

Genetic analysis of genes found on  
the 4th chromosome of *Drosophila*  
- emphasizing the developmental context  
of *Pax6*

Jesper Kronhamn

Umeå, 2004

Department of Molecular Biology  
Umeå University  
Umeå, Sweden



Akademisk avhandling

Som med vederbörligt tillstånd av rektorsämbetet vid Umeå Universitet för  
erhållande av filosofie doktorsexamen i genetik framlägges till offentlig  
granskning fredagen den 7 maj 2004, klockan 13.00 i sal E 04, byggnad 6E,  
Norrlands Universitetssjukhus, Umeå.

Examinator: Prof. Åsa Rasmuson-Lestander

Fakultetsopponent: Assoc. Prof. Kevin Moses, Emory University,  
Atlanta, USA

**Front cover:** Scanning electron micrograph of a homozygote *toy<sup>hal</sup>* fly lacking all structures from the eye-antennal discs. The picture shows the proboscis derived from the labial and clypeo-labral discs protruding from the thorax.

Printed in Sweden by  
Solfjärden Offset AB  
Umeå 2004  
ISBN: 91-7305-603-0

Till mina älskade  
Anna-Karin och Signe

**Organisation**

Umeå University  
Department of Molecular Biology  
SE-901 87 Umeå  
Sweden

**Document Name**

DOCTORAL DISSERTATION

**Date of issue**

May 2004

**Author**

Jesper Kronhamn

**Title**

Genetic analysis of genes found on the 4th chromosome of *Drosophila*  
- emphasizing the developmental context of *Pax6*

**Abstract**

The small size and lack of recombination set the fourth chromosome of *Drosophila melanogaster* apart from the other chromosomes.

I have shown that the Minute gene on chromosome 4, earlier named *Minute-4*, encodes the ribosomal protein *RpS3A*. Two *Pax6* genes, *eyeless (ey)* and *twin of eyeless (toy)* are also located on chromosome 4.

*Pax6* genes are important in head and eye development in both mammals and *Drosophila*. I have focused much of the study on *ey* and *toy*. The first mutant of *toy* that was characterized showed a headless phenotype. This indicates that Toy is important for the development of both the eye and antennal discs. The phenotype of the null mutation in *toy* is temperature sensitive due to that transcription of *ey* is temperature dependent in the eye-antennal primordium in absence of Toy. This temperature dependence was used to find out that the phenocritical period for *ey* in the adult head development is during embryonic stage 12-16 when *ey* first is expressed in the eye-antennal primordium. I also conclude that *ey* is activated by Toy in the eye-antennal primordium.

The strong *ey<sup>D</sup>* mutation was molecularly characterized and it was finally settled that it is an allele in the *ey* locus. I also show that *ey<sup>D</sup>* homozygotes have a headless phenotype, much stronger than the earlier *ey* mutations.

**Key words:** *Pax6*, *twin of eyeless*, headless flies, head development, eye development, *eyeless*, Minute, *RpS3A*, regulatory region, *Drosophila*

**Language:** English    **ISBN:** 91-7305-603-0    **Number of pages:** 46

**Date:** 14 April 2004

## Table of Contents

<i>List of papers</i> .....	6
<i>Abbreviations</i> .....	7
<i>Introduction</i> .....	8
<b>Chromosome 4</b> .....	8
<b>Minute-4</b> .....	11
<b>Pax6 genes</b> .....	11
Modular structure of Pax proteins.....	12
Evolution of <i>Pax</i> genes .....	13
<b>The Embryonic Head Development in Drosophila</b> .....	13
Procephalic region.....	15
Neuroblasts of the embryonic brain .....	16
Development of the embryonic visual primordium .....	16
<b>The Eye-antennal discs</b> .....	18
Eye-antennal primordium.....	18
The developing eye .....	18
<b>Adult head</b> .....	26
<b>Pax6 in vertebrate eye development</b> .....	27
The developing mouse eye.....	27
Expression of <i>Pax6</i> in the mouse eye .....	28
<i>Pax6</i> mutations.....	29
<i>Conclusions</i> .....	31
<b>Paper I</b> .....	31
<b>Paper II</b> .....	31
<b>Paper III</b> .....	32
<b>Paper IV</b> .....	33
<i>Future perspectives</i> .....	34
<i>Acknowledgements</i> .....	35
<i>References</i> .....	37
<i>Papers I - IV</i> .....	46

## List of papers

- I) **Kronhamn, J. and Rasmuson-Lestander, A.** (1999). Genetic organization of the ci-M-pan region on chromosome IV in *Drosophila melanogaster*. *Genome* **42**, 1144-9.
- II) **Kronhamn, J., Frei, E., Daube, M., Jiao, R., Shi, Y., Noll, M. and Rasmuson-Lestander, A.** (2002). Headless flies produced by mutations in the paralogous Pax6 genes *eyeless* and *twin of eyeless*. *Development* **129**, 1015-26.
- III) **Kronhamn, J. and Rasmuson-Lestander, A.** (2004) Analysis of the cis-regulatory region of *twin of eyeless* in *Drosophila*. *Manuscript*
- IV) **Kronhamn, J. and Rasmuson-Lestander, A.** (2004) *Pax6* gene redundancy in *Drosophila melanogaster*. *Manuscript*

## Abbreviations

<i>ANTC</i>	<i>Antennapedia Complex</i>
<i>atonal</i>	<i>ato</i>
<i>btd</i>	<i>buttonhead</i>
<i>BXC</i>	<i>Bithorax Complex</i>
<i>cnc</i>	<i>cup'n'collar</i>
<i>ci</i>	<i>cubitus interruptus</i>
<i>ct</i>	<i>cut</i>
<i>dac</i>	<i>dachshund</i>
<i>Dll</i>	<i>Distal-less</i>
<i>dpp</i>	<i>decapentaplegic</i>
<i>ems</i>	<i>empty spiracle</i>
<i>ey</i>	<i>eyeless</i>
<i>eya</i>	<i>eyes absent</i>
<i>eyg</i>	<i>eyegone</i>
<i>gt</i>	<i>giant</i>
<i>hh</i>	<i>hedgehog</i>
<i>hsp</i>	<i>heat shock protein</i>
<i>N</i>	<i>Notch</i>
<i>mam</i>	<i>mastermind</i>
<i>otd</i>	<i>orthodenticle</i>
<i>pan</i>	<i>pangolin</i>
<i>pntP1</i>	<i>pointedP1</i>
<i>PD</i>	<i>Paired domain</i>
<i>rp gene</i>	<i>ribosomal protein gene</i>
<i>RpS3A</i>	<i>Ribosomal protein S3A</i>
<i>sal</i>	<i>spalt</i>
<i>Shh</i>	<i>Sonic hedgehog</i>
<i>slp</i>	<i>sloppy paired</i>
<i>so</i>	<i>sine oculis</i>
<i>sv</i>	<i>shaven</i>
<i>tll</i>	<i>tailless</i>
<i>toy</i>	<i>twin of eyeless</i>

## Introduction

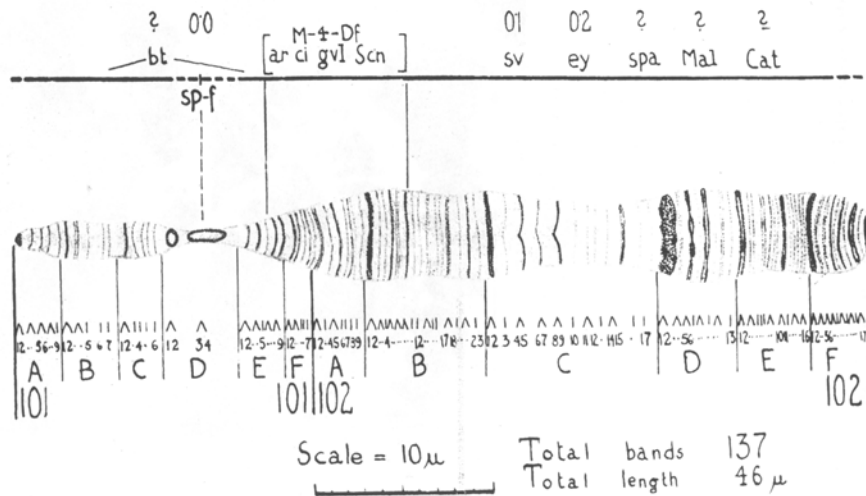
It is the fruit fly *Drosophila melanogaster*, more than any other organism that has promoted our understanding of how genes govern the patterning of our body. *Drosophila* has been used as an experimental organism for over a century, but it was Morgan and his students who used *Drosophila* for genetic experiments from 1909 (Morgan, 1910). These studies have yielded a cumulative knowledge and a framework that gives good opportunities to ask questions concerning gene function and interaction and to get interesting answers.

### Chromosome 4

Its small size, lack of recombination and dispersed heterochromatin set the fourth chromosome of *Drosophila melanogaster* apart from the other chromosomes. The size of the fourth chromosome is 5-6 Mb and it contains 3,5 % of the *Drosophila* genome (Locke and McDermid, 1993). It is made up of two major regions, the centromeric region of 3-4 Mb which is heterochromatic and contains primarily short satellite repeats. The remaining 1.2 Mb constitutes the banded region 101E - 102F on the salivary gland polytene chromosomes and contains the identified genes, shown in Fig. 1 (Locke et al., 2000). This small chromosome is not known to recombine under ordinary laboratory conditions (Ashburner, 1989; Carr et al., 2001; Hochman, 1976) but see, however Wang (2002). The small size also makes cytogenetic localization of genes difficult and the lack of crossing over precludes the construction of a typical genetic map. Often genetic screens include only the two major autosomes and the X chromosome and consequently, many of the genes on 4 are uncharacterized at present.

Dispersed heterochromatin in the 1.2 Mb banded region is probably the cause of diffuse banding of the polytene chromosome 4 and underlies the variegation of many P-elements inserted in the region (Locke et al., 1999b). Heterochromatin, defined as the portion of the eukaryotic genome that remains condensed as the cell progresses from metaphase to interphase, has high proportion of repetitious DNA, contains relatively few genes and is characteristically replicated late during S phase (Sun et al., 2000; Weiler and Wakimoto, 1995). The 1.2 Mb banded region displays several features

typical of  $\beta$ -heterochromatin. This term was first used in *Drosophila* by Heitz (1934) to describe the diffuse and poorly banded regions that comprise much of the chromocenter of the *Drosophila virilis* polytene chromosome set. The chromocenter of *D. virilis* contains a very strongly staining material that he called  $\alpha$ -heterochromatin that forms the pericentric, satellite-rich DNA. The classical view of  $\beta$ -heterochromatin put forward by Heitz is that it represents the transition between the  $\alpha$ -heterochromatin and the euchromatin at the base of the polytene chromosome arms. Gatti and Pimpinelli (1992) suggest that there are two distinct molecular organizations of  $\beta$ -heterochromatin that are cytologically distinguishable. "Proximal"  $\beta$ -heterochromatin comprises the low copy sequence embedded within the satellite arrays in the pericentric regions of each chromosome, whereas "distal"  $\beta$ -heterochromatin is comprised of the diffuse regions that lie at the base of most of the chromosome arms. The latter form is a mosaic of middle and low-copy repetitive DNA, often transposons, interspersed with unique DNA (Locke et al., 1999b; Miklos et al., 1988). Chromosome 4 exhibits a further similarity to  $\beta$ -heterochromatin in that the chromosomal protein HP1, thought to be an important constituent of heterochromatin, binds to several sites along the chromosome (James et al., 1989). Using a P-element containing the *hsp70-white* gene and a copy of *hsp26*, Sun and coworkers (2000) identified domains in the 1.2 Mb banded region that allow full expression of the *white* marker, euchromatinlike domains, and other, heterochromatinlike domains, that induce a variegating phenotype. In the former case the *white* marker shows a heatshock-inducible activity and a uniform red-eye phenotype that is typical for P-elements in euchromatic domains, whereas in the latter case, accessibility to transcription factors and inducible expression are reduced and the eyes show a variegated eye color phenotype. When these P-elements are mapped, it shows that the euchromatinlike and heterochromatinlike domains are interspersed and closely associated within the 1.2 Mb region of the 4th chromosome (Sun et al., 2000).



**Figure 1.** An early drawing of the polytene chromosome 4 by Slizynski (1944). The map shows both the strength and weakness of old time genetics trying to disclose the nature of chromosome 4. To be able to see that chromosome 4 is acrocentric from the polytene chromosomes demands an enormous endurance, due to the small size of the left arm that almost always becomes embedded in the chromocenter. The gene order was harder to determine, since crossing over does not occur under normal laboratory conditions and deficiencies are rare. One can observe that every second gene in region 102C-E, *sv*, *spa* and *Cat*, are now known to be alleles of the gene *shaven*, mapped at 102D4. The number of bands is too high on the map, Saura and coworkers conclude that there are approximately 35 bands on the 4th chromosome (Lefevre, 1976; Saura et al., 2002). Reprinted by permission of Oxford University Press.

