/+&lt;/sup&gt;</td>
<td>----</td>
<td>No effect (3)</td>
<td>----</td>
<td>----</td>
<td>Enh arista (8)</td>
</tr>
<tr>
<td>yan&lt;sup&gt;FR33&lt;/sup&gt;/+</td>
<td>----</td>
<td>No effect (12)</td>
<td>----</td>
<td>Enh (18)</td>
<td>Sup? (2)</td>
</tr>
<tr>
<td>yan&lt;sup&gt;FR433&lt;/sup&gt;/+</td>
<td>Sup arista? (3)</td>
<td>No effect (5)</td>
<td>----</td>
<td>No effect (8)</td>
<td>Mild Sup (5)</td>
</tr>
<tr>
<td>phyl&lt;sup&gt;2248&lt;/sup&gt;/+</td>
<td>No effect (4)</td>
<td>No effect (4)</td>
<td>----</td>
<td>No effect (13)</td>
<td>Sup (5)</td>
</tr>
<tr>
<td>phyl&lt;sup&gt;3366&lt;/sup&gt;/+</td>
<td>----</td>
<td>No effect (10)</td>
<td>----</td>
<td>----</td>
<td>Sup (10)</td>
</tr>
<tr>
<td>pnt&lt;sup&gt;7825,882&lt;/sup&gt;/+</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>Mild enh (35)</td>
<td>----</td>
</tr>
<tr>
<td>ttk&lt;sup&gt;6e&lt;/sup&gt;/+</td>
<td>----</td>
<td>No effect (15)</td>
<td>No effect (10)</td>
<td>No effect (23)</td>
<td>----</td>
</tr>
<tr>
<td>svp&lt;sup&gt;67842&lt;/sup&gt;/+</td>
<td>----</td>
<td>----</td>
<td>No effect (5)</td>
<td>No effect (10)</td>
<td>----</td>
</tr>
<tr>
<td>sp&lt;sup&gt;P1&lt;/sup&gt;/+</td>
<td>----</td>
<td>No effect (6)</td>
<td>----</td>
<td>----</td>
<td>----</td>
</tr>
<tr>
<td>sp&lt;sup&gt;P2&lt;/sup&gt;/+</td>
<td>----</td>
<td>Lethal? (0/76)</td>
<td>----</td>
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</tr>
<tr>
<td>r&lt;sup&gt;SR-135&lt;/sup&gt;/+</td>
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<td>No effect (10)</td>
<td>----</td>
<td>No effect (3)</td>
<td>Enh arista (16)</td>
</tr>
<tr>
<td>top&lt;sup&gt;+&lt;/sup&gt;/+</td>
<td>Enh arista (20)</td>
<td>----</td>
<td>----</td>
<td>Enh arista (14)</td>
<td>----</td>
</tr>
<tr>
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<td>----</td>
<td>No effect (3)</td>
<td>M en arista (25)</td>
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<tr>
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<td>----</td>
<td>----</td>
<td>----</td>
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<td>E eye, s aris (2)</td>
</tr>
<tr>
<td>mam&lt;sup&gt;C8&lt;/sup&gt;/+</td>
<td>Enh arista (5)</td>
<td>Enh arista (10)</td>
<td>----</td>
<td>----</td>
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</tr>
<tr>
<td>eya&lt;sup&gt;+&lt;/sup&gt;/+</td>
<td>----</td>
<td>----</td>
<td>No effect (126)</td>
<td>No effect (262)</td>
<td>----</td>
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</tbody>
</table>

Eye clones were induced by FLP/FRT (see Chapter 3 for details). Mutations on the third were introduced by recombination onto a *rno, FRT80B* chromosome. For mutations on the second, double balanced stocks were established. Numbers of flies scored are in parentheses. “Arista” means that the *rno* arista peda phenotype was affected, but not the eye.
Table C.2  Tests for dominant modification by rno alleles

<table>
<thead>
<tr>
<th>Homozygous allelic combination</th>
<th>rno$^{G4}/+$</th>
<th>rno$^{AD2}/+$</th>
<th>rno$^{CF2}/+$</th>
<th>rno$^{CM3}/+$</th>
<th>rno$^{CT1}/+$</th>
<th>rno$^{CV1}/+$</th>
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</thead>
<tbody>
<tr>
<td>Gap1/Gap1</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>Sup</td>
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<tr>
<td>pnt$^{762X5E3}$/pnt$^{358O2L1}$</td>
<td>----</td>
<td>Mild enh</td>
<td>----</td>
<td>Mild enh</td>
<td>Mild enh</td>
<td>----</td>
</tr>
<tr>
<td>sina$^{a}$/sina$^{3}$</td>
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<td>No effect</td>
<td>----</td>
<td>No effect</td>
<td>No effect</td>
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<tr>
<td>Cyo,GMR-sina</td>
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<td>Enh</td>
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<tr>
<td>sdk$^{DI4}/Y$</td>
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<td>----</td>
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<td>No effect</td>
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<td>No effect</td>
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<tr>
<td>Dl$^{B2}/+$</td>
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<td>----</td>
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<td>No effect</td>
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<td>No effect</td>
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<tr>
<td>spi$^{PI}$/spi$^{PI}$</td>
<td>----</td>
<td>No effect</td>
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<tr>
<td>spi$^{P2}$/spi$^{P2}$</td>
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<td>No effect</td>
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<td>sev-Ras$^{v12}$ (T2B)</td>
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<tr>
<td>sev-Gal4, UAS-CtermspenNLS</td>
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<td>Enh</td>
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<tr>
<td>Yan$^{E2ae2}/+$</td>
<td>Mild enh</td>
<td>Mild enh</td>
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<td>sev-Yan$^{AC}$ (SCY)</td>
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<td>Mild enh</td>
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<td>sev-Notch$^{DN}$ (lines 5,6)</td>
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<td>No effect</td>
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<tr>
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<td>No effect</td>
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<td>No effect</td>
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<tr>
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<td>No effect</td>
<td>----</td>
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</table>