Molecular genetic analysis of a *sine oculis* enhancer

and

The *leventina* gene as a model system to study human macular degeneration in *Drosophila*

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1. Abbreviations

aa	amino acid
AMD	age-related macular degeneration
APP	Amyloid precursor protein
bHLH	basic helix–loop–helix
BMP	bone morphogenetic protein
BOR	branchiootorenal dysplasia
BSA	Bovine serum albumine
cDNA	complementary deoxyribonucleic acid
dach	*dachshund*
DNA	deoxyribonucleic acid
DSL	Delta, Serrate, Lag-2
E(spl)	Enhancer of split locus
EFEMP1	EGF-containing fibrillin-like extracellular matrix protein 1
EFEMP2	EGF-containing fibrillin-like extracellular matrix protein 2
EGF	epidermal growth factor
ey	*eyeless*
eya	*eyes absent*
eyg	*eye gone*
GFP	green fluorescent protein
GMR	Glass Multimer Reporter
gro	*groucho*
GST	Glutathione-S-transferase
hh	*hedgehog*
HRP	Horseradish peroxidase
lz	lozenge
MF	Morphogenetic furrow
ML	Malattia Leventinese
N^{IC}	Notch intracellular
ninaE	*neither inactivation nor afterpotential E*
Pax	*paired box*
PBS	phosphate-buffered saline
PCR	Polymerase chain reaction
RGC	retinal ganglion cell
RPE	retinal pigment epithelium
RT	room temperature
S^{35} HN-1^{EC}	S^{35} labeled extracellular part of human Notch-1
SDS	sodium dodecyl sulfate
Six	sine oculis homeobox
smo	*smoothened*
so	*sine oculis*
soAE	sine oculis autoregulatory element

Su(H)	Suppressor of Hairless
TGF-β	transforming growth factor-β
toy	*twin of eyeless*
w	*white*
wt	wildtype
y	*yellow*
β-Gal	β-Galactosidase

The introduction as well as the results presented in 2 (The *leventina* gene as a model system to study human macular degeneration in *Drosophila*), subtitles 2.4.1 - 2.4.10 were used in the framework of the MD-PhD program of the university of Basel to obtain the degree of Doctor from the Medical Faculty.

2. The leventina gene as a model system to study human macular degeneration in *Drosophila*

2.1. Summary

Age-related macular degeneration (AMD) is one of the most frequent reasons for blindness of the elderly people and accounts for approximately 50% of registered blindness in the industrial world. AMD pathogenesis is poorly understood and there is no beneficial medical or surgical treatment possible in most cases.

The autosomal dominant retinal disease Malattia Leventinese (ML) has a similar phenotype to AMD and seems to be an early onset form. It has recently been shown that ML is caused by a single point mutation (Arg345Trp) that affects the extracellular matrix protein EFEMP1.

Studying the molecular function of EFEMP1 could therefore be helpful to understand the pathomechanism of both, ML and AMD.

In the last decade striking homologies between *Drosophila* and vertebrate eye development have been revealed. In addition, the fly model was useful to gain insight into the molecular mechanisms that lead to neurodegenerative diseases like Alzheimer, Parkinson and Chorea Huntington.

In this thesis, *Drosophila melanogaster* was used as a model system in an attempt to reveal the function of EFEMP1 and its putative *Drosophila* homologue the *leventina* gene, in the molecular mechanisms of eye development and its function in the retina of adult flies.

Pax-6, the master control gene of eye development, is able to induce ectopic eyes in *Drosophila* and ectopic eye structures in vertebrates. In the ribbonworm *Lineus* (Nemertini) *Pax-6* is also important for regeneration and maintenance of the retina. This is in agreement with our working hypothesis that *Pax-6* is involved in maintenance and regeneration of the human retina.

The transmembrane receptor Notch genetically lies upstream of *Pax-6*. The Notch signaling cascade appears in many steps of eye development in human, mouse and

Drosophila. Activation of Notch increases the expression of the transcription factor *Pax-6* in frogs.

EFEMP1 displays a high amino acid similarity to the ligands of the Notch receptor. The point mutation in EFEMP1 that causes ML could result in a loss of its ability to activate the Notch signaling cascade leading to a inappropriate *Pax-6* transcription and therefore cause ML. Based on this hypothesis one of our approaches was to test whether EFEMP1 interacts with Notch. If this is the case, EFEMP1 as a soluble Notch ligand could be a therapeutic tool to activate Notch, increase the transcription of *Pax-6* and thereby slow down retinal degeneration.

With the so far used methods we were not able to get any evidence that EFEMP1 is indeed a Notch ligand despite the convincing sequence homology to known Notch ligands.
By overexpressing EFEMP1 and *lvt*, its putative fly homologue, in different developmental stages in *Drosophila* we could not detect any specific phenotypic alteration. Overexpressing the mutated form of EFEMP1 that causes Malattia Leventinese did not show any mutant phenotype in the fly nor did it cause degeneration of photoreceptor cells in the retina of aging flies.

Even though we do not have any evidence whether we can use the fly as a model system to study the function of EFEMP1, the powerful genetics and the high conservation of the genetic regulatory network between vertebrate and *Drosophila* eye development makes it clear, that *Drosophila* is a very attractive *in vivo* system to study molecular mechanisms that lead to human diseases.

2.2. Introduction

2.2.1. Malattia Leventinese

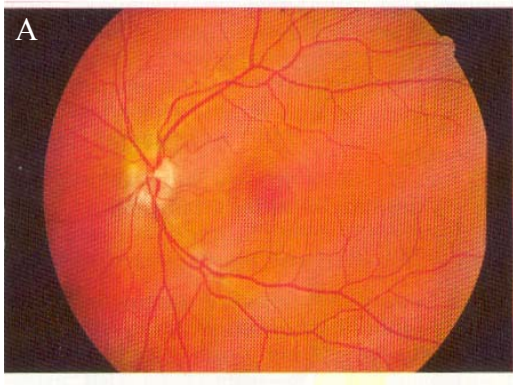
Malattia leventinese (ML) is a rare, autosomal-dominant retinal dystrophy that was first described in patients living in the Leventina Valley in southern Switzerland, hence, its name. Clinically, patients usually present with slow, progressive visual loss, typically in the third decade of life. The fundi of affected individuals are characterized by the presence of yellow/white deposits of extracellular debris (drusen) arranged in a spoke-wheel or radial distribution centered on the fovea (Figure 2.1). Recently, the gene responsible for this disorder was identified as the EFEMP1 gene (Stone et al., 1999) encoding a fibrillin-like, extracelllular matrix protein.

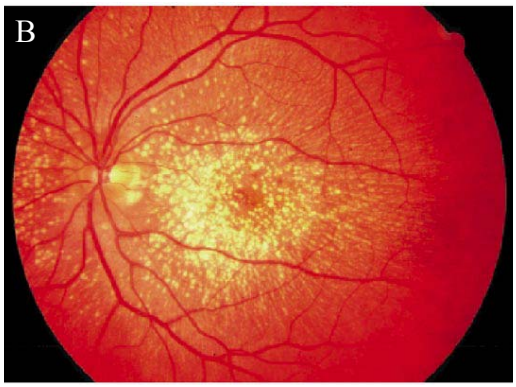
The importance of Malattia Leventinese is due in large part to their close phenotypic similarity to age-related macular degeneration (AMD), a disorder with a strong genetic component that accounts for approximately 50% of registered blindness in the Western world. AMD pathogenesis is poorly understood and there is no beneficial medical or surgical treatment possible in most cases (Bressler et al., 1988).