The nature and distribution of repetitive elements on chromosome 4 could play a role in the establishment of this unusual chromatin configuration. *DINE-1* is a short, interspersed repetitive sequence with euchromatic locations almost exclusively confined to chromosome 4. In situ hybridization with a *DINE-1* probe confirms the preferential distribution along 4, in addition to its presence in the centric heterochromatin (Locke et al., 1999a). Another transposable element that is almost exclusively distributed along chromosome 4 is the *hoppel* element (Locke et al., 1999a). Although the 1.2 Mb banded region of chromosome 4 is characterized by  $\beta$ -heterochromatin, Hochman (1976) estimated the number of genes to be 75 and Adams et al., (2000) to 74. This is a density about equal to that in other euchromatic regions of the genome.

Another unique feature for the 4th chromosome is that flies survive with one copy of chromosome 4 (haplo-4), three copies (triplo-4) or even in a few cases with four copies (Bridges, 1935a; Hochman, 1976). Aneuploidy for the large autosomes is invariably lethal.

There is evidence suggesting a closer kinship of chromosome 4 with the X chromosome than with the other autosomes (Ashburner, 1989). First, in contrast to autosomes, but in a fashion similar to the X chromosome, chromosome 4 has "female tendencies", shifting 2X:3A intersexes toward female development when its dosage is increased and toward male development when its dosage is decreased (Bridges, 1925; Fung and Gowen, 1960). Second, most *Drosophila* species have a similar microchromosome, but in *Drosophila busckii* the karyotype and location of mutants suggest that *D. busckii* lacks an independent chromosome 4, and that the homologous region is probably located on the proximal part of X (Krivshenko, 1959). Lastly, the 4th is, as the X chromosome, associated with chromosome-specific proteins (Larsson et al., 2001).

### ***Minute-4***

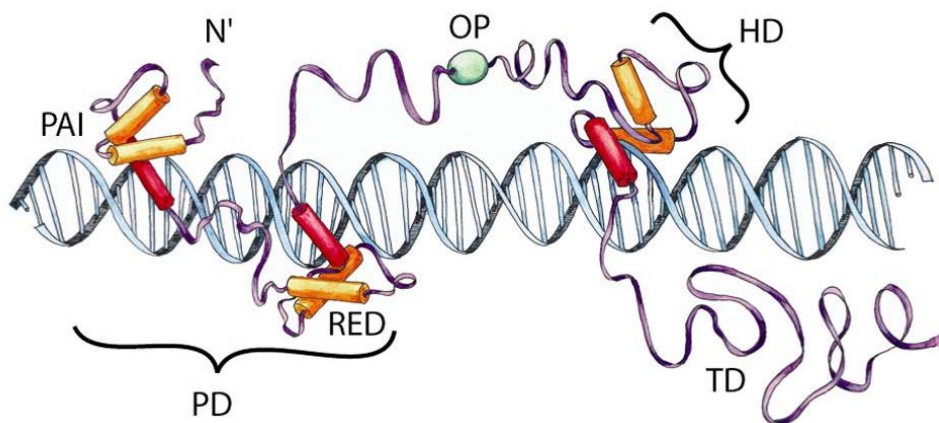
The Minute mutation on chromosome 4 was named *Minute-4* by Bridges (1935b). The *Minute-4* was shown to encode the ribosomal protein *RpS3A* (Kronhamn and Rasmuson-Lestander, 1999; van Beest et al., 1998). In a Minute fly, the amount of rp gene mRNA is reduced to ~ 50 % of the normal amount of gene product, and becomes rate limiting for ribosome biogenesis, cell proliferation and growth. The Minute phenotype results from mutations at more than 50 loci scattered throughout the genome of *Drosophila*. They are associated with a similar dominant visible phenotype which is characterized by short, thin bristles, delayed development, small body size and low fertility (Lambertsson, 1998). The chief weakness of haplo-4 flies is due to the Minute phenotype. Homozygous Minute mutations are lethal and die during late embryogenesis, at about time of hatching.

### ***Pax6 genes***

Although genes containing a paired-box known as *Pax* genes, were first found and described in *Drosophila* (Bopp et al., 1986) the *Pax6* gene was first isolated in humans (Ton et al., 1991) and mice (Walther and Gruss, 1991). There are 3 *Pax* genes on chromosome 4 in *D. melanogaster* two of them are *Pax6* genes: *eyeless (ey)* (Quiring et al., 1994) and *twin of eyeless (toy)* (Czerny et al., 1999) and the third is a *Pax2* gene called *shaven (sv)*.

## Modular structure of Pax proteins

*Pax* genes are defined by the presence of a paired-box, which encodes a paired-domain (PD), a highly conserved 128 amino acid DNA binding domain, see Fig. 2 (Breitling and Gerber, 2000). Crystal structure data indicate that the PD consists of two independent subdomains, the amino-terminal PAI domain and the carboxy terminal RED domain. Structurally both domains resemble a helix-turn-helix (HTH) motif that can bind DNA, either independently or synergistically, see Fig. 2 (Czerny et al., 1993). These two motifs are always found together except in the Pax6-like *Drosophila* protein Eye gone (Eyg), which lacks the amino-terminal PAI domain (Jun et al., 1998). In addition to their PD, Pax proteins often contain other conserved domains such as a complete or partial paired-type homeodomain (HD). The HD is a 60 amino acid DNA-binding domain, found in all *Hox* gene products, the specificity of which depends on a crucial residue found at the position 50. The HD found in *Pax* genes is always characterized by a S<sub>50</sub>, and these proteins can bind as homo- or heterodimers with any paired-class HD protein to a palindromic DNA sequence (Wilson et al., 1995). Cooperative dimerization allows these HD proteins to recognize sequences not bound by other HDs. Recent evidence suggests that intramolecular interactions between distinct DNA-binding domains can also function as protein-protein interaction domains (Plaza et al., 2001), which suggests caution in using Pax protein function as a simple summation of discrete modular activities (Pichaud and Desplan, 2002).



**Figure 2.** A Pax protein containing a paired domain (PD), composed of an aminoterminal PAI domain and a carboxy-terminal RED domain, each of which is composed of three  $\alpha$ -helices. An octapeptide motif (OP) is followed by a homeodomain with a helix-turn-helix structure. Finally, a carboxy-terminal transactivation domain (TD) is shown. Pax6 proteins contain a PD, a HD and probably also TD, but no OP. Modified from Chi and Epstein (2002) Reprinted with permission from Elsevier.

## Evolution of *Pax* genes

Miller et al. (2000) state that the *Pax* family has a monophyletic origin. Miller also place the nine human *Pax* genes in four phylogenetic groups: (1) *Pax1* and *Pax9*; (2) *Pax3* and *Pax7*; (3) *Pax4* and *Pax6*; (4) *Pax2*, *Pax5* and *Pax8*. In addition to these four groups the Cnidarian *PaxA* and the *Drosophila Pox-neuro* may constitute a fifth group, with an uncertain phylogenetic position (Miller et al., 2000; Sun et al., 1997). The ten *Drosophila Pax* genes are related to the human orthologs as follows: (1) *POXmeso*; (2) *gooseberry*, *gooseberry neuro* and *paired*; (3) *eyeless* and *twin of eyeless*; (4) *shaven*. The *Pax6*-like genes *eyegone* and *twin of eyegone* cluster with *Pox-neuro* (Breitling and Gerber, 2000). No homologs of the *eyg* or *toe* are found in vertebrates (Jang et al., 2003). Jun and coworkers (1998) state in their molecular analysis of *Eyg* that the closest relative seems to be *Pax6*. *Pox-neuro* was, however, not included in this analysis. We have concluded that both *eyg* and *twin of eyegone (toe)* are *Pax6* genes (Kronhamn et al., 2002) with a perspective of how *Eyg* is functioning. It is likely that the best vertebrate equivalent is the vertebrate *Pax6-5a* isoform (Jang et al., 2003). Homologs to *toy* or two *Pax6* genes can be found in holometabolous insects (Czerny et al., 1999); i.e. insects that go through full metamorphosis, while mammals have only one *Pax6* gene.

## The Embryonic Head Development in *Drosophila*

*Drosophila* development is a continuous process that, perhaps unfortunately, must be divided into discrete steps to describe it. In the remainder of this introduction I will focus on *toy* in particular and eye development in general.

The establishment of the basic body plan of the *Drosophila* embryonic head requires the informational input of three of the four maternal coordinate systems: the anterior, the terminal and the dorso-ventral systems (St Johnston and Nusslein-Volhard, 1992). The anterior system determines the head and thorax region, the terminal system is responsible for the formation of most anterior head and the dorsoventral system defines positional information along the dorsoventral axis (Grossniklaus et al., 1994). The anterior system acts through *bicoid* mRNA localized to the anterior pole and a gradient of Bicoid is formed by diffusion of the protein. The homeodomain-containing transcription factor Bicoid then regulates target genes like the gap genes *hunchback* and *orthodenticle* in a concentration dependent manner (Schaeffer et al., 2000). The terminal system requires the

localized generation of an extracellular ligand that activates the receptor tyrosine kinase Torso (Casanova and Struhl, 1993). The ligand-bound Torso acts through the Ras signaling pathway to activate MAPK (Rollover MAP kinase), and results in the expression of zygotic target genes such as *tailless* at both ends of the embryo (Pignoni et al., 1990). The dorsoventral system is established by the gradient of Dorsal, a transcription factor homologous to NF- $\kappa$ B. Unlike Bicoid, Dorsal forms a gradient over a field of cells that is established as a consequence of cell-to-cell interactions. The initial dorsalizing signal from the oocyte nucleus appears to be the product of the *gurken* gene (Forlani et al., 1993).

In the central trunk region, gap genes activate the pair-rule genes and the pair-rule genes initiate the expression of segment polarity genes. Segment identity is conferred by the regionalized expression of homeotic selector genes from the *Antennapedia (ANTC)* and *Bithorax complexes (BXC)*, activated by a combined action of gap and pair-rule genes.

The head segments are defined early in the embryonic development. The number of segments and where to divide the anterior and posterior head has, however, been a matter of debate. Urbach and Technau (2003b) state that the head of the *Drosophila* embryo consists of four pregnathal and three gnathal segments. The pregnathal segments are (from anterior): the labral, the ocular, the antennal and the intercalary segments. The gnathal segments are concise and clear-cut: the mandible, the maxilla and the labium.

The maternally dependent Bicoid gradient is necessary for the whole head. The maternal Torso in the terminal pathway is initially defining the three most anterior segments.

The posterior head or the gnathal segments are subdivided in a fashion similar to the trunk, but the anterior head or pregnathal segments are patterned by a significantly different molecular mechanism. The formation of pregnathal head segments requires the expression of head gap genes: *giant (gt)*, *tailless (tll)*, *orthodenticle (otd)*, *empty spiracle (ems)*, *buttonhead (btd)* and *sloppy paired (slp)* (Finkelstein and Perrimon, 1991; Gallitano-Mendel and Finkelstein, 1997; Grossniklaus et al., 1994; Mohler et al., 1995). Since the pair-rule genes do not contribute to segmentation in the pregnathal head, it has been proposed that the head gap genes directly activate segment polarity gene expression in this domain (Cohen and Jurgens, 1990; Gallitano-Mendel and Finkelstein, 1997). Crozatier and coworkers (1999) have however found parasegment-specific second order regulators in the head, at a level similar to that of pair-rule genes in the trunk. The gene (*Knot*) is expressed in parasegment 0, or in posterior

intercalary and anterior mandibular segment. The intercalary segment belongs to the pregnathal segments (Campos-Ortega and Hartenstein, 1997; Urbach and Technau, 2003b).

The ocular segment comprises most of the dorsal head capsule, most of the visual primordium and protocerebrum. *ill* defines the ocular segment (or the nonsegmented acron) (Campos-Ortega and Hartenstein, 1997).

The homeotic selector genes of the *ANTC* are expressed in the gnathal head segments as well as in the trunk. Although, in the head, the *ANTC* genes are expressed in non-overlapping domains that in some cases encompass two morphologically distinct segments. Among the pregnathal segments only the *ANTC* gene *labial* is expressed in the intercalary segment. Accordingly, specification of segment identity must involve other genes acting independent of or together with the genes of the *ANTC*. Mutations in other genes have been described that cause homeotic transformations and are segment specific homeotic genes, acting in parallel to the *BXC* and *ANTC* genes. Examples of this group that are expressed in the head are *spalt (sal)*, *cup'n'collar (cnc)* and *forkhead (fkh)*. None of these gene products contain a homeodomain (Mohler et al., 1995) as do all of the homeotic selector genes in *ANTC* and *BXC*.

### **Procephalic region**

At the onset of gastrulation, the anlage that gives rise to the anterior brain (protocerebrum) and the visual primordium, is roughly defined by the expression of *otd* (Chang et al., 2001), *ill* (Rudolph et al., 1997) or *toy* (Czerny et al., 1999). The expression of *otd* extends from the cephalic furrow to the anlage of the foregut. In the dorsoventral axis, the anlage crosses the dorsal midline; laterally it reaches to ~50% of the embryo diameter, where it is bounded by the ventral neuroectoderm. During gastrulation and germband elongation the anlage splits up into different compartments that can be recognized as three domains with molecular markers. Except for the head ectoderm, the protocerebral neuroectoderm will form the anterior embryonic brain (Younossi-Hartenstein et al., 1996) and the visual system (Green et al., 1993). The most ventral part of the embryonic head give rise to mesodermal tissue (Lebestky et al., 2000; Tepass et al., 1994).

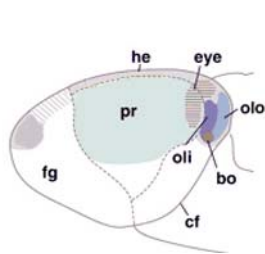
## Neuroblasts of the embryonic brain

Progenitors of the central nervous system, called neuroblasts, typically delaminate from the neuroectoderm as individual cells, whereas neighboring cells that stay behind in the neuroectoderm form epidermal progenitors. During stage 9-11 about 100 neuroblasts segregate from the procephalic neuroectoderm on each side (Urbach and Technau, 2003a; Younossi-Hartenstein et al., 1996). As in the trunk *ey* is expressed in a reiterated pattern in head neuroblasts. At stage 11 six neuroblasts per hemi-segment are *Ey* positive. *toy* expression during stage 9 encompasses the dorsal ocular and the anterodorsal part of the antennal ectoderm. All neuroblasts that delaminate from this part express *Toy*. By stage 11, these include 40 protocerebral neuroblasts, most of which derive from the ocular ectoderm. *Toy* and *Ey* seem only to be co-expressed in the *Ey* positive neuroblasts deriving from the ocular segment (Urbach and Technau, 2003a). *toy* and *ey* are also expressed in a segmentally reiterated pattern in the ventral nerve cord (Kammermeier et al., 2001).

## Development of the embryonic visual primordium

The visual primordium is defined by *sine oculis (so)* expression (Cheyette et al., 1994), which is expressed slightly after *toy* translation is detected. The primordium is wedged between the midline ectoderm and the protocerebral neuroectoderm in the posterior head (Chang et al., 2001; Cheyette et al., 1994). During gastrulation and germ band extension, cells of the visual primordium move laterally and are divided into the eye-antennal primordium (adult eye) and the optic placode that consists of the inner and outer optic lobe (visual center in the brain) and Bolwig's organ (larval eye), see Fig. 3. The optic lobe placode invaginates and by the end of germ band retraction, the optic lobe primordium forms a deep pouch containing approximately 85 cells that loses contact with the outer surface of the embryo and forms an epithelial vesicle attached to the brain. Bolwig's organ arises from the ventralmost portion of the optic placode, but its 12 cells remain in the head epidermis until they, in conjunction with head involution, reach their final position alongside the pharynx. Unlike the adult eye, pigment cells or any other type of support cells do not surround the photoreceptors of Bolwig's organ (Green et al., 1993). The eye-antennal discs develop from the posteriormost extension of the pouch in late embryo. The eye-antennal primordium is derived from the maxillary and antennal segment in particular as well as from the ocular segment, but may also be derived from mandibular, intercalary and labial segment. The optic placode

is derived from the ocular segment (Campos-Ortega and Hartenstein, 1997).



**Figure 3.** The *Drosophila* brain/eye field and its derivatives at the time of gastrulation, lateral view. bo: Bolwig's organ or larval eye, cf: cephalic furrow, eye: adult eye or eye antennal primordium, fg: foregut, he: dorsal head epiderm, oli: inner optic lobe, olo: outer optic lobe and pr: protocerebral neuroectoderm (Chang et al., 2001). Redrawn with permission from the Company of Biologists LDT.

An early signaling molecule regulating eye field partitioning is Decapentaplegic (Dpp, a BMP4 homolog). Dpp activates the expression of the "early eye genes" *sine oculis* (*so*) and *eyes absent* (*eya*) in visual primordia during early gastrulation. In *dpp*-null flies neither *so* nor *eya* are expressed in the visual primordium (Chang et al., 2001). Reduction in the Dpp signaling results in a cyclopia phenotype, and the entire eye field remains unpaired. Thus, the Dpp gradient plays a role in the *Drosophila* eye field that resembles the role of Sonic hedgehog (Shh) in the vertebrate head (Chang et al., 2003). The *Drosophila* homolog of *shh*, *hedgehog* (*hh*) is expressed slightly later together with *so* and *eya* to induce the transcription factor Atonal (Ato) in the Bolwig's organ primordium. According to Suzuki and Saigo (2000) *ato* is a "master gene" in Bolwig's organ. *Egfr* is also activated in the precursors of Bolwig's organ and the adjacent optic lobe at a stage preceding optic lobe invagination and larval eye separation. *Egfr* activation is required to phosphorylate the cadherin-catenin complex that connects the membrane to the actin cytoskeleton and thereby allows optic lobe invagination and Bolwig's organ separation to occur. The most prominent phenotype of loss of *Egfr* is cell death and if apoptosis is blocked by a deficiency in the Reaper-complex, cells of the optic lobe fail to invaginate and Bolwig's organ fails to separate (Dumstrei et al., 2002).

*ey* is not expressed either in the optic lobe or Bolwig's organ at any stage of embryogenesis (Daniel et al., 1999). Nevertheless, Kammermeier and coworkers (2001) argue (without showing it) that *ey* is expressed in a small subset of cells in the optic lobe of the embryo. After germband retraction Toy is detected in the optic lobe primordia (Czerny et al., 1999). Toy is also expressed in Bolwig's organ when the 12 cells still are in the head epidermis (personal observation). Kammermeier et al. (2001) also state that *toy* is expressed in a small subset of cells in the optic lobe. Suzuki and Saigo (2000) have analyzed *null-4* embryos and found that both *Pax6* are

dispensable for Bolwig's organ formation, since *ato* is expressed in Bolwig's organ primordium (stage10) and in Bolwig's organ at stage 16.

## **The Eye-antennal discs**

### **Eye-antennal primordium**

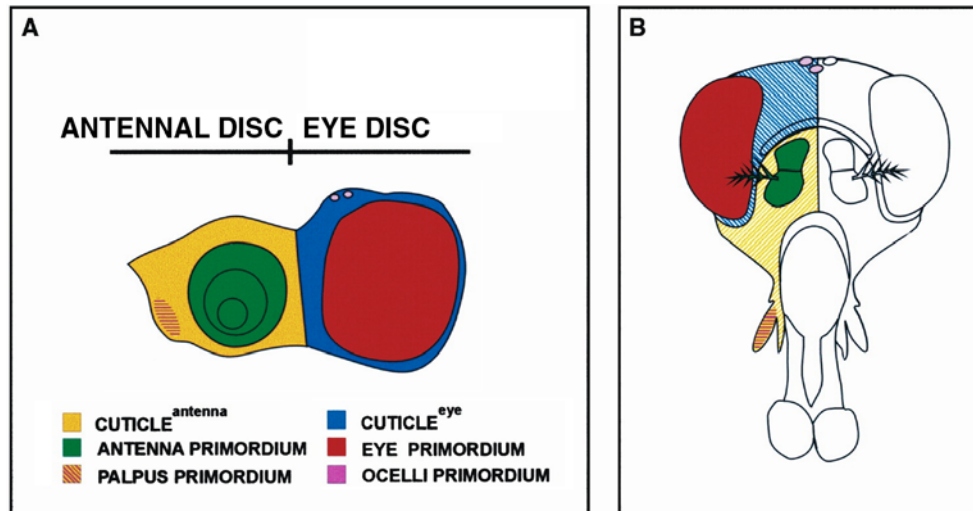
*toy* is first expressed in the eye-antennal primordium after germband retraction and activates *ey* (Czerny et al., 1999; Kronhamn et al., 2002). In the eye-antennal primordium Hh is activating *ey* (directly or indirectly), since *hh*-null embryos lack *ey* expression in the eye-antennal primordium (Chang et al., 2001). *eyg* is expressed in the primordium from stage 15 and is expressed independently of *toy* and *ey* (Dominguez et al., 2004; Jang et al., 2003). The other "early eye genes" *so*, *eya* or *dachshund* (*dac*) are not expressed in the embryonic eye-antennal primordium (Jiao et al., 2001; Kumar and Moses, 2001). Strong mutations in *ey*, *toy* and *eyegone* (*eyg*) all generate headless flies, losing all derivatives from the eye-antennal discs (Jang et al., 2003; Kronhamn et al., 2002). At this stage it is obvious from the mutant phenotype that the task for the expressed "eye-specification genes" is to give identity to the whole eye-antennal primordium, and not to specify the eye disc alone.

### **The developing eye**

During first instar *ey* is expressed in the entire eye-antennal discs. *ey* expression retracts during (mid and late-) second instar to the eye disc (or posterior part) of the eye-antennal discs, see Fig 4. *toy* has not been as extensively studied but appears to have a similar spatio-temporal expression pattern. Cut (a transcription factor) is an early marker for the antennal disc. It is activated when *ey* is downregulated at mid second instar and is complementary to the Ey-positive domain of the eye-antennal discs. Ey and Cut serve as markers for the eye disc and antennal disc identity, respectively, during mid second instar, see Fig. 4 (Halder et al., 1998; Kenyon et al., 2003).

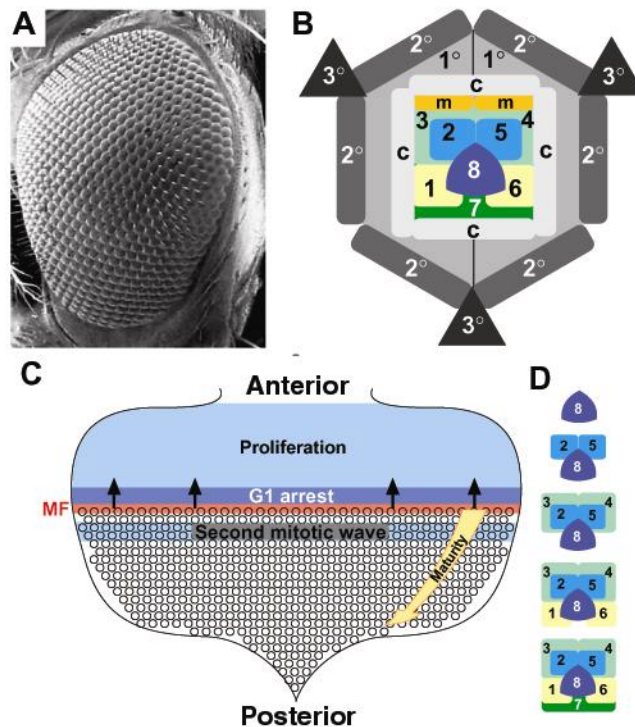
*eya* expression is initiated first in early second instar, earlier than *so* or *dac*. *Eya* expands from the posterior margin of the eye disc toward the center and anterior regions of the eye disc and persists in this pattern until neurogenesis starts in early third instar. *Eya* is the earliest marker of the eye primordium

and *Distal-less (Dll)* expression reflect antennal primordium, shown in Fig. 4 (Kenyon et al., 2003).



**Figure 4.** Fate map of the third instar eye-antennal discs and corresponding structures of adult fly head. A large portion of the fly head originates from a pair of eye-antennal imaginal discs. **(A)** A fate map of the late third instar eye-antennal epithelium and **(B)** corresponding structures of the fly head. The ocelli and palpus originate also form from this tissue. The eye and antennal discs respectively, give rise to substantial portions of the head cuticle. Discs are shown with anterior to the left. Redrawn from (Kenyon et al., 2003) with permission from Elsevier.

The compound eye (shown in Fig. 5) and the ocelli (shown in Fig. 7) develop from the eye disc, an epithelial monolayer. The eye disc grows from the approximately 20 cells that are set aside during embryogenesis to roughly 2000 cells in third larval instar. Photoreceptor differentiation is initiated in early third instar at the posterior margin of the eye disc and proceeds anteriorly following a synchronous wave of cellular changes called the morphogenetic furrow, shown in Fig. 5 (C) and (D) (Ready et al., 1976). These changes ultimately generate 750 - 800 differentiated ommatidia organized in a precise hexagonal array. Each ommatidium consists of eight photoreceptors, twelve accessory cells, including four cone cells, six pigment cells and one mechanosensory bristle, see Fig. 5 (B) (Pappu et al., 2003).