Just as in ML, the early hallmark of AMD is the presence of Drusen (Figure 2.1). Drusen are yellow/white hyaline deposits of extracellular debris consisting mostly out of lipids and proteins - but not EFEMP1 - and are located between the retinal pigment epithelium (RPE) and the Bruch membrane.

Besides the similar distribution of drusen, the pattern of EFEMP1 accumulation in ML and AMD is analogous. In ML, EFEMP1^{Arg345Trp} is not properly secreted from the retinal pigment epithelium (RPE) and accumulates in the cytoplasma of the RPE and underneath the RPE overlaying the drusen. In patients with AMD, the normal form of EFEMP1 is secreted properly but accumulates at the same localization underneath the RPE (Marmorstein et al., 2002). Therefore it is suggestive that ML is an early onset form of AMD.

Studying EFEMP1 could be important to get insights into the molecular mechanisms that lead to ML and hence to AMD and possibly give some new ideas for the design of a treatment.





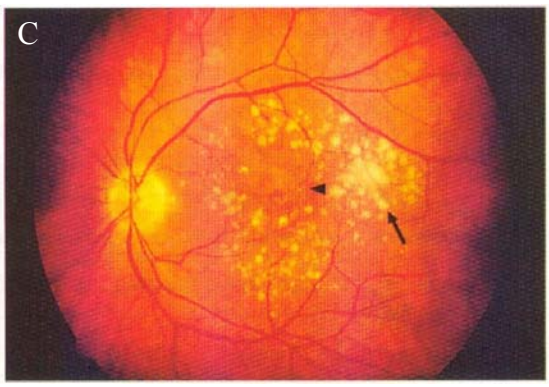


Figure 2.1 Phenotypic comparison of ML and AMD
A Fundus of a normal human eye
B Fundus of a patient affected with Malattia Leventinese (ML). The yellow drusen in the area of the macula are the characteristic of ML.
C Fundus of a patient with age related macular degeneration (AMD). Like in ML the center of the retina is characterized by the presence of drusen (arrow).
A and C from (Lang, 1998), B from (Stone et al., 1999)

2.2.2. EFEMP1

The human protein EFEMP1 (also called fibulin-3 or S1-5) is a 490 amino acids (54 kDa) extracellular matrix protein with a signal peptide at its amino terminal end and 9 EGF like repeats. The gene spans approximately 18kb of genomic DNA, consists of 12 exons and is located on chromosome 2p16 (Ikegawa et al., 1996).

EFEMP1 was originally isolated from a subtractively enriched cDNA library established from a subject with Werner syndrome (WS), a disease of premature aging (Lecka-Czernik et al., 1995). Microinjection of EFEMP1 mRNA into human fribroblasts is followed by the stimulation of DNA synthesis in a autocrine and paracrine manner (Lecka-Czernik et al., 1995).

mRNA from EFEMP1 is found in various human tissues (Lung, ovary, small intestine, colon) except for the brain and the peripheral leukocytes (Ikegawa et al., 1996). In the human eye, EFEMP1 is secreted by the retinal pigment epithelium and is localized to the outer retinal segment, surrounding the photoreceptor cells. In patients with ML EFEMP1^{Arg345Trp} is found in the tissue closest to the site of drusen formation (but not

in the drusen) (Stone et al., 1999) and gets accumulated in and underneath the retinal pigment epithelium.

The type of EGF-like domain present in EFEMP1 shows high homology to EGF-like domains in several previously described proteins. These include the transforming growth factor β1–binding protein (Kanzaki et al., 1990), a variety of extracellular matrix proteins such as fibulin (Argraves et al., 1990), fibrillin (Maslen et al., 1991) and nidogen (Mann et al., 1989), specific proteins involved in the blood coagulation cascade (protein S, factor IX) (Furie and Furie, 1988) and the transmembrane receptor Notch and its ligands Delta and Serrate. All of these proteins are secreted to the extracellular space or bind to the plasma membrane, where they interact with other proteins. The importance of the EGF repeats is due to their role in specific protein-protein interactions. Indeed, point mutations in EGF repeats of fibrillin and factor IX cause severe diseases like Marfan syndrom (Dietz et al., 1992; Hewett et al., 1993) and a distinct kind of hemophilia (Handford et al., 1991).

Similar to these diseases, a single exchange of an amino acid in the last EGF repeat of EFEMP1 (Arg345Trp) leads to the retinal dystrophy Malattia Leventinese (ML) (Stone et al., 1999).

The interaction of Notch with its ligands is also mediated by specific EGF repeats (Rebay et al., 1991). The EGF domains 11 and 12 of Notch specifically interact with the DSL domain of its ligands. This DSL domain is related to EGF repeats (Tax et al., 1994). From our studies, EFEMP1 contains a putative DSL domain at its aminoterminal end.

EFEMP1, which contains a signal peptide, 5 EGF domains and is able to stimulate DNA synthesis via autocrine and paracrine modes, would appear, therefore, to be an extracellular factor involved in cell proliferation. In addition, its role in the eye disease ML, its structural similarity to Notch ligands, its putative DSL domain and the fact that Notch is important in eye development as explained below makes it conceivable that EFEMP1 acts on the retina by modulating the Notch receptor.

2.2.3. The Notch receptor

Notch signaling is an evolutionarily conserved mechanism that is used in organisms ranging from *Drosophila* to humans to control cell fates through local cell-cell

interactions. Signals transmitted through the Notch receptor, in combination with other cellular factors, influence differentiation, proliferation, and apoptotic events at all stages of development. Notch signaling modulates the ability on non-terminally differentiated cells to respond to differentiation and proliferation signals in order to progress into the next developmental stage. Activation of Notch in immature precursor cells suppresses their differentiation and delays cell fate specification.

The *Notch* gene, first characterized in *Drosophila melanogaster*, encodes a 300-kD single-pass transmembrane receptor. The large extracellular domain contains 36 tandem EGF–like repeats and 3 cysteine-rich Notch/LIN-12 repeats. Six tandem ankyrin repeats, a glutamine-rich domain (opa), and a PEST sequence are found within the intracellular domain (Wharton et al., 1985). Notch-like proteins have been identified and extensively characterized in *Caenorhabditis elegans* (LIN-12 and GLP-1) (Kimble and Simpson, 1997; Greenwald, 1998) sea urchins, and many different vertebrates, including humans (Gridley, 1997; Sherwood and McClay, 1997). In all animal models tested, mutations in the Notch receptor invariably result in developmental abnormalities. In human, three disorders including a neoplasia (a T-cell acute lymphoblastic leukemia/lymphoma), a late onset neurological disease (CADASIL) and a developmental disorder (the Alagille syndrome) are associated with mutations in the *Notch1*, *Notch3* and *Jagged1* (the vertebrate homologue of *Serrate*) genes (Li et al., 1997; Joutel and Tournier-Lasserve, 1998).