**Figure 5.** The structure and development of the *Drosophila* eye. **(A)** The fly's eye is composed of a regular array of 750 - 800 ommatidia; anterior is to the left. **(B)** Each ommatidium has the same internal structure. This cartoon shows most of the cells in one layer and the photoreceptors (1-8) are arranged in the order they develop rather than their adult position. The 'mystery' cells (m) are only present in the third instar eye disc - they later leave the developing ommatidium. c: cone cell, 1°, 2°, 3°, primary, secondary and tertiary pigment cells, respectively. **(C)** The third instar imaginal disc, anterior is at the top, as indicated. The morphogenetic furrow (MF, red) sweeps anteriorly, leaving developing ommatidia (small circles) in its wake. There is therefore a gradient of maturing cell clusters, indicated by the yellow arrow. Cells ahead of the furrow (pale blue) are proliferating, but they enter G<sub>1</sub> arrest (dark blue) in front of the furrow. **(D)** The stereotype pattern of differentiation of photoreceptors, with clusters of increasing maturity from the top. R8 is the first photoreceptor that is recruited. Redrawn from (Freeman, 1997) with permission from the Company of Biologists LDT.

## Notch

Notch signaling, in combination with other cellular factors, controls cell fates through local cell interactions. Notch (N) is a transmembrane receptor activated by the partially redundant ligands Delta and Serrate. The transcription factor Suppressor of Hairless (Su(H)) appears to function as the major downstream effector of Notch signaling and the genes of the Enhancer of split complex are the primary targets of Notch signaling

(Artavanis-Tsakonas et al., 1999). When inducing ectopic eyes with *UAS-N<sup>act</sup>*, a constitutive active Notch (N) protein, both *ey* and *toy* are co-expressed in the growing eye-antennal discs indicating that N is upstream of *ey* and *toy* (Kurata et al., 2000). When expressing a *UAS-N<sup>DN</sup>* in the eye disc (under *ey-Gal4*) the eye is transformed into an antenna, indicating that N is a homeotic determinant of eye specification. The phenocritical period for *N<sup>DN</sup>* in eye disc development is during the second half of the second larval instar. The phenocritical period for removing N coincides with the first time when the 6 early eye genes (*toy*, *ey*, *eyg*, *eya*, *so* and *dach*) are expressed in the eye disc (Jang et al., 2003; Kumar and Moses, 2001). This strongly suggests that the posterior half of the eye-antennal discs gets an eye identity during this stage through Notch signaling. When other components in the Notch pathway were expressed in the developing eye disc as dominant negatives, both Delta and Serrate gave eye to antenna transformations, but neither Su(H) nor many members of the Enhancer of split complex gave any effect. An eye to antenna transformation was however also obtained with a dominant negative construct of *mastermind (mam)* (Kumar and Moses, 2001). Mam is a transcription factor and a neurogenic gene that show most prominent expression in the eye disc posterior to the morphogenetic furrow (Bettler et al., 1996). Interference of this late expression is most likely not causing the transformation, but an earlier *mam* expression during the second instar.

Kenyon et al. (2003) oppose the view that N promotes eye identity by regulating *ey* or *eya*, because in *N* mutant clones in the eye disc neither *ey* nor *eya* expression are affected. Notch can instead influence the establishment of the eye primordium through its control of proliferation in the eye disc. Notch and its effector Suppressor of Hairless (Su(H)) work also upstream of *eyg* in the eye disc, where the role of *Eyg* is to regulate eye proliferation (Dominguez et al., 2004) see also (Mann, 2004), shown in Fig. 6. Dominguez and coworkers (Dominguez et al., 2004) state that N is not upstream *ey* and *toy*. Their primary result behind this statement could be questioned in the light of results in paper IV in this thesis.

### *Egfr*

The *Drosophila* EGF receptor homolog, commonly called DER or *Egfr*, is a transmembrane receptor tyrosine kinase and has a complex biology. In addition to numerous functions outside the topic of this thesis, it is involved in dorsal-ventral, anterior-posterior patterning (Ray and Schupbach, 1996), optic lobe formation (Dumstrei et al., 2002), in the developing retina (Kumar et al., 1998) and in the formation of adult head vertex (Amin et al., 1999). The principal ligand of *Egfr* is Spitz and together they activate the Ras pathway. The segregation of *toy* and *ey* expression to the eye disc, and

antennal specific protein(s) to the antennal disc are an important step in eye-antennal discs development. Notch and Egfr signaling act antagonistically upstream of *toy* and *ey*: Egfr signaling promotes an antennal fate and blocks eye fate, while Notch signaling is acting in the opposite way. When  $Egfr^{DN}$ , is expressed under *ey-Gal4* both eye and antennal discs are deleted, so either Egfr is needed early in eye-antennal primordium and/or it is needed later in development in both discs. A constitutively active Egfr gives an eye to antenna transformation. Overexpression of wild-type or activated forms in the eye disc of other components of the Ras pathway including Spitz and the downstream elements Ras, Raf and PointedP1 (PntP1) gave same type of transformations (Kumar and Moses, 2001). See Fig. 6. *pointed* is expressed in the embryonic head, eye-antennal primordium and in the eye disc. A mutant fly has a pointed head (Jurgens et al., 1994).

### ***hedgehog (hh), decapentaplegic (dpp) and wingless (wg)***

The products from these genes are all secreted signaling molecules that regulate the size and shape of many embryonic and adult structures. Ectopic expression of *ey* in the wing disc is restricted to areas where *hh* and *dpp* are expressed and co-expression shows that Ey needs to collaborate with high levels of Hh and Dpp to induce ectopic eye formation. Ectopic *ey* induction does, however, not induce *hh* expression and the induced level of Dpp is too low for inducing ectopic eyes (Kango-Singh et al., 2003). *dpp* expression precede the induction of *eya*, but not the restriction of Ey to the eye disc during second instar. In *dpp* mutants Eya, but not Ey, is absent (Kenyon et al., 2003). When overexpressing Dpp under *ey-Gal4*, the *eya* expression is upregulated throughout most of the Ey-positive field. Therefore, *eya* expression and formation of eye primordium seems to be Dpp dependent (Kenyon et al., 2003). During morphogenetic furrow initiation *dpp* is the sole target of Hh and the main role for Hh signaling is to alleviate the repression of *dpp* and *eya* by  $Ci^{rep}$ ,  $Ci^{act}$  has little or no role in this context. The critical target of Hh signaling is *eya* (Pappu et al., 2003).

Overexpression of wild-type Ci in the developing eye gives an eye to antenna transformation (Kumar and Moses, 2001).  $Ci^{act}$  or  $Ci^{rep}$  ought to have a repressive role on eye disc identity; since for a transformation to occur it is probably not enough only to turn off eg. *dpp* and *eya*. On the other hand it may be enough given that Kenyon and coworkers (2003) are right when they state that the transformed antenna originates from one enlarged antennal primordium. The wingless pathway functions through Sloppy paired (Slp). Expressing *slp2* in the developing eye gives no eye (Kumar and Moses, 2001) and this may be interpreted as if the wingless pathway would block eye induction. (See Fig 6.)

## *toy* and *ey*

Both *toy* and *ey* are expressed in the entire eye disc in the second and early third instar but in late third instar they are expressed in undifferentiated cells anterior to the morphogenetic furrow and downregulated in cells undergoing differentiation (Czerny et al., 1999; Halder et al., 1998).

What phenotypes do the ectopic expression of the two Pax6 genes produce? Both induce ectopic eyes on legs and wings with the similar strength when misexpressed (Czerny et al., 1999; Halder et al., 1995). Ectopic expression of Toy induces *ey* expression, but Ey does not induce *toy*, placing Toy upstream of *ey* during (ectopic) eye induction. Furthermore, ectopic expression of Toy is unable to induce eyes in an *ey*<sup>2</sup> mutant (which is mutated in the eye specific enhancer in the *ey* gene). The driver used to express *UAS-Toy* is *dpp*<sup>disk</sup>-*Gal4* (Czerny et al., 1999). When Punzo et al. (2002) express *toy* ectopically with *dpp*<sup>blink</sup>-*Gal4*, *so* is induced in an *ey*<sup>2</sup> mutant background.

Expression of *ey* induces the expression of *so* and *eya* (Halder et al., 1998). Ey protein regulates *so* directly, so when this regulatory region is deleted in the *so*<sup>l</sup> mutation, Ey fails to induce *so* and to induce ectopic eyes. In contrast, ectopic Toy induces both So and ectopic eyes in homozygous *so*<sup>l</sup> mutants (*so*<sup>l</sup> is mutated in the eye specific enhancer in the *so* gene) (Niimi et al., 1999). This indicates that Toy either can induce *so* by other cis-acting regions or that Toy can induce *so* indirectly.

Several *toy* mutations were generated in Hochman's systematic screens of X-ray and EMS induced mutations on the fourth chromosome (Hochman et al., 1964). One was named *l(4)8* (the others are lost) and was kept in the stock centers for decades; in 2002 the phenotypic and molecular analysis of *l(4)8* or *toy*<sup>hdl</sup> was published showing it to be a null mutation of *toy* (Kronhamn et al., 2002).

The first mutant in *ey* was isolated in 1914 by Hoge (1915). The mutations *ey*<sup>2</sup> and *ey*<sup>R</sup> are both hypomorphs, but expression of *ey* is not detected in the eye-antennal primordium in any of them (Czerny et al., 1999; Qiring et al., 1994). The *ey*<sup>D</sup> mutant was induced by X-irradiation by Muller (Patterson and Muller, 1930). Until the molecular characterization of *ey*<sup>D</sup> in 2002 there was some doubt whether this mutation belonged to the *ey* locus (Kronhamn et al., 2002; Lindsley and Zimm, 1992). *ey*<sup>D</sup> is a mutation in *ey* but it is still not clear why it has a dominant phenotype, while deletions uncovering *ey* are phenotypically normal. *ey*<sup>J5.71</sup> and *ey*<sup>C7.20</sup> are the only mutations recorded as *ey* null alleles (Kammermeier et al., 2001; Kurusu et al., 2000; Punzo et

al., 2002). The *ey*<sup>J5.71</sup> allele is reported to be caused by a 9-kb deletion in the 5' region of the gene, and to be an RNA and protein null mutant (Punzo et al., 2001). Complementation analysis of *ey*<sup>J5.71</sup> seems, however, to contradict the proposition that it is a null mutation (*ey*<sup>C7.20</sup> was not analyzed) (Callaerts et al., 2001). Unfortunately, no molecular analysis of these mutants has been published to support any of the assertions.

### ***eyg***

*eyg* is, as stated above, a *Pax* gene encoding a truncated Paired domain. *eyg* is expressed in the eye-antennal primordium and has a headless loss-of-function phenotype, similar to *ey* and *toy* (Jang et al., 2003). *eyg* expression is detected at second instar in the eye disc and it has its phenocritical period during late second instar (Dominguez et al., 2004; Jang et al., 2003). Ectopic expression of *eyg* induces extra eyes only in the eye disc. The driver used was *dpp-Gal4*, indicating the need of Dpp for ectopic eye induction. *ey* and *eyg* can induce ectopic eyes independently of each other, are transcriptionally independent, but have a synergistic effect in ectopic eye induction when coexpressed (Jang et al., 2003).

Dominguez and coworkers (2004) conclude that the role of *Eyg* is in regulating eye proliferation, but that it is dispensable for specification and differentiation of the eye. Misexpression of the anti-apoptosis baculoviral protein P35 driven by *ey-Gal4* fail to rescue the 'no eye' phenotype of *eyg* mutants suggesting that apoptosis is not the major cause of the eye phenotype (Jang et al., 2003). In the second instar *eyg* is expressed along the midline of the disc induced by Notch signaling (Dominguez et al., 2004).

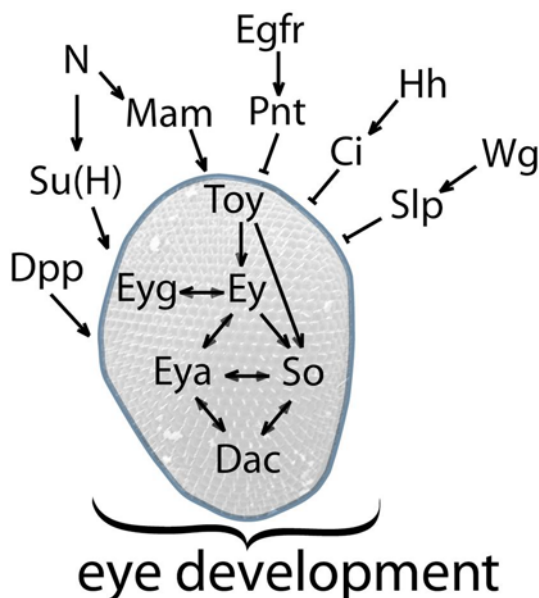
### **Core retinal determination genes**

Positive feed back loops exist whereby *ey* together with *eya*, *so*, and *dac* mutually sustain each other's expression (Chen et al., 1997; Pignoni et al., 1997). These four genes are sometimes called "core retinal determination genes" (Pappu and Mardon, 2002). *Ey* initiate expression of both *eya* and *so*, and cannot induce ectopic eyes in neither *eya*<sup>l</sup> nor *so*<sup>l</sup> (both have mutations in eye specific enhancer) (Halder et al., 1998). *Eya* protein is first to define the area in the eye disc that develops into the eye (eye primordium) and *So* is expressed slightly later during second instar (Kenyon et al., 2003). *Eya* is likely to function upstream of *so* since *so* expression is absent third instar eye disc in *eya*<sup>l</sup> mutant, while *So* is not required for *eya* expression (Halder et al., 1998). Both *eya*<sup>l</sup> and *so*<sup>l</sup> mutant are without compound eyes, *so*<sup>l</sup> is also without ocelli (Bonini et al., 1993; Cheyette et al., 1994). *Eya* can (only) induce ectopically eyes in the eye-antennal discs (Bonini et al., 1997). Co-expression of *eya* and *so* increased the development of ectopic

eyes drastically. Pignoni et al. (1997) also show that Eya and So have direct protein-protein interaction. Furthermore, *eya* can not induce ectopic eyes in *ey<sup>2</sup>* homozygous mutants, and *ey* is expressed in the ectopic eyes in the eye-antennal discs induced by Eya (Bonini et al., 1997), with underline the importance of the feed back loop.

*dac* is downstream of Eya and So based on the observation that Dac is not required for *eya* or *so* expression and that So and Eya are required for *dac* expression (Chen et al., 1997). Otherwise, it is a similar story for *dac* as for *eya*. (1) A mutation specific for eye development in *dac* is without compound eyes (Mardon et al., 1994). (2) *dac* is, as *eya*, expressed along the posterior margin of the second instar eye disc (Mardon et al., 1994). (3) *dac* is able to induce ectopic eyes when misexpressed. (4) *ey* are activated and needed for ectopic eye induction (Chen et al., 1997). Additionally, Dac and Eya have protein-protein interaction (Chen et al., 1997).

*optix* and *teashirt* are both good candidates for being "early eye genes". Both have been shown to induce ectopic eyes and they are expressed in second instar larval eye discs. However, mutations in *teashirt* do not show any eye phenotype and no mutations are known for the *optix* gene (Pan and Rubin, 1998; Seimiya and Gehring, 2000).

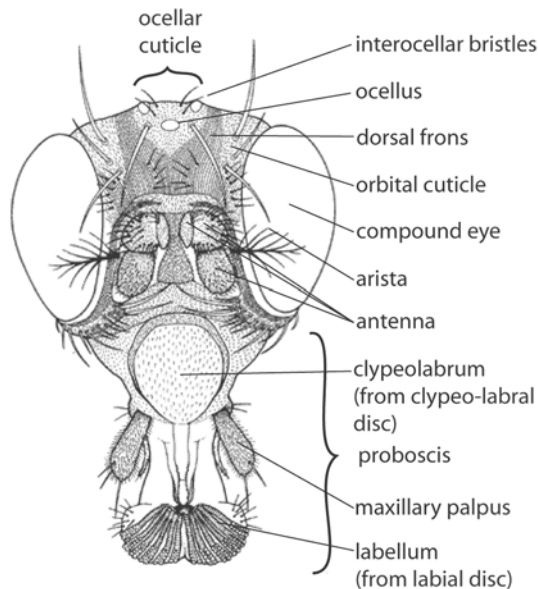


**Figure 6.** A model for the genetic network controlling eye determination and a summary of the text in "The developing eye". The proteins indicated in the eye are from what is called in this introduction "early eye genes". They are all expressed in the eye disc during the second larval instar and can on there own or together induce ectopic eyes.

## Adult head

With the exception of the central nervous system, most of the structures of the adult fly develop during the larval period and take their final form during the pupal stage.

The external structures of the adult head develop from three imaginal discs. The main part of the head, including the head capsule with the ocelli, the antenna, the compound eyes, the maxillary palpi and the upper proboscis develop from the fusion of the paired eye-antennal discs, shown in Fig. 4. The distal part of the proboscis, including the labellum develops from the labial disc. The clypeolabrum develops from the clypeolabral disc (See Fig 7) (Hartenstein, 1993).



**Figure 7.** The anterior part of the adult *Drosophila* head. Compare the figure with figure 4. Redrawn from (Bryant, 1978) with permission from Elsevier.

Weak *orthodenticle* (*otd*; Flybase: *ocelliless*) mutant alleles have a head phenotype reminiscent of the weak *toy* phenotype, with defects in the dorsal region of the head, the head vertex. This region that lies between the compound eyes can be divided into three morphological domains. The ocellar cuticle, the most medial domain, contains the three ocelli, between the ocelli are located six to eight small interocellar bristles and outside the ocellar triangle are two sets of larger bristles, only the ocellar bristles are shown in Fig. 7. The second domain, the dorsal frons lies mediolaterally and consists of a series of closely spaced parallel ridges. The orbital cuticle is the third most lateral domain. It occupies the space between the frons and the compound eyes (Royet and Finkelstein, 1995) (see Fig. 7). *otd* is expressed in the part of the eye-antennal discs that later develops into the

dorsal part of the head. A progressive reduction of *otd* during third instar eye disc development causes the graded loss of structures along the mediolateral axis starting with the ocellar cuticle. *otd* mutants also affect the frons cuticle but even the strongest of the *ocelliless* alleles in the *otd* locus do not have an effect on the lateral orbital cuticle. In the ocellar cuticle, the domains of *en* and *hh* are coexpressed, but the *hh* expression precedes *En*. *Hh* is suggested to activate *otd* in the head vertex primordium (Royet and Finkelstein, 1996). *otd* acts through the segment polarity gene *engrailed* (*en*) to pattern the ocellar cuticle (Royet and Finkelstein, 1995)

### ***Pax6* in vertebrate eye development**

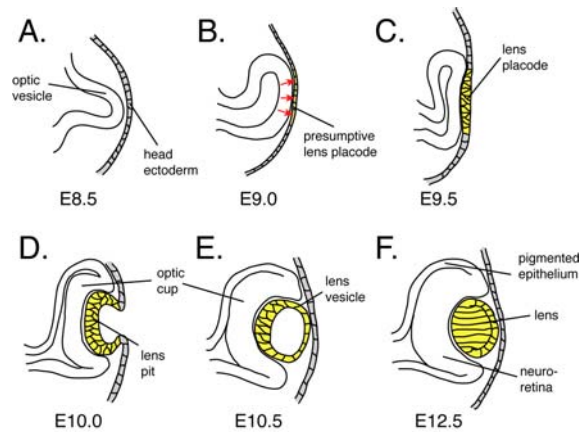
*Pax6* homologues are required for eye or light sensing structure development in a wide variety of chordates ranging from sea squirts (Tunicates) to humans (Glaridon et al., 1997; Tomarev et al., 1997). *Pax6* is, however, not found to be important for triggering the eye development in flatworms (Pineda et al., 2002). The camera-type eye with a single lens projecting onto a retina that is found in vertebrates and cephalopods is strikingly different from the compound eye of insects. The differences lie not only in the basic morphology of the two types of eye, but also in their different embryonic origins, their photoreceptors and their phototransduction pathways. Nevertheless, *Pax6* plays a central role in eye development in both vertebrates and invertebrates.

### **The developing mouse eye**

*Pax6* expression has been studied in many vertebrates including mouse and humans (Callaerts et al., 1997), and the expression is comparable in all species examined. The following description is based on the expression in mouse.

The eye develops as a two-component system, with the neural ectoderm on the one hand and the head surface ectoderm on the other. Morphological development of the eye begins with the formation of an outpouching of the neural ectoderm/diencephalon called the optic vesicle at the embryonic day 8.5 or stage E8.5, see Fig. 8A. The optic vesicle subsequently contacts the head ectoderm (E9.0; see Fig. 8B) and signals the induction of a pseudostratified thickening of the ectoderm called the lens placode (E9.5; see Fig. 8C). The lens placode invaginates and separates from the surrounding ectoderm to form a lens vesicle (E10; see Fig. 8D and E). Eventually, the cells of the lens vesicle differentiate into fiber cells characteristic of the adult lens (E12.5; see Fig. 8F). In parallel, the optic

vesicle folds inward on itself, surrounding the lens vesicle and forming the optic cup (E10), which will eventually form the neural and pigmented layers of the adult retina f(E12.5).(Wawersik and Maas, 2000).

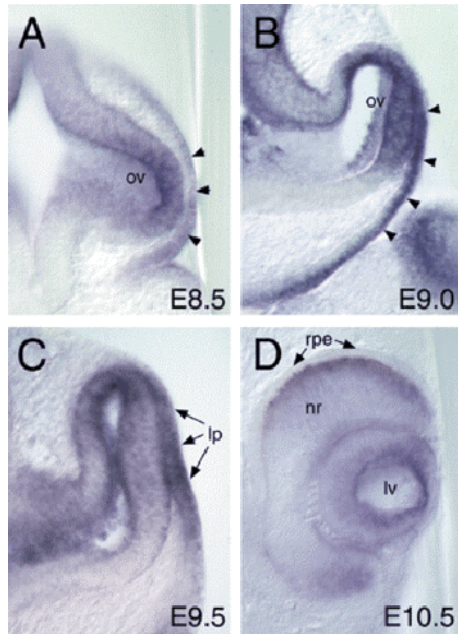


**Figure 8.** Vertebrate eye development. **(A)** The optic vesicle forms as an outpouching of the forebrain at embryonic day 8.5, compare with Fig. 9A. **(B)** The out-pouching contacts the ectoderm of the head at E9.0. **(C)** Signals from the optic vesicle, see arrows, induce the lens placode to form. **(D)** The lens placode invaginates to form a lens pit, whereas the optic vesicle invaginates to create the optic cup. **(E)** Invagination of the lens pit to form the lens vesicle is complete, and the lens vesicle detaches from the overlying ectoderm. **(F)** Differentiation of the optic cup continues, with the inner layer forming the neuroretina and the outer layer comprising the retinal pigment epithelium. Reprinted from (Wawersik and Maas, 2000) by permission of Oxford University Press.

### Expression of *Pax6* in the mouse eye

The failure of homozygous *Pax6* mutants to form a lens placode indicates an early role for *Pax6* in eye development. *Pax6* is first expressed in a broad ectodermal anterior surface, covering the prosencephalon (which later divided into the telencephalon and the diencephalon) and in an extensive part of head neural ectoderm (E8.0). The latter is resolved into bilateral fields under the influence of sonic hedgehog (Belloni et al., 1996) resembling the early *toy* expression in neuroblasts in the protocerebrum in *Drosophila* (Urbach and Technau, 2003a). *Pax6* is expressed during all stages shown in Fig. 8. The developing lens cells goes through three stages, first the Bias stage (E8.5; see Fig. 9A), then the Specification stage (E9, see Fig. 9B) finally the Differentiation stage (E10-11, see Fig. 9 D). Experiments, in which *Pax6* has been exclusively deleted from the surface ectoderm show that *Pax6* is first essential for the Bias stage where *Pax6* activates *sox2* in the ectoderm. Then *Pax6* activity is essential for the initiation of lens differentiation. During this stage *Pax6* controls the

expression of other regulatory genes such as the homeobox genes *Six3*, *Drosophila* homolog: *so* (Ashery-Padan et al., 2000).



**Figure 9.** *In situ* hybridization with *Pax6* in the developing mouse eye. **(A)** *Pax6* is expressed in the optic vesicle (ov) and in the overlying surface ectoderm (arrowhead). Compare with Fig. 8A. **(B)** The optic vesicle (ov) contacts the surface ectoderm and *Pax6* is expressed in a broad domain of the head ectoderm (arrowheads) and in the optic vesicle (ov). **(C)** Prior to thickening of the head ectoderm, *Pax6* expression becomes restricted to the presumptive lens placode (lp). **(D)** *Pax6* is detected in the lens vesicle (lv) and in the optic cup. nr: neuroretina, rpe: retinal pigmented epithelium. Reprinted from (Wawersik and Maas, 2000) by permission of Oxford University Press.