In *Drosophila*, the two single-pass transmembrane proteins, Delta and Serrate, have been identified as partially redundant Notch ligands (Delta and Jagged in vertebrates, LAG-2 and APX-1 in *C. elegans*) (Gu et al., 1995; Gridley, 1997; Greenwald, 1998). The transcription factor Suppressor of Hairless [Su(H)] (CBF1/RJBk in mammals, LAG-1 in *C. elegans*) appears to function as the major downstream effector of Notch signaling and the genes of the *Enhancer of split* [*E(spl)*] locus, which encode nuclear basic helix-loop-helix (bHLH) proteins, are primary targets of Notch signaling (Egan et al., 1998; Greenwald, 1998).

Activation of the Notch receptor requires at least three proteolytic cleavages (Weinmaster, 2000). The first cleavage of the Notch receptor occurs during intracellular trafficking at the prospective extracellular side by a furin-like convertase. After this primary cleavage the receptor is presented as a heterodimeric transmembrane protein (Blaumueller et al., 1997; Logeat et al., 1998). Interaction with a ligand

induces a second extracellular cleavage, close to the plasma membrane (Brou et al., 2000) which, in turn, leads to a third cleavage in the transmembrane region that releases the intracellular part of Notch ($Notch^{IC}$) from the membrane (Kopan et al., 1996). $Notch^{IC}$ translocates to the nucleus where it acts in conjunction with the DNA-binding protein Su(H). On its own, Su(H) acts as a transcriptional repressor whereas together with $Notch^{IC}$ Su(H) turns into a transcriptional activator (Furriols and Bray, 2001) and drives expression of downstream target genes like the genes from the *E(spl)* complex.

In *Drosophila*, expression of a dominant activated form of the Notch receptor comprising only its intracellular part under the control of an eye specific enhancer leads to a huge overproliferation of eye tissue. Conversely expression of a dominant repressor of Notch signaling during eye development produces flies without eyes (Sun and Artavanis-Tsakonas, 1997; Kurata et al., 2000).

In vertebrates, retinal progenitor cells (RPCs) seem to be retained in a progenitor state by the action of Notch-Delta signaling (Tomita et al., 1996; Henrique et al., 1997). Together with other extrinsic signals, Notch signaling triggers retinal differentiation in *Drosophila* and vertebrates (Dorsky et al., 1997).
Notch acts upstream of *Pax-6* and is able to induce ectopic retinal tissue (Kurata et al., 2000; Kumar and Moses, 2001; Onuma et al., 2002).
It is thinkable that the action of the Notch receptor is required for both, retaining RPCs in their undifferentiated state and, together with other extrinsic signals, trigger the differentiation of RPCs into retinal cells by activating the transcription of *Pax-6* and other retinal specifications genes. Therefore, a soluble Notch ligand that acts as an activator of Notch signaling could be a powerful tool to prevent retinal degeneration.

2.2.4. Pax-6

The *Pax-6* gene was first cloned from mouse (Walther and Gruss, 1991). Since then, *Pax-6* genes have been cloned from representatives species of eight animal phyla, including homologs of human (Ton et al., 1991) and *Drosophila* (Quiring et al., 1994a; Czerny et al., 1999).

Pax-6 genes encode a transcription factor with two DNA binding domains, a homeodomain and a paired domain, both of which have been highly conserved during evolution. In mammals, congenital diseases known as *Aniridia* (humans) and *Small eye* (mice and rats) are caused by loss-of-function mutations of *Pax-6* in heterozygotes, whereas homozygous embryos lack eyes and nostrils completely, have brain and spinal cord malformations, and die prior to birth (Hill et al., 1991). In *Drosophila*, loss-of-function mutations in the *eyeless* (*ey*) locus, found to encode a *Pax-6* homologous gene (Quiring et al., 1994a), also show hypomorphic eye defects. In gain-of-function *Drosophila* mutants, ectopic eyes are formed on the antennae, legs, wings and halteres of the fly (Halder et al., 1995). Due to the fact that ectopic expression of *Pax-6* homologs from human, mouse, squid and sea squirts (ascidians) are capable of inducing ectopic eyes in *Drosophila* (Halder et al., 1995; Glardon et al., 1997; Tomarev et al., 1997) and conversely, *eyeless* and *twin of eyeless* (the two *Drosophila* homologs of *Pax-6*) are able to induce ectopic eye structures in Xenopus (Onuma et al., 2002), it was proposed that *Pax-6* may serve as a universal master control gene for eye morphogenesis in metazoa (Gehring and Ikeo, 1999).
In addition it has been demonstrated that *Pax-6* has not only a function during development of the eye but also for its maintenance and regeneration: In the ribbonworm *Lineus sanguineus,* injection of *Pax-6* double-stranded RNA (RNAi) which impedes translation of *Pax-6* leads to the disappearance of the eyes, whereas the eyes reappear by giving the worm time to recover without any new injections of RNAi (M.Tarpin, pers.comm.).

In vertebrates, *Pax-6* is required for the development of the lens and the retinal primordia (Glaser et al., 1994; Grindley et al., 1995; Ashery-Padan et al., 2000; Collinson et al., 2000).
In the retina of vertebrates *Pax-6* mediates the full retinogenic potential of retinal progenitor cells (RPC) by directly controlling the transition from uncommitted RPC toward a lineage-restricted RPC intermediate. RPCs from mice, where *Pax-6* is conditionally knocked-out, lack the ability to differentiate into all of the 6 different cell types present in a normal retina except for amacrine cells (Marquardt et al., 2001).

In the retina of adult humans and mice *Pax-6* and Notch-1 are still expressed (F.Hafezi, pers. comm.). This is in perfect agreement with our working hypothesis

which is, that Notch-1 and *Pax-6* are important for the maintenance and regeneration of the retina in the adult, Notch controls *Pax-6*, and the reason for retinal degeneration could be a decrease in Notch activity leading to a reduced transcription of *Pax-6*.

2.2.5. Eye development in *Drosophila* and vertebrates

The compound eye of *Drosophila* consists of approximately 800 single units - the ommatidia - that form a highly regular hexagonal array (Wolff and Ready, 1993). Each ommatidium, being somehow an eye of its own, consists of a lens, secreted by the cone cells, eight photoreceptor cells and pigment cells that surround the light gathering rhabdomers of the photoreceptor cells (Figure 2.2).

The *Drosophila* retina, which gives rise to the compound eye, is derived from the eye-antennal imaginal disc (Figure 2.2). This monolayer epithelium is formed by an involution from the head ectoderm during embryonic stages. In the third larval stage (the last before pupation) a wave of differentiation, the morphogenetic furrow, moves from posterior to anterior across the eye imaginal disc. This wave, visible as an intendation in the imaginal disc, transforms the unpatternd and undifferentiated cells in front of the furrow into a precise pattern of determined and differentiated cells behind the furrow. Hence, the undifferentiated eye imaginal disc develops into the compound eye during pupation.

The above described compound eye is from a morphological point of view completely different from the camera type eye of vertebrates. The vertebrate eye consists of a light focusing system including the cornea, iris and lens that projects the incoming light on the neuroretina which is optically isolated from the surroundings by the retinal pigmentepithelium (RPE).

The development of the vertebrate eye starts as a protrusion of the diencephalon (forebrain) in the middle of the fourth week of pregnancy resulting in a blister called the optic vesicle. Through mutual interaction of this optical vesicle with the lens placode of the overlaying head ectoderm, the lens placode starts to invaginate, forming the lens vesicle. At the same time when the lens is formed, the outer surface of the optic vesicle invaginates and forms the optic cup where the outer layer will become the RPE and the inner surface gives raise to the neural retina (Figure 2.2).

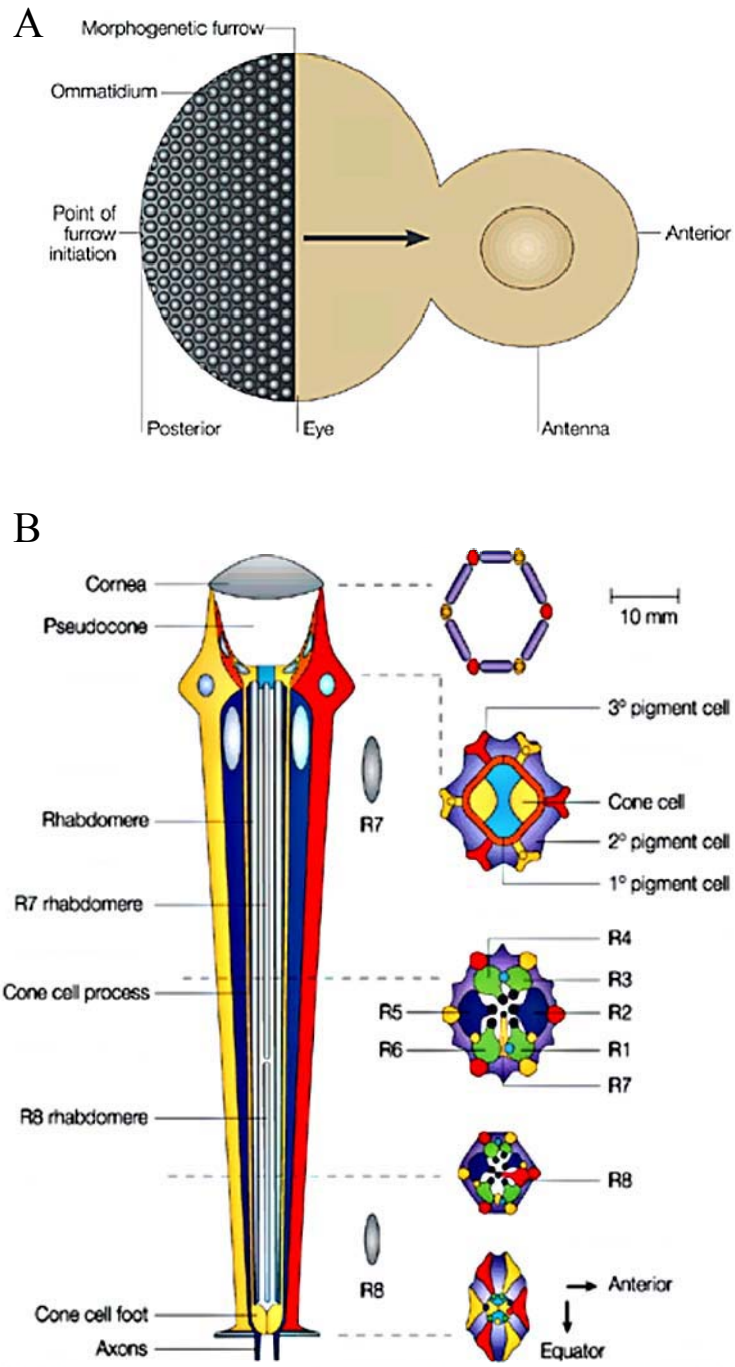


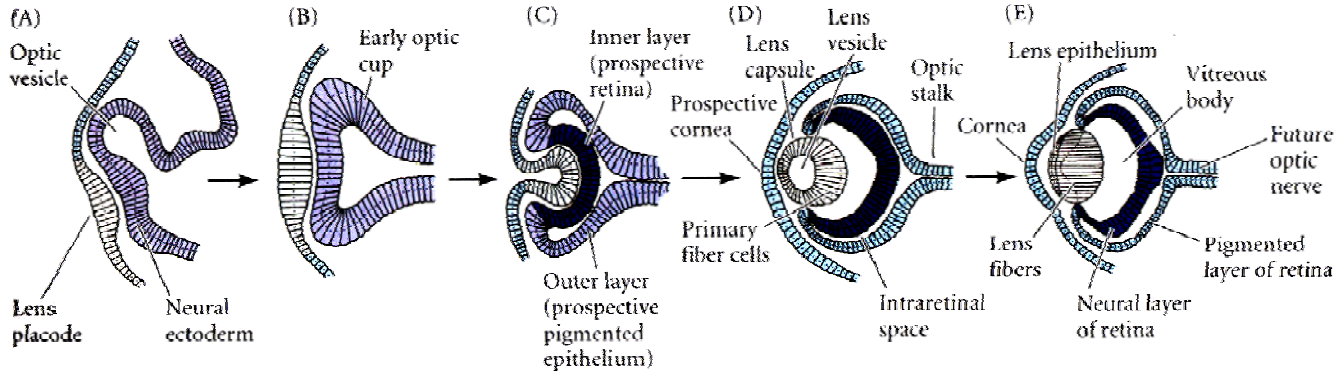
Figure 2.2 *Drosophila* and vertebrate eye development

A Eye-antennal imaginal disc
Schematic view of a larval eye-antennal imaginal disc which gives raise to the eye and the antenna of the adult fly during pupation. Differentiation occurs in a wave like manner form posterior to anterior across the disc leaving behind a precisely formed pattern, the ommatidia.

B An ommatidium of the adult fly
Longitudinal section through one of the 800 ommatidia of the compound eye of an adult fly.
The cornea is secreted by the cone cells. The rhabdomers are the light gathering parts of the photoreceptor cells (R1-R8).
Both pictures are from (Kumar, 2001)

C Development of the vertebrate eye
(A) The optic vesicle, a protuberance of the forebrain (diencephalon), extends towards the surface ectoderm. The lens placode (the prospective lens) appears as a local thickening of the surface ectoderm near the optic vesicle.
(B) The optic vesicle becomes an optic cup.
(C) The lens placode invaginates and forms the lens vesicle while the two layers of the optic cup become distinguished as neuroretina and RPE.
(D) The lens vesicle induces the development of the cornea.
(E) Cross-section through the developed vertebrate eye
From (Cvekl and Piatigorsky, 1996)

C



2.2.6. Homologies between human and *Drosophila* eye development

In spite of the different appearance of the compound eye and the camera type eye, several studies revealed astonishing homologies between *Drosophila* and human considering the molecular mechanisms of early eye development.

As mentioned above, the Notch receptor and the transcription factor *Pax-6* are crucial for eye development in both species. In addition, a set of seven nuclear factors have been identified in *Drosophila* that are implicated in a highly conserved network that directs the initiation of eye development (Halder et al., 1995; Oliver and Gruss, 1997; Gehring and Ikeo, 1999).

These 'eye-specification genes' (*eyeless* (*ey*), *twin of eyeless* (*toy*), *sine oculis* (*so*), *eyes absent* (*eya*), *dachshund* (*dac*), *eye gone* (*eyg*) and *optix*) have all homologues genes in vertebrates where they are also involved in eye specification (Table 2.1). Experiments both *in vivo* and *in vitro* indicate that these genes do not function as a linear biochemical or enzymatic pathway but, upon interacting with each other bulding up a complicated interwoven regulatory network (Figure 2.3) (Hunt, 1970; Chen et al., 1997; Halder et al., 1998a; Hazelett et al., 1998; Czerny et al., 1999; Niimi et al., 1999; Bui et al., 2000b; Seimiya and Gehring, 2000). In *Drosophila*, removal of any of these seven 'core' eye-specification genes in the eye primordium results in a drastic reduction or deletion of the adult compound eye, whereas ectopic expression of these genes (except *so*) results in the induction of retinal development outside the normal eye tissue (Halder et al., 1995; Bonini et al., 1997; Shen and Mardon, 1997; Czerny et al., 1999; Seimiya and Gehring, 2000). In humans, several retinal disorders have been attributed to mutations in the human homologues of these fly eye-specification genes; for example, aniridia and bilateral anopthalmia are due to mutations in the human homologues of *ey* and *so*, respectively (Table 2.1) (Jordan et al., 1992; Hanson et al., 1993; Gallardo et al., 1999).

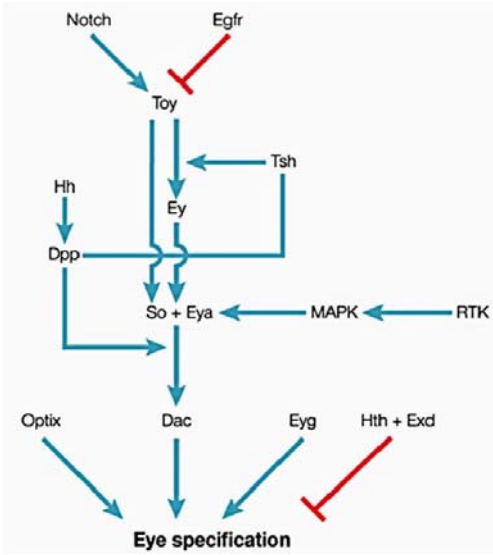


Figure 2.3 Eye specification in *Drosophila*
Several nuclear factors, patterning molecules and signaling cascades orchestrate in an intriguing interplay the specification and development of the eye.
The arrows show the direction of the relationship. Blue arrow means activator whereas red arrow indicate inhibition.
Dac, Dachshund; Dpp, Decapentaplegic; Egfr, Epidermal growth factor receptor; Exd, Extradenticle; Ey, Eyeless; Eya, Eyes absent; Eyg, Eye gone; Hh, Hedgehog; Hth, Homothorax; MAPK, Mitogenactivated protein kinase; RTK, receptor tyrosine kinase; So, Sine oculis; Toy, Twin of eyeless; Tsh, Teashirt. From (Kumar, 2001) modified after (C. Punzo, pers. comm.)

Besides the molecular mechanisms that specify the tissue to adopt an eye fate, different genes and their function in patterning the retina are conserved between insects and mammals.

In *Drosophila* the first cells to become committed to a neuronal fate are the R8 photoreceptor cells. Each R8 recruits surrounding undifferentiated cells to form an ommatidium and is therefore called 'the ommatidium founding cell'. To adopt their fate, R8 cells need the expression of the basic helix-loop-helix transcription factor *atonal* (Jarman et al., 1994). *Atonal* expression is detectable as a stripe on the imaginal disc that coincides with the morphogenetic furrow and signifies R8 competence in front of this wave of differentiation. By lateral inhibition, where again Notch-Delta signaling is involved, the expression of *atonal* gets refined to an evenly spaced grid of cells that become R8 (Figure 2.4).

Table 2.1 Factors involved in vertebrate and *Drosophila* eye development

Fly genes	Protein	Vertebrate gene(s)	Loss-of-function phenotype	Expression pattern
twin of eyeless (*toy*) and *eyeless* (*ey*)	Homeodomain/ paired domain	Small eye (*Pax6*)	Aniridia	Anterior neural plate, optic vesicle, lens placode and cornea
sine oculis (*so*) and *optix*	Homeodomain/ Six domain	Six family (*Six3*, *Six6*)	Bilateral anophthalmia	Anterior neural plate, optic vesicle and stalk, neural retina and lens
eyes absent (*eya*) (*clift* (*cli*))	Novel, Proteinphosphatase	*Eya1–Eya4*	BOR syndrome	Eya1 in lens placode; Eya3 in vesicle, lens vesicle and retina
dachshund (*dac*)	Novel	Dachshund homologue 1/2 (*Dach1*, *Dach2*)	Unknown	Dach1 in optic vesicle, optic cup and retina; Dach2 in retina and surrounding mesenchyme
eye gone (*eyg*)	Homeodomain	Unknown, Pax6 splice variant	Unknown	Unknown
hedgehog (*hh*)	Secreted morphogen	Sonic hedgehog (*Shh*), Tiggy winkle hedgehog (*Twhh*), Indian hedgehog (*Ihh*) and Desert hedgehog (*Dhh*)	Cyclopia	Shh and Twhh in neural retina and retinal pigmented epithelium
atonal (*ato*)	bHLH transcription factor	Mouse atonal homologue 5 (*Math5*)	Loss of RGC	Optic cup, RGC precursor
EGF receptor (Egfr)	Receptor tyrosine kinase	*Waved2*	Anterior segment dysgenesis, loss of anterior chamber, corneal scarring	Perioptic mesenchyme, eyelid epithelium, corneal epithelium
Notch (*N*)	Transmembrane receptor	*Notch1–Notch4* (*N1–N4*)	Retrolentricular hyperplasia, bilateral microphthalmia	N1 in neural retina; N2 in lens and retinal pigmented epithelium; N3 in lens and neural retina
decapentaplegic (*dpp*)	TGF-β-secreted morphogen	BMP family (*BMP4*, *BMP7*)	Block in lens induction, bilateral anophthalmia	BMP4 in optic vesicle, lens placode; BMP7 in surface ectoderm, lens placode, optic vesicle and stalk
Crumbs (*crb*)	Transmembrane protein	CRB1	Retinitis pigmentosa (human)	Retina
Sparkling (*spa*)	homeodomain	*Pax2*	Optic nerve coloboma (mouse, human)	Optic stalk
Orthodentical (*otd*)	homeodomain	*Crx*	Cone-rod dystrophie, Leber congenital amaurosis (mouse)	Neural retina

bHLH, basic helix–loop–helix; BMP, bone morphogenetic protein; BOR, branchiootorenal dysplasia; EGF, epidermal growth factor; Pax, paired box; RGC, retinal ganglion cell; Six, sine oculis homeobox; TGF-β, transforming growth factor-β. From (Kumar, 2001) and (Wawersik and Maas, 2000) modified after (Seimiya and Gehring, 2000)

In eyes of different vertebrates, close homologues of *atonal* have been identified that are involved in neurogenesis: *Xath5* in *Xenopus*, *Math5* in Mice (Kanekar et al., 1997; Brown et al., 1998). Math5 knockout mice lack almost all retinal ganglion cells, suggesting that Math5 is the proneural gene for this class of neurons. The significance of this is that the ganglion cells are the first neurons to differentiate in vertebrate eyes. Thus, in mice as in *Drosophila*, the differentiation of the first retinal neurons require an *atonal* homologue. Furthermore, like in the fly, neurogenesis in the vertebrate retina has been found to occur in a wave like manner with its starting point near the optic stalk (Figure 2.4) (McCabe et al., 1999; Masai et al., 2000).