The growing optic vesicle contains bipotential progenitors that could give rise to both pigmented and neural layered cell types. *Pax6* is expressed in the cells that will give rise to the optic vesicle. Surprisingly, *Pax6* function seems to be dispensable for the formation of the optic vesicle, as indicated by expression of early retinal markers in homozygote mutant *Pax6* optic rudiments (Ashery-Padan and Gruss, 2001; Grindley et al., 1995). Marquardt et al., (2001) have shown that *Pax6* is important late in retinal development to maintain a pluripotent phenotype in the retinal progenitor cells. Tissue specific inactivation of *Pax6* just after the retinal progenitor cells have formed restricts the fate of these precursor cells such that only amacrine cells (a type of retinal neuron) continue to develop at the expense of five other retinal neuron cell types.

### ***Pax6* mutations**

Homozygous *PAX6* mutations in humans result in anophtalmia (complete lack of eyes), nasal hypoplasia and central nervous system defects (Wawersik and Maas, 2000). Homozygous mutations in mouse *Pax6* are lethal and the mouse embryo completely lacks eyes, nasal cavities and has severe brain defects. The *PAX6* gene dosage is also important: haploinsufficiency in humans causes aniridia (absence of iris), a heritable

panocular disorder characterized by iris and foveal hypoplasia, often accompanied by cataracts, corneal opacification and progressive glaucoma. Like aniridia, mutations in the mouse *Pax6* cause the Small eye (*Sey*) phenotype and are also inherited in a semi-dominant fashion, with heterozygotes exhibiting corneal and lenticular (crystalline lens) abnormalities (Hill et al., 1991; Wawersik and Maas, 2000). Overexpression of *Pax6* is also deleterious, leading to reduced eye size in mice transgenic for five copies of a human *PAX6* (Schedl et al., 1996), underlining the dosage sensitivity of PAX6 in eye development. The close similarities in phenotype and mode of inheritance between aniridia and *Sey* suggest that Pax6 functions equivalently in the regulation of both humans and mouse eye development.

It is fascinating that despite the radically different architecture in vertebrates and fly eye patterning, a similar molecular scaffold underlies both structures, suggesting that the power of *Drosophila* genetics can be harnessed to study mammalian ocular development and used to answer questions underlying human diseases.

## Conclusions

### **Paper I**

The genes *cubitus interruptus (ci)*, *Ribosomal protein S3A (RpS3A)* and *pangolin (pan)* are located within 73 kb in the cytological region 101F-102A on chromosome 4 in *Drosophila melanogaster*. Both *ci* and *pan* are encoding segment polarity protein. A region of 13 kb harbors the regulatory region of both *ci* and *pan* transcribed in opposite directions and the gene *RpS3A*. This clustering gives rise to very complicated complementation patterns and we have investigated the region genetically and molecularly, see Fig 2 and 3 in paper I.

The nature of newly generated and old Minute mutants of RpS3A was thoroughly characterized. The mutants were found to have a ~50% reduction in *RpS3A* transcript. The developmental delays with and without maternally derived Minute chromosomes were characterized. Paternally derived Minute chromosomes resulted in a ~24-25 h delay, while maternally derived Minute chromosomes augmented the delay to 29-37 h.

The early zygotic expression pattern of *pan* was elucidated with the enhancer trap line *lacZ* expression of *IA5*. Staining against  $\beta$ -gal show expression in a segmentally reiterated pattern in stage 10-12 embryos and in brain lobes and in gut in later embryos. The homozygote *IA5* embryos exhibit severe brain lobe defects, indicating that a wingless signal transduction mechanism is necessary for brain lobe formation.

The characterization of the P-element insert *IA5* is useful for the *Drosophila* researchers. It is, to my knowledge, the best recessive lethal  $\beta$ -gal "balancer" for the 4th chromosome.

### **Paper II**

The first mutant of *toy* was characterized. It showed a headless phenotype, and this indicates that Toy is important for both the eye and antennal disc development. The temperature sensitivity of the *toy* mutant is due to the transcription of *ey* in the eye-antennal primordium, that is temperature

dependent in absence of Toy. This means that activation of *ey* is not strictly dependent on the Toy Protein. This temperature sensitivity is used to find out that the phenocritical period for *ey* in the adult head development is during embryonic stage 12-16 when *ey* is first expressed in the eye-antennal primordium.

The strong *ey<sup>D</sup>* mutation is molecularly characterized and it is finally settled that it belongs to the *ey* locus. It has also been shown that *ey<sup>D</sup>* homozygotes are headless i.e. have a much stronger phenotype than earlier *ey* mutations.

Finally, by inhibiting apoptosis by misexpression the baculovirus P35 protein under *ey-Gal4*, we could partially rescue the headless, but not the eyeless phenotype of *ey<sup>D</sup>* homozygotes. This implies that the headless phenotype of *ey<sup>D</sup>* is a result of considerably cell death in the eye-antennal discs, a process that is inhibited by the wild-type Ey protein.

### **Paper III**

This paper dissects the 5 kb cis-regulatory region of *toy*, by making transgenic lines containing reporter constructs driven by the upstream *toy* sequences and by *in silico* analysis of possible binding sites in the same region. A 170 bp region was found to be necessary for both the expression of an RNAi transgene interfering with the translational machinery to generate headless pharate adults and for the expression of the reporter construct in the larval eye disc. Using *in silico* analysis of the same region a number presumptive binding sites were found of which Broad and Eip74EF looked most interesting. Furthermore, a 280 bp might be important for expression in the eye-antennal primordium.

We also report the identification of a new transcription start site of the *toy* gene, which could be translated into a protein lacking the paired domain. Reporter constructs containing the region upstream of the new transcription start is expressed in only a pair of cells in the *Drosophila* embryo. This pair of cells is most likely a non-neuronal cell in the labial sensory complex.

## **Paper IV**

Homozygous *toy<sup>hdl</sup>* was rescued with *toy-Gal4>UAS-toy*. To our surprise, even the heterozygous *toy<sup>hdl</sup>* containing the rescue constructs had problems to survive at 29°C, the temperature at which the *Gal4>UAS* system is normally optimal. When the temperature was successively lowered, a rescue of 70% was achieved at 15°C. At this temperature we could see a positive effect of the toy driver, showing that we have cloned an important regulatory sequence. Homozygous *ey<sup>D</sup>* was also rescued with *ey-Gal4>UAS-ey*. This shows that the lethality of *ey<sup>D</sup>* is not caused by an additional gene since the eyeless protein alone is sufficient to rescue the lethality.

An enhanced level of cell death in the eye disc of the *ey<sup>2</sup>* mutant has previously been reported. It is more surprising that the predominant place of cell death in the third instar larval eye-antennal discs in the *toy<sup>hdl</sup>* mutant is in the anterior part of the antennal disc. Lack of antennae has been assigned to *toy<sup>hdl</sup>* weaker phenotype, unless in the headless phenotype. Since the head cuticle phenotype of *toy<sup>hdl</sup>* is rescuable with the anti-apoptotic P35 protein, the central role of Toy in the antennal disc has to be related to cell survival.

We also found a strong redundancy between *ey* and *toy*. Two strong *ey* mutations were rescued to viability by *toy-Gal4>UAS-toy*, to the same levels as when using the transgenic *ey<sup>+</sup>* gene. Similarly, *toy<sup>hdl</sup>* and *toy<sup>G7.39</sup>* were rescued by *ey-Gal4>UAS-ey*. When trying to rescue the observed cell death in the eye-antennal discs of *toy* and *ey* mutants, the lethality was rescued to a moderate degree but the compound eyes of the *ey<sup>D</sup>* mutant and ocelli of the *toy<sup>hdl</sup>* mutant were almost never rescued, concluding that the redundancies are only partial.

## Future perspectives

The cis regulatory region of *toy* will be further scrutinized. We have currently seven more independent insertions of construct *toy1-Gal4*, since 4 of the 7 constructs we have analyzed showed a variegated  $w^+$  expression (and in 2 of the remaining strains the P-element was inserted in the *TMS*,  $\Delta 2-3$  *Sb* chromosome). These strains will be analyzed in the same way as *toy1-Gal4(a-f)*. The analysis of these will hopefully underline the results from our current strains and confirm the direction of the analysis of the cis regulatory region.

The *in silico* analyses are not intended to be published but is a first step to find potentially upstream regulators of the *toy* gene. From the current analysis we would like to do gel shift analyses for Broad, Eip74EF (Ecdysone-induced protein 74EF) and possibly also Engrailed. If positive results are found, foot printing will be pursued. Furthermore, analyses of the expression of *toy* cis regulating constructs in mutant *broad*, *Eip74EF* and *engrailed* background, respectively, would further elucidate their interaction.

To get a picture of the new *toy* transcript that starts at exon 1' and which results in a protein lacking the paired domain, we are currently analyzing if Toy is expressed in the pair cells that are positive for the reporter constructs. In situ hybridization using a probe from exon 1' would reveal the full expression pattern of the transcript and we will use markers for the different cell types in labial sensory organ to elucidate what type of cells this is.

One new question is in which way Toy is responsible for the antennal disc development. Does Toy induce *cut* that in turn inactivates *toy*?

## Acknowledgements

My time as a graduate student started at the Department for Genetics, Umeå University and will finish at Molecular Biology, Umeå University, which "Genetics" has merged with. Some part of this project has also been done at Markus Noll's laboratory, Institute for Molecular Biology, in Zürich, Switzerland. I would like to thank all friends and co-workers during my time as a graduate student, especially:

My supervisor **Åsa Rasmuson-Lestander** for allowing me to be an individual with an individual way to work and to solve problems. You also had belief in my abilities and gave me a great degree of freedom in my work, which has helped me in maturing as a scientist. I appreciate you very much as a friend and as a scientist.

**Markus Noll** and all the members in his laboratory, especially Erich Frei that took such good care of me in Zürich, both showing me the lab and Switzerland. Markus - I appreciate your enthusiasm, knowledge and that you took me under your wings. Renjie for being so open-hearted and for sharing my interest in headless flies and with whom I found the way back to *l(4)8*. Yandong for being a good friend and co-worker on *DPax2*. Werner - next time I come to Zürich we will have a fermented herring party.

**Christos Samakovlis** and his (former) graduate students: Camilla, Per and Johanna. My first year of research was in your lab and I have much of the things that have worked well in my hands to thank you for.

**Anssi Saura** for always being generous with your time, knowledge and encouragement.

Stefan and Janne for taking care of Department of Genetics.

All (former) graduate students: Ammie, Anna, Bettan, Magnus, Malin, Markus, Per, Sa and Tor-Mikael, it has been rewarding to get to know you.

Anna-Sara, Astrid, Gunilla, Helena, Karin, Kerstin, Maggan and Marianne for making me long for the coffee breaks. To Kerstin for having fun in the lab and for all the gels. The fly kitchen - for all the tasty flyfood.

**Dan Hultmark's group:**

AnnaKarin (I come back to you), Dan, Calle, Ines, Ingrid, Karin, Magdalena, Michael, Pia, Shannon, Svenja and Thomas. A big hug to you all that nearly have "adopted" me to your group. And thanks Michael for your efforts to ameliorate my English.

**Ruth Palmer's group:**

Camilla, Caroline, Christina and Ruth for your openness to mix *toy* with the mysteries PTK's in our journal club.

**Molecular Biology:**

I really appreciate coming here. Peter, David and Regina - for sharing the heat and square meters, with Sa, Kerstin and me in our office and all you who appreciate the 2 o'clock coffee-break in "ölhörnan".

**Outside the lab:**

Kent, KF and Bertil - for being such good and honest friends.

**Family:**

Agneta and Thommy, Ulf and Angelica, Andreas and Åsa, and Nilla - for being there and trying to understand what I am doing.

Harry and Monica, and Louise for letting me be a part of your family and for all your support.

Finally, **AnnaKarin** and **Signe** - You have shown me what love is. Words cannot express my feelings for You - I love You!

## References

- Adams, M. D., Celniker, S. E., Holt, R. A., Evans, C. A., Gocayne, J. D., Amanatides, P. G., Scherer, S. E., Li, P. W., Hoskins, R. A., Galle, R. F. et al.** (2000). The genome sequence of *Drosophila melanogaster*. *Science* **287**, 2185-95.
- Amin, A., Li, Y. and Finkelstein, R.** (1999). Hedgehog activates the EGF receptor pathway during *Drosophila* head development. *Development* **126**, 2623-30.
- Artavanis-Tsakonas, S., Rand, M. D. and Lake, R. J.** (1999). Notch signaling: cell fate control and signal integration in development. *Science* **284**, 770-6.
- Ashburner, M.** (1989). *Drosophila. A laboratory handbook*. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.
- Ashery-Padan, R. and Gruss, P.** (2001). Pax6 lights-up the way for eye development. *Curr Opin Cell Biol* **13**, 706-14.
- Ashery-Padan, R., Marquardt, T., Zhou, X. and Gruss, P.** (2000). Pax6 activity in the lens primordium is required for lens formation and for correct placement of a single retina in the eye. *Genes Dev* **14**, 2701-11.
- Belloni, E., Muenke, M., Roessler, E., Traverso, G., Siegel-Bartelt, J., Frumkin, A., Mitchell, H. F., Donis-Keller, H., Helms, C., Hing, A. V. et al.** (1996). Identification of Sonic hedgehog as a candidate gene responsible for holoprosencephaly. *Nat Genet* **14**, 353-6.
- Bettler, D., Pearson, S. and Yedvobnick, B.** (1996). The nuclear protein encoded by the *Drosophila* neurogenic gene mastermind is widely expressed and associates with specific chromosomal regions. *Genetics* **143**, 859-75.
- Bonini, N. M., Bui, Q. T., Gray-Board, G. L. and Warrick, J. M.** (1997). The *Drosophila* eyes absent gene directs ectopic eye formation in a pathway conserved between flies and vertebrates. *Development* **124**, 4819-26.
- Bonini, N. M., Leiserson, W. M. and Benzer, S.** (1993). The eyes absent gene: genetic control of cell survival and differentiation in the developing *Drosophila* eye. *Cell* **72**, 379-95.
- Bopp, D., Burri, M., Baumgartner, S., Frigerio, G. and Noll, M.** (1986). Conservation of a large protein domain in the segmentation gene paired and in functionally related genes of *Drosophila*. *Cell* **47**, 1033-40.
- Breitling, R. and Gerber, J. K.** (2000). Origin of the paired domain. *Dev Genes Evol* **210**, 644-50.
- Bridges, B. C.** (1925). Sex in relation to chromosomes and genes. *Am Nat* **59**, 127-137.

- Bridges, B. C.** (1935a). Cytological data on chromosome four of *Drosophila melanogaster*. *Trud Dinam Razvit* **10**, 463-474.
- Bridges, B. C.** (1935b). The mutants and linkage data of chromosome four of *Drosophila melanogaster*. *Biol. Zh. (Mosc.)* **4**, 401-420.
- Bryant, P. J.** (1978). Pattern formation in imaginal discs, (ed. M. Ashburner and T. R. F. Wright), pp. 230-335. London: Academic press Inc. Ltd.
- Callaerts, P., Halder, G. and Gehring, W. J.** (1997). PAX-6 in development and evolution. *Annu Rev Neurosci* **20**, 483-532.
- Callaerts, P., Leng, S., Clements, J., Benassayag, C., Cribbs, D., Kang, Y. Y., Walldorf, U., Fischbach, K. F. and Strauss, R.** (2001). *Drosophila* Pax-6/eyeless is essential for normal adult brain structure and function. *J Neurobiol* **46**, 73-88.
- Campos-Ortega, J. A. and Hartenstein, V.** (1997). The Embryonic development of *Drosophila melanogaster*. Berlin, Heidelberg: Springer-Verlag.
- Carr, M., Soloway, J. R., Robinson, T. E. and Brookfield, J. F.** (2001). An investigation of the cause of low variability on the fourth chromosome of *Drosophila melanogaster*. *Mol Biol Evol* **18**, 2260-9.
- Casanova, J. and Struhl, G.** (1993). The torso receptor localizes as well as transduces the spatial signal specifying terminal body pattern in *Drosophila*. *Nature* **362**, 152-5.
- Chang, T., Mazotta, J., Dumstrei, K., Dumitrescu, A. and Hartenstein, V.** (2001). Dpp and Hh signaling in the *Drosophila* embryonic eye field. *Development* **128**, 4691-704.
- Chang, T., Shy, D. and Hartenstein, V.** (2003). Antagonistic relationship between Dpp and EGFR signaling in *Drosophila* head patterning. *Dev Biol* **263**, 103-13.
- Chen, R., Amoui, M., Zhang, Z. and Mardon, G.** (1997). Dachshund and eyes absent proteins form a complex and function synergistically to induce ectopic eye development in *Drosophila*. *Cell* **91**, 893-903.
- Cheyette, B. N., Green, P. J., Martin, K., Garren, H., Hartenstein, V. and Zipursky, S. L.** (1994). The *Drosophila* sine oculis locus encodes a homeodomain-containing protein required for the development of the entire visual system. *Neuron* **12**, 977-96.
- Chi, N. and Epstein, J. A.** (2002). Getting your Pax straight: Pax proteins in development and disease. *Trends Genet* **18**, 41-7.
- Cohen, S. M. and Jurgens, G.** (1990). Mediation of *Drosophila* head development by gap-like segmentation genes. *Nature* **346**, 482-5.
- Crozatier, M., Valle, D., Dubois, L., Ibsouda, S. and Vincent, A.** (1999). Head versus trunk patterning in the *Drosophila* embryo; collier

requirement for formation of the intercalary segment. *Development* **126**, 4385-94.

**Czerny, T., Halder, G., Kloter, U., Souabni, A., Gehring, W. J. and Busslinger, M.** (1999). twin of eyeless, a second Pax-6 gene of *Drosophila*, acts upstream of eyeless in the control of eye development. *Mol Cell* **3**, 297-307.

**Czerny, T., Schaffner, G. and Busslinger, M.** (1993). DNA sequence recognition by Pax proteins: bipartite structure of the paired domain and its binding site. *Genes Dev* **7**, 2048-61.

**Daniel, A., Dumstreit, K., Lengyel, J. A. and Hartenstein, V.** (1999). The control of cell fate in the embryonic visual system by atonal, tailless and EGFR signaling. *Development* **126**, 2945-54.

**Dominguez, M., Ferres-Marco, D., Gutierrez-Avino, F. J., Speicher, S. A. and Beneyto, M.** (2004). Growth and specification of the eye are controlled independently by Eyegone and Eyeless in *Drosophila melanogaster*. *Nat Genet* **36**, 31-9.

**Dumstreit, K., Wang, F., Shy, D., Tepass, U. and Hartenstein, V.** (2002). Interaction between EGFR signaling and DE-cadherin during nervous system morphogenesis. *Development* **129**, 3983-94.

**Finkelstein, R. and Perrimon, N.** (1991). The molecular genetics of head development in *Drosophila melanogaster*. *Development* **112**, 899-912.

**Forlani, S., Ferrandon, D., Saget, O. and Mohier, E.** (1993). A regulatory function for K10 in the establishment of dorsoventral polarity in the *Drosophila* egg and embryo. *Mech Dev* **41**, 109-20.

**Freeman, M.** (1997). Cell determination strategies in the *Drosophila* eye. *Development* **124**, 261-70.

**Fung, S.-T. C. and Gowen, J. W.** (1960). Role of autosome-IV in *Drosophila melanogaster* sex balance. *Genetics* **45**, 988-989.

**Gallitano-Mendel, A. and Finkelstein, R.** (1997). Novel segment polarity gene interactions during embryonic head development in *Drosophila*. *Dev Biol* **192**, 599-613.

**Gatti, M. and Pimpinelli, S.** (1992). Functional elements in *Drosophila melanogaster* heterochromatin. *Annu Rev Genet* **26**, 239-75.

**Gardon, S., Callaerts, P., Halder, G. and Gehring, W. J.** (1997). Conservation of Pax-6 in a lower chordate, the ascidian *Phallusia mammillata*. *Development* **124**, 817-25.

**Green, P., Hartenstein, A. Y. and Hartenstein, V.** (1993). The embryonic development of the *Drosophila* visual system. *Cell Tissue Res* **273**, 583-98.

**Grindley, J. C., Davidson, D. R. and Hill, R. E.** (1995). The role of Pax-6 in eye and nasal development. *Development* **121**, 1433-42.

- Grossniklaus, U., Cadigan, K. M. and Gehring, W. J.** (1994). Three maternal coordinate systems cooperate in the patterning of the *Drosophila* head. *Development* **120**, 3155-71.
- Halder, G., Callaerts, P., Flister, S., Walldorf, U., Kloter, U. and Gehring, W. J.** (1998). Eyeless initiates the expression of both sine oculis and eyes absent during *Drosophila* compound eye development. *Development* **125**, 2181-91.
- Halder, G., Callaerts, P. and Gehring, W. J.** (1995). Induction of ectopic eyes by targeted expression of the eyeless gene in *Drosophila*. *Science* **267**, 1788-92.
- Hartenstein, V.** (1993). Atlas of *Drosophila* development. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.
- Heitz, E.** (1934). Über  $\alpha$ - und  $\beta$ -heterochromatin sowie Konstanz und Bau der Chromomeren bei *Drosophila*. *Biol. Zentbl.* **54**, 588-609.
- Hill, R. E., Favor, J., Hogan, B. L., Ton, C. C., Saunders, G. F., Hanson, I. M., Prosser, J., Jordan, T., Hastie, N. D. and van Heyningen, V.** (1991). Mouse small eye results from mutations in a paired-like homeobox-containing gene. *Nature* **354**, 522-5.
- Hochman, B.** (1976). The fourth chromosome of *Drosophila melanogaster*. In *The Genetics and Biology of Drosophila*, vol. 1 b (ed. A. M and N. E), pp. 903-928. London: Academic Press.
- Hochman, B., Gloor, H. and Green, M. M.** (1964). Analysis of Chromosome 4 in *Drosophila melanogaster*. I. Spontaneous and X-Ray-Induced Lethals. *Genetica* **35**, 109-26.
- Hoge, M. A.** (1915). Another gene in the fourth chromosome of *Drosophila*. *Am Nat* **49**, 47-49.
- James, T. C., Eissenberg, J. C., Craig, C., Dietrich, V., Hobson, A. and Elgin, S. C.** (1989). Distribution patterns of HP1, a heterochromatin-associated nonhistone chromosomal protein of *Drosophila*. *Eur J Cell Biol* **50**, 170-80.
- Jang, C. C., Chao, J. L., Jones, N., Yao, L. C., Bessarab, D. A., Kuo, Y. M., Jun, S., Desplan, C., Beckendorf, S. K. and Sun, Y. H.** (2003). Two Pax genes, eye gone and eyeless, act cooperatively in promoting *Drosophila* eye development. *Development* **130**, 2939-51.
- Jiao, R., Daube, M., Duan, H., Zou, Y., Frei, E. and Noll, M.** (2001). Headless flies generated by developmental pathway interference. *Development* **128**, 3307-19.
- Jun, S., Wallen, R. V., Goriely, A., Kalionis, B. and Desplan, C.** (1998). Lune/eye gone, a Pax-like protein, uses a partial paired domain and a homeodomain for DNA recognition. *Proc Natl Acad Sci U S A* **95**, 13720-5.

- Jurgens, G., Wieschaus, E., Nusslein-Volhard, C. and Kluding, H.** (1994). Mutations affecting the pattern of the larval cuticle in *Drosophila melanogaster*. *Roux Arch. dev. Biol.* **193**, 283-295.
- Kammermeier, L., Leemans, R., Hirth, F., Flister, S., Wenger, U., Walldorf, U., Gehring, W. J. and Reichert, H.** (2001). Differential expression and function of the *Drosophila* Pax6 genes *eyeless* and *twin of eyeless* in embryonic central nervous system development. *Mech Dev* **103**, 71-8.
- Kango-Singh, M., Singh, A. and Henry Sun, Y.** (2003). *Eyeless* collaborates with Hedgehog and Decapentaplegic signaling in *Drosophila* eye induction. *Dev Biol* **256**, 49-60.
- Kenyon, K. L., Ranade, S. S., Curtiss, J., Mlodzik, M. and Pignoni, F.** (2003). Coordinating proliferation and tissue specification to promote regional identity in the *Drosophila* head. *Dev Cell* **5**, 403-14.
- Krivshenko, J.** (1959). New evidence for the homology of the short euchromatic elements of the *X* and *Y* chromosomes of *Drosophila busckii* with the micro-chromosome of *Drosophila melanogaster*. *Genetics* **44**, 1027-1040.
- Kronhamn, J., Frei, E., Daube, M., Jiao, R., Shi, Y., Noll, M. and Rasmuson-Lestander, A.** (2002). Headless flies produced by mutations in the paralogous Pax6 genes *eyeless* and *twin of eyeless*. *Development* **129**, 1015-26.
- Kronhamn, J. and Rasmuson-Lestander, A.** (1999). Genetic organization of the *ci-M-pan* region on chromosome IV in *Drosophila melanogaster*. *Genome* **42**, 1144-9.
- Kumar, J. P. and Moses, K.** (2001). EGF receptor and Notch signaling act upstream of *Eyeless/Pax6* to control eye specification. *Cell* **104**, 687-97.
- Kumar, J. P., Tio, M., Hsiung, F., Akopyan, S., Gabay, L., Seger, R., Shilo, B. Z. and Moses, K.** (1998). Dissecting the roles of the *Drosophila* EGF receptor in eye development and MAP kinase activation. *Development* **125**, 3875-85.
- Kurata, S., Go, M. J., Artavanis-Tsakonas, S. and Gehring, W. J.** (2000). Notch signaling and the determination of appendage identity. *Proc Natl Acad Sci U S A* **97**, 2117-22.
- Kurusu, M., Nagao, T., Walldorf, U., Flister, S., Gehring, W. J. and Furukubo-Tokunaga, K.** (2000). Genetic control of development of the mushroom bodies, the associative learning centers in the *Drosophila* brain, by the *eyeless*, *twin of eyeless*, and *Dachshund* genes. *Proc Natl Acad Sci U S A* **97**, 2140-4.
- Lambertsson, A.** (1998). The minute genes in *Drosophila* and their molecular functions. *Adv Genet* **38**, 69-134.

- Larsson, J., Chen, J. D., Rasheva, V., Rasmuson-Lestander, A. and Pirrotta, V.** (2001). Painting of fourth, a chromosome-specific protein in *Drosophila*. *Proc Natl Acad Sci U S A* **98**, 6273-8.
- Lebestky, T., Chang, T., Hartenstein, V. and Banerjee, U.** (2000). Specification of *Drosophila* hematopoietic lineage by conserved transcription factors. *Science* **288**, 146-9.
- Lefevre, G. j.** (1976). A photographic representation and interpretation of the polytene chromosomes of *Drosophila melanogaster* salivary glands. In *The genetics and Biology of Drosophila*, vol. 1a (ed. M. Ashburner and E. Novitski), pp. 32-66. London: Academic Press.
- Lindsley, D. L. and Zimm, G. G.** (1992). The genome of *Drosophila melanogaster*. London: Academic Press.
- Locke, J., Howard, L. T., Aippersbach, N., Podemski, L. and Hodgetts, R. B.** (1999a). The characterization of DINE-1, a short, interspersed repetitive element present on chromosome and in the centric heterochromatin of *Drosophila melanogaster*. *Chromosoma* **108**, 356-66.
- Locke, J. and McDermid, H. E.** (1993). Analysis of *Drosophila* chromosome 4 using pulsed field gel electrophoresis. *Chromosoma* **102**, 718-23.
- Locke, J., Podemski, L., Aippersbach, N., Kemp, H. and Hodgetts, R.** (2000). A physical map of the polytenized region (101EF-102F) of chromosome 4 in *Drosophila melanogaster*. *Genetics* **155**, 1175-83.
- Locke, J., Podemski, L., Roy, K., Pilgrim, D. and Hodgetts, R.** (1999b). Analysis of two cosmid clones from chromosome 4 of *Drosophila melanogaster* reveals two new genes amid an unusual arrangement of repeated sequences. *Genome Res* **9**, 137-49.
- Mann, R. S.** (2004). Two Pax are better than one. *Nat Genet* **36**, 10-1.
- Mardon, G., Solomon, N. M. and Rubin, G. M.** (1994). dachshund encodes a nuclear protein required for normal eye and leg development in *Drosophila*. *Development* **120**, 3473-86.
- Marquardt, T., Ashery-Padan, R., Andrejewski, N., Scardigli, R., Guillemot, F. and Gruss, P.** (2001). Pax6 is required for the multipotent state of retinal progenitor cells. *Cell* **105**, 43-55.
- Miklos, G. L., Yamamoto, M. T., Davies, J. and Pirrotta, V.** (1988). Microcloning reveals a high frequency of repetitive sequences characteristic of chromosome 4 and the beta-heterochromatin of *Drosophila melanogaster*. *Proc Natl Acad Sci U S A* **85**, 2051-5.
- Miller, D. J., Hayward, D. C., Reece-Hoyes, J. S., Scholten, I., Catmull, J., Gehring, W. J., Callaerts, P., Larsen, J. E. and Ball, E. E.** (2000). Pax gene diversity in the basal cnidarian *Acropora millepora* (Cnidaria, Anthozoa): implications for the evolution of the Pax gene family. *Proc Natl Acad Sci U S A* **97**, 4475-80.

- Mohler, J., Mahaffey, J. W., Deutsch, E. and Vani, K.** (1995). Control of Drosophila head segment identity by the bZIP homeotic gene *cnc*. *Development* **121**, 237-47.
- Morgan, T. H.** (1910). Sex limited inheritance in Drosophila. *Science* **32**, 120-122.
- Niimi, T., Seimiya, M., Kloter, U., Flister, S. and Gehring, W. J.** (1999). Direct regulatory interaction of the eyeless protein with an eye-specific enhancer in the sine oculis gene during eye induction in Drosophila. *Development* **126**, 2253-60.
- Pan, D. and Rubin, G. M.** (1998). Targeted expression of teashirt induces ectopic eyes in Drosophila. *Proc Natl Acad Sci U S A* **95**, 15508-12.
- Pappu, K. and Mardon, G.** (2002). Retinal Specification and Determination in Drosophila. In *Drosophila Eye Development*, (ed. K. Moses). Berlin: Springer-Verlag.
- Pappu, K. S., Chen, R., Middlebrooks, B. W., Woo, C., Heberlein, U. and Mardon, G.** (2003). Mechanism of hedgehog signaling during Drosophila eye development. *Development* **130**, 3053-62.
- Patterson, J. T. and Muller, H. J.** (1930). Are 'progressive' mutations produced by X-rays? *Genetics* **15**, 495-577.
- Pichaud, F. and Desplan, C.** (2002). Pax genes and eye organogenesis. *Curr Opin Genet Dev* **12**, 430-4.
- Pignoni, F., Baldarelli, R. M., Steingrimsson, E., Diaz, R. J., Patapoutian, A., Merriam, J. R. and Lengyel, J. A.** (1990). The Drosophila gene *tailless* is expressed at the embryonic termini and is a member of the steroid receptor superfamily. *Cell* **62**, 151-63.
- Pignoni, F., Hu, B., Zavitz, K. H., Xiao, J., Garrity, P. A. and Zipursky, S. L.** (1997). The eye-specification proteins *So* and *Eya* form a complex and regulate multiple steps in Drosophila eye development. *Cell* **91**, 881-91.
- Pineda, D., Rossi, L., Batistoni, R., Salvetti, A., Marsal, M., Gremigni, V., Falleni, A., Gonzalez-Linares, J., Deri, P. and Salo, E.** (2002). The genetic network of prototypic planarian eye regeneration is Pax6 independent. *Development* **129**, 1423-34.
- Plaza, S., Prince, F., Jaeger, J., Kloter, U., Flister, S., Benassayag, C., Cribbs, D. and Gehring, W. J.** (2001). Molecular basis for the inhibition of Drosophila eye development by *Antennapedia*. *Embo J* **20**, 802-11.
- Punzo, C., Kurata, S. and Gehring, W. J.** (2001). The eyeless homeodomain is dispensable for eye development in Drosophila. *Genes Dev* **15**, 1716-23.
- Punzo, C., Seimiya, M., Flister, S., Gehring, W. J. and Plaza, S.** (2002). Differential interactions of *eyeless* and *twin of eyeless* with the *sine oculis* enhancer. *Development* **129**, 625-34.

- Quiring, R., Walldorf, U., Kloter, U. and Gehring, W. J.** (1994). Homology of the eyeless gene of *Drosophila* to the Small eye gene in mice and Aniridia in humans. *Science* **265**, 785-9.
- Ray, R. P. and Schupbach, T.** (1996). Intercellular signaling and the polarization of body axes during *Drosophila* oogenesis. *Genes Dev* **10**, 1711-23.
- Ready, D. F., Hanson, T. E. and Benzer, S.** (1976). Development of the *Drosophila* retina, a neurocrystalline lattice. *Dev Biol* **53**, 217-40.
- Royet, J. and Finkelstein, R.** (1995). Pattern formation in *Drosophila* head development: the role of the orthodenticle homeobox gene. *Development* **121**, 3561-72.
- Royet, J. and Finkelstein, R.** (1996). hedgehog, wingless and orthodenticle specify adult head development in *Drosophila*. *Development* **122**, 1849-58.
- Rudolph, K. M., Liaw, G. J., Daniel, A., Green, P., Courey, A. J., Hartenstein, V. and Lengyel, J. A.** (1997). Complex regulatory region mediating tailless expression in early embryonic patterning and brain development. *Development* **124**, 4297-308.
- Saura, A. O., Cuenca, J. B., Heino, T. I., de Frutos, R. and Sorsa, V.** (2002). The polytene dot chromosome of *Drosophila*: *D. melanogaster* and *D. subobscura*. *Chromosoma* **111**, 273-83.
- Schaeffer, V., Killian, D., Desplan, C. and Wimmer, E. A.** (2000). High bicoid levels render the terminal system dispensable for *Drosophila* head development. *Development* **127**, 3993-9.
- Schedl, A., Ross, A., Lee, M., Engelkamp, D., Rashbass, P., van Heyningen, V. and Hastie, N. D.** (1996). Influence of PAX6 gene dosage on development: overexpression causes severe eye abnormalities. *Cell* **86**, 71-82.
- Seimiya, M. and Gehring, W. J.** (2000). The *Drosophila* homeobox gene *optix* is capable of inducing ectopic eyes by an eyeless-independent mechanism. *Development* **127**, 1879-86.
- Slizynski, B. M.** (1944). A revised map of salivary gland chromosome 4 of *Drosophila melanogaster*. *The Journal of Heredity* **35**, 323-325.
- St Johnston, D. and Nusslein-Volhard, C.** (1992). The origin of pattern and polarity in the *Drosophila* embryo. *Cell* **68**, 201-19.
- Sun, F. L., Cuaycong, M. H., Craig, C. A., Wallrath, L. L., Locke, J. and Elgin, S. C.** (2000). The fourth chromosome of *Drosophila melanogaster*: interspersed euchromatic and heterochromatic domains. *Proc Natl Acad Sci U S A* **97**, 5340-5.
- Sun, H., Rodin, A., Zhou, Y., Dickinson, D. P., Harper, D. E., Hewett-Emmett, D. and Li, W. H.** (1997). Evolution of paired domains: isolation and sequencing of jellyfish and hydra Pax genes related to Pax-5 and Pax-6. *Proc Natl Acad Sci U S A* **94**, 5156-61.

- Suzuki, T. and Saigo, K.** (2000). Transcriptional regulation of atonal required for Drosophila larval eye development by concerted action of eyes absent, sine oculis and hedgehog signaling independent of fused kinase and cubitus interruptus. *Development* **127**, 1531-40.
- Tepass, U., Fessler, L. I., Aziz, A. and Hartenstein, V.** (1994). Embryonic origin of hemocytes and their relationship to cell death in Drosophila. *Development* **120**, 1829-37.
- Tomarev, S. I., Callaerts, P., Kos, L., Zinovieva, R., Halder, G., Gehring, W. and Piatigorsky, J.** (1997). Squid Pax-6 and eye development. *Proc Natl Acad Sci U S A* **94**, 2421-6.
- Ton, C. C., Hirvonen, H., Miwa, H., Weil, M. M., Monaghan, P., Jordan, T., van Heyningen, V., Hastie, N. D., Meijers-Heijboer, H., Drechsler, M. et al.** (1991). Positional cloning and characterization of a paired box- and homeobox-containing gene from the aniridia region. *Cell* **67**, 1059-74.
- Urbach, R. and Technau, G. M.** (2003a). Molecular markers for identified neuroblasts in the developing brain of Drosophila. *Development* **130**, 3621-37.
- Urbach, R. and Technau, G. M.** (2003b). Segment polarity and DV patterning gene expression reveals segmental organization of the Drosophila brain. *Development* **130**, 3607-20.
- Walther, C. and Gruss, P.** (1991). Pax-6, a murine paired box gene, is expressed in the developing CNS. *Development* **113**, 1435-49.
- van Beest, M., Mortin, M. and Clevers, H.** (1998). Drosophila RpS3a, a novel Minute gene situated between the segment polarity genes cubitus interruptus and dTCF. *Nucleic Acids Res* **26**, 4471-5.
- Wang, W., Thornton, K., Berry, A. and Long, M.** (2002). Nucleotide variation along the Drosophila melanogaster fourth chromosome. *Science* **295**, 134-7.
- Wawersik, S. and Maas, R. L.** (2000). Vertebrate eye development as modeled in Drosophila. *Hum Mol Genet* **9**, 917-25.
- Weiler, K. S. and Wakimoto, B. T.** (1995). Heterochromatin and gene expression in Drosophila. *Annu Rev Genet* **29**, 577-605.
- Wilson, D. S., Guenther, B., Desplan, C. and Kuriyan, J.** (1995). High resolution crystal structure of a paired (Pax) class cooperative homeodomain dimer on DNA. *Cell* **82**, 709-19.
- Younossi-Hartenstein, A., Nassif, C., Green, P. and Hartenstein, V.** (1996). Early neurogenesis of the Drosophila brain. *J Comp Neurol* **370**, 313-29.

## **Papers I - IV**