Besides the transcription factor *atonal* the promotion of the *Drosophila* morphogenetic furrow requires the signaling molecule Hedgehog (HH) (Heberlein and Moses, 1995). The first neurons induced close to the optic stalk secrete Hedgehog protein, which diffuses anteriorly to promote *atonal* expression and neurogenesis in immediately adjacent undifferentiated epithelium. These new neurons then also synthesize hedgehog and promote furrow progression.

The vertebrate Hedgehog homologous gene, sonic hedgehog (Shh), is also a short-range signaling molecule. Shh expression in Zebrafish spreads in a wave like manner over the retina similar to the morphogenetic furrow in the fly (Figure 2.4) (Neumann and Nuesslein-Volhard, 2000).

In both, *Drosophila* and Zebrafish, ectopic Hedgehog leads to the initiation of an ectopic wave of differentiation, whereas lack of hedgehog signaling prevents the progression of the furrow respectively the wave.

In summary, findings of the last decade indicate that the selector genes like *Pax-6* which are involved in specifying tissue to adopt an eye fate and the mechanisms of early retinal patterning are conserved from *Drosophila* to humans despite the different appearance of the two eye types. Therefore, the fly seems suitable as a model system to study the molecular mechanisms of eye development that lead to a human eye disease.

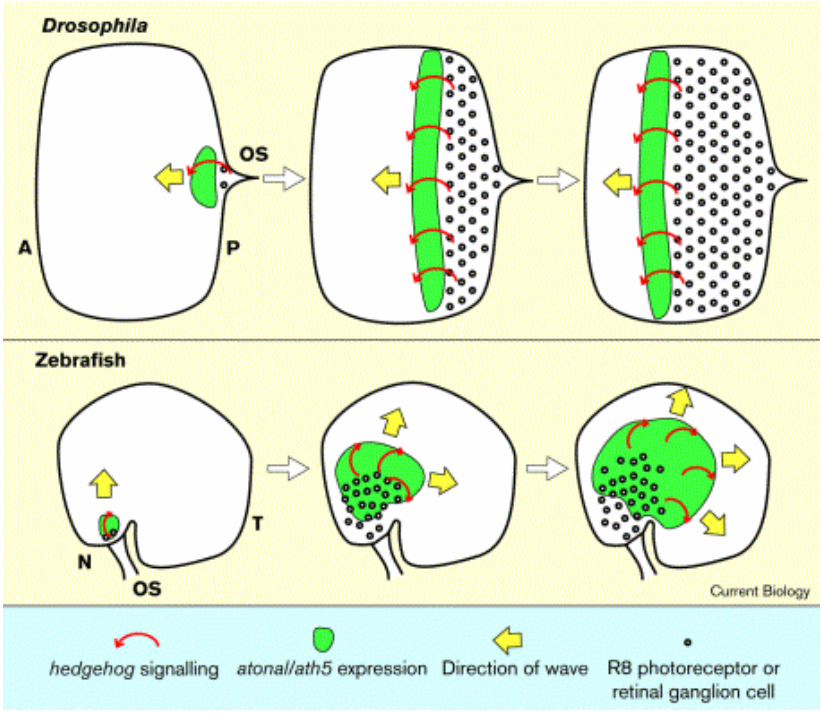


Figure 2.4 ‚The mexican wave of differentiation'

In *Drosophila* and Zebrafish (vertebrate) the first neurons of the retina that differentiate require the basic helix-loop-helix protein *atonal/ath5*.

In both animals the short range signaling protein hedgehog appears to drive the wave of neurogenesis.
A, anterior; P, posterior; N, nasal; T, temporal; OS, optic stalk

From (Jarman, 2000)

2.2.7. Why using *Drosophila* as a model system to study a human eye disease

Working with *Drosophila* has the advantage of the very powerful tools that allow the discovery of the basic molecular and cell-biological mechanisms that underlie biological processes in a complex organism. As an example, ectopic expression of a gene - from the start by cloning the cDNA until the homozygous transgenic fly - can easily be done in less than four months.

In addition several genetic studies revealed that the basic cell-biological pathways are remarkably conserved between invertebrates and vertebrates.

The recently completed genome sequence of the fly (Adams et al., 2000) and the human genome (Lander et al., 2001; Venter et al., 2001) indicated a high degree of interrelatedness between the two species. With the complete sequences of *Drosophila* and Human in hand, it was possible to show that out of 287 well defined inherited human diseases 178 (62%) have a homologous gene in *Drosophila* (Fortini et al., 2000).

In this chapter some examples are given to show how the fly is useful to study the molecular pathways that lead to human neurodegenerative diseases.

Parkinson's disease is a movement disorder of late adult onset. The clinical main features are resting tremor, rigidity and bradykinesia. In the brains of patients that were affected with this disease specific changes like degeneration of dopamine neurons in the substantia nigra and the presence of cytoplasmic neuronal inclusions known as Lewy bodies are found. Most cases are sporadic, but there are also families with autosomal dominant Parkinson's disease where missense mutations (A53T and A30P) in the Alpha-synuclein gene have been identified (Polymeropoulos et al., 1997; Kruger et al., 1998).

To produce the fly model the wild type and the mutated forms of the human alpha synuclein were overexpressed in *Drosophila* (Feany and Bender, 2000). The transformed flies showed several of the neuropathological features that are specific for parkinson: They had an age related loss of dopamine neurons, they developed alpha-synuclein-immunoreactive cytoplasmatic aggregates with a fibrillar appearance like the Lewy bodies and the flies showed age related motoric deficits.

Alzheimer's disease is the most frequent reason for dementia worldwide. The disease is defined pathologically by extracellular amyloid plaques and intracellular neurofibrillary tangles, accompanied by neuronal loss.

Amyloid is produced by the action of γ–secretase (encoded by presenilin) on the amyloid precursor protein (APP).

Improper cleavage of APP causes the production of the strong amylogenic aβ42. The accumulation of aβ42 is increased by mutations either in presenilin or in APP. Mutations in these genes are found in familial early onset forms of Alzheimer's disease.

Neurofibrillary tangles are composed of aggregated, hyperphosphorylated forms of the microtubule-associated protein TAU. Mutations in the TAU gene are involved in neurodegenerative diseases that are related to Alzheimer's disease (Hutton et al., 1998).

In *Drosophila*, human APP is cleaved by the fly presenilin at exactly the same aa position like in vertebrates (Ramabhadran et al., 1993; Fossgreen et al., 1998).

With mutated forms of APP, presenilin and TAU, fly models were created that showed similarities to the human disease (Fossgreen et al., 1998; Wittmann et al., 2001).

From studies with *Drosophila* it was recently reported that APP might function as a receptor and that overexpression of APP causes axonal transport defects (Gunawardena and Goldstein, 2001).
Studies of presenilin in *C. elegans* and *Drosophila* have revealed its function in the proteolysis of the Notch receptor (Levitan and Greenwald, 1995; Struhl and Greenwald, 1999; Ye et al., 1999). The importance of this receptor raises the question whether γ–secretase inhibitors are the appropriate means to prevent progression of Alzheimer's disease.

Huntington's disease is an autosomal-dominant disorder. Patients present with chorea, dementia and neuropsychiatric symptoms. All patients have an expansion of greater than 35 repeats in the polyglutamine segment of the mutant huntingtin protein (Gusella, 1993). The neuropathology is characterized by progressive neuronal loss from the striatum and frontal cortex, in association with the presence of neuronal intranuclear inclusions (DiFiglia et al., 1997). Expression of expanded (120 glutamines), but not unexpanded, huntingtin protein under an eye-specific promoter in the *Drosophila* retina produces age-related progressive neurodegeneration accompanied by nuclear inclusions (Jackson et al., 1998). The human disease and the fly model are related to each other by the degenerative nature of the pathology, the presence of characteristic intranuclear inclusions and the correlation between the number of the polyglutamine repeats and the severity of the disease.
In vitro, the expanded huntingtin peptide interacts with a histon acetyltransferase and impairs its activity. By feeding flies with histon deacetylase (HDAC) inhibitors the insufficient acetylation of histons in flies that express the mutant huntingin peptide is prevented and the flies show a less severe neurodegenerative phenotype (Steffan et al., 2001).

The above mentioned fly models for neurodegenerative diseases are ideal entry points to dissect the molecular mechanisms underlying those complex diseases and to find new targets for drug development. Therefore, we would like to produce a fly model for the early onset of AMD.

2.2.8. Model for Malattia Leventinese

The retina of the adult mammalian eye still contains retinal stem cells, localized to the pigmented ciliary margin (Tropepe et al., 2000). *Pax-6* and Notch-1 are expressed in the retina of aged humans and mice (F. Hafezi, personal communications). The function of *Pax-6* and Notch-1 in the adult eye is not explored so far but, from the knowledge of the function of the Notch receptor and the transcription factor *Pax-6* in eye development and the involvement of *Pax-6* during regeneration and maintenance of the eyes in lower invertebrates, we assume that they are also important for maintenance and regeneration in the adult vertebrate eye.

Furthermore we believe that Malattia Leventinese is not only caused by the accumulation of EFEMP1 underneath the retinal pigment epithelium but also by a loss of function caused by the point mutation in EFEMP1^{Arg345Trp}. We hypothesize that EFEMP1 acts on the retina of adult individuals by modulating the Notch receptor. Its convincing similarity to known Notch ligands makes it conceivable that EFEMP1 is a direct activator of the Notch receptor.

As a modulator of Notch, a loss of function due to the mutation in EFEMP1 that causes Malattia Leventinese or an age related downregulation of EFEMP1 like in AMD would lead to an reduced activation of the Notch receptor and therefore to an insufficient expression of *Pax-6*. A reduction of *Pax-6* in the retina of adult individuals could have the following consequences:

Retinal progenitor cells (RPC) lose their full retinogenic potential. The replacement of lost retinal cells would be impaired.

Differentiated retinal cells might ‘forget’ their identity and undergo apoptosis.

The consequences of this hypothesis would be, that EFEMP1, or any soluble Notch ligand, could be a therapeutic means to prevent progression of retinal degeneration of aged individuals.

In this thesis we tested whether EFEMP1 is a Notch ligand and if we can use the fly to study the function of EFEMP1 and its putative fly homologs in the development and the maintenance of the retina of *Drosophila melanogaster*.

2.3. Materials and Methods

2.3.1. Fly work

All stocks were maintained as homozygous viable or as balanced strains and kept at 18°C in tubes with standard cornmeal medium supplemented with a drop of live yeast. Monthly the flies have been transferred into fresh tubes. Amplification of the flies for experimental use was performed in tubes or bottles at 25°C.

General fly stocks

Stock	Source
dpp ^{blink} *-Gal4*	(Staehling-Hampton and Hoffmann, 1994)
so10-Gal4	(Niimi et al, 1999)
ey-Gal4	(Halder *et a*l., 1998)
UAS-Nact	(Shoichiro Kurata 1999)
UAS-Ndn	(Shoichiro Kurata 1999)
UAS-HN-1	(Spyros Artavanis-Tsakonas)
UAS-Notch	(Spyros Artavanis-Tsakonas)
UAS-GFP	(K. Basler)
ELAV-Gal4	(Lin and Goodman 1994)
GMR-Gal4	(M. Freeman)
Rh1-Gal4	(B. Mollerau)
dpp ^{blink} -Gal4, UAS-Gal4	(D. Felix)
yw^{67c23}	(U. Kloter)
yw ac	(Bruno Bello)
w; Sp/CyO; rf10/TM6b Hu	(Urs Kloter)
yw 67c23; SM1 Cy / Gla	(Urs Kloter)
yw 67c23; TM3 (Sb, Ser) / ?	(Urs Kloter)
Gbe Su(H)$_4$ *LacZ*	(Jennings et al 1994)

Genotypes generated

Crosses to generate new genotypes were done according standard procedures. A detailed explanation on fly genetics can be found in “FLY Pushing” (Greenspan, 1997).

Stock

yw; UAS-EFEMP1; UAS-Notch

yw; UAS-EFEMP1; GMR-Gal4

yw; UAS-EFEMP1; ey-Gal4

yw; UAS-EFEMP1; UAS-HN-1

yw; UAS-EFEMP1; UAS-chimera

(chimera = extracellular Human Notch-1, intracellular *Drosophila* Notch as described below)

Transformants

We generated a minimum of two to ten transgenic lines. For lines that showed no mutant phenotype a minimum of 5 independent lines were tested. All transgenic lines were generated in a yw^{67c23} background using pUAST (Brand and Perrimon, 1993) as vector.

Stock

yw^{67c23}; UAS-EFEMP1

yw^{67c23}; UAS-EFEMP2

yw^{67c23}; UAS-EFEMP1^{Arg345Trp}

yw^{67c23}; UAS-EFEMP1-sp (-sp = without signal peptide)

yw^{67c23}; UAS-*chimera*

Injections were done according to standard procedures: Collection of embryos on grape juice plates for 45 minutes; dechorionation with 4% chlorox; rinsing with washing solution and tap water; mounting on grape juice plates and transfer on double stick tape; drying with hair drier using cold air for about 4 minutes; covering them with Voltalef 10S oil; injected embryos were kept in a humid box at 18°C until hatching.

Material

Injection buffer 10X: 1mM sodium phosphate buffer; 50mM KCl

Injection solution, DNA: 200ng/µl Plasmid and 150ng/µl helper (pp25.7 D 2-3 wc)

Washing solution: 0.7% NaCl; 0.03% Triton X-100

Voltalef 10S oil: Distributed by Elf Atochem (Switzerland)

Grape juice plate: Autoclave solution A (500ml water; 27gr agar) and solution B (250ml water; 250ml grape-juice; 12.5gr sugar; 2gr. Nipagin) mix and poor in petridishes.

2.3.2. Constructs

For cloning standard protocols described in (Sambrook and Russel, 2001) were used. The additional kits used for DNA purification were obtained from Sigma or Nucleobond. Constructs were verified by sequencing with 310 ABI sequencer.

List of cDNAs

EFEMP1 a commercially available clone covering the whole cDNA of EFEMP1 was ordered from the I.M.A.G.E consortium (clone #380914)

EFEMP2 was cloned as described below

HN-1 (Human Notch-1) kindly provided by Spyros Artavanis-Tsakonas cloned in pcDNA3 (*Eco*RI - *Xho*I)

Drosophila Notch kindly provided by Spyros Artavanis-Tsakonas

Inserted into pUAST vector in a 3 way ligation: *Eco*RI-*Asp*718(5'), *Asp*718-*Xba*I(3'), *Eco*RI-*Xba*I (vector)

EFEMP1 - sp (EFEMP1 without signal peptide) was cloned as described below

Chimeric Notch was cloned as described below

cDNA of EFEMP2

EFEMP2 was cloned using a human Liver cDNA library (kindly provided by Michael Podvimec).

Primers were designed as follows: two primers next to each other were created from upstream of the translation start of EFEMP2 (EFEMP2 5'1, EFEMP2 5'3); another two primers were created at the 3' end also only a few base pairs away from each other (EFEMP2 3'1, EFEMP2 3'2).

A first PCR amplification step was done with the outer pair of primers (30 cycles with Adv 2 HF (Advantage-2 High Fidelity PCR kit from CLONTECH (Cat# K1914-1))) which led to an unspecific amplification of several DNA pieces. A second amplification step with the inner primer pair from the product of the first amplification resulted in the expected 2kb DNA piece, which was confirmed to be EFEMP2 by sequencing.
EFEMP2 was subcloned with TOPO TA Cloning® Kit from Invitrogen and cloned into the pUAST vector for fly transformation (*Not*I-*Asp*718).

Primers

EFEMP2 5'1	5-3	5' CAAGCTTGGCACGAGGGCAGGCATTGCCCG 3'
EFEMP2 5'3	5-3	5' GCCAGCCGAGCCGCCAGAGCCGCG 3'
EFEMP2 3'	3-5	5' CCAGTTGCCTCGTTTTATAGAAAAACAGGCCCAGG 3'
EFEMP2 3'2	3-5	5' GGAATGGAACCCAGGGCCTCCTGGCGC 3'

EFEMP1 without signal peptide (EFEMP1-sp)

The sequence from the *Eag*I site upstream of the translation start as far as the *Bsa*AI site in the sequence of EFEMP1 was deleted and replaced by a designed linker DNA that replaces the deleted sequence except for the signal peptide. Thus, all the sequence upstream of the start codon remained unchanged making it likely, that this construct is expressed similar to the wildtype EFEMP1 construct used before.

Amino acid sequence that is deleted in this construct
LKALFLTMLTLALVK
Oligos to create the linker DNA (*Eag*I-*Bsa*AI)
EF1woSP 5'3'
5'GGCCGACAGATTCACAATGTCACAGGACACCGAAGAAACCATCAC3'
EF1woSP 3'5'
5'GTGATGGTTTCTTCGGTGTCCTGTGACATTGTGAATCTGTC3'

Construction of Chimeric Notch

A linker DNA piece was created by PCR using *Drosophila* Notch as a template and the chim 5' and chim 3' primers with the Adv 2 HF. This linker was sub cloned with

the TOPO TA Cloning® Kit from Invitrogen. The linker DNA was cleaved with *Aat*II and *Xmn*I.

Human Notch-1 (Human Notch-1 (*Eco*RI - *Xho*I) in pBluescript II KS (from Stratagene)) was cleaved with *Eco*RI and *Xmn*I, producing a 4.5kB DNA fragment corresponding to the 'extracellular' part of Human Notch-1.

Drosophila Notch (*Drosophila* Notch (*Eco*RI - *Xba*I) in pBluescript II KS) was cleaved with *Aat*II and *Xba*I, producing a 3.2kB DNA fragment corresponding to the 'transmembrane and intracellular' part of *Drosophila* Notch.

All three pieces were ligated at the same time into the pUAST vector (*Eco*RI - *Xba*I).

Chimeric Notch is a protein that comprises the extracellular part from Human Notch-1 and the transmembrane and intracellular part from *Drosophila* Notch.

Primers

Chim 5'	5-3	5' CGGCCAACGAGTGCTG**GAAGTACTTC**AAGAACGGC
Chim 3'	3-5	5' CGTC**GACGTC**GCGACATAACCGCCGC

GAAGTACTTC *Xmn*I recognition site; **GACGTC** *Aat*II recognition site

Directed mutagenesis of EFEMP1

Two primers were designed (MutEFEMP1 5', MutEFEMP1 3'), one in 5'-3' and the other in 3'-5' direction, covering the region of EFEMP1 which harbours the mutation responsible for Malattia Leventinese. The primers included one base pair mismatch so that the copies produced by PCR of EFEMP1 coded the mutated form $EFEMP^{Arg345Trp}$.

As template the whole plasmid (EFEMP1 in KS II) was used in a 10 cycle PCR reaction with Adv 2 HF.

To destroy the template after the PCR reaction I made use of the fact that PCR amplified DNA is not methylated, whereas DNA amplified from bacteria is. Thus, by cleaving the product from the PCR reaction with *Dpn*I, which only cleaves methylated DNA, the wildtype template gets destroyed.

After the cleavage step the PCR product was directly used to transform E. coli.

To control whether the mutagenesis had worked, DNA from different clones was cleaved by *Nci*I, which only cleaves DNA that has the mutation in it.

The mutagenised plasmid EFEMP1 was named $EFEMP1^{Arg345Trp}$

PCR-Primers for directed mutagenesis of EFEMP1:

```
MutEFEMP1 5'              5' GAATGCTGGGAGGATGAAATGTGTTGG 3'
MutEFEMP1 3'   3' CTGGTGTTTACTTACGACCCTCCTAC 5'
```

Constructs used in the *pull-down* assay

The fragments of EFEMP1 that were used for the *pull-down* assay were cloned by PCR (Adv 2 HF). The primers were created with an *Eco*RI cleavage site at their 5' end and a *Xho*I site at their 3' end. The PCR fragments were cloned into the pGEX Vector (Pharmacia):

For the fragments the following primer pairs were used:

DSL	TranslStrt 5', DSL Domain 3'
EFEMP1-DSL	EGFstart 5', EFEMP1 3'
DSL+2EGF	TranslStrt 5', DSL2EGF 3'
EFEMP1	TranslStrt 5', EFEMP1 3'

Primers

EGFstart 5'	5-3	5' CCACC**GAATTC**AGTGTGCAGCAGGCTAC
TranslStrt 5'	5-3	5' GCTAA**GAATTC**CAATGTTGAAAGCCC
EFEMP1 3'	3-5	5' G**CTCGAG**CTAAAATGAAAATGGCCCCAC
DSL Domain 3'	3-5	5' GC**CTCGAG**CTGGATACGGTGGGAAGGG
DSL2EGF 3'	3-5	5' CT**CTCGAG**GCAATAAAAAGTCCGGGTTG

CTCGAG *Xho*I recognition site; **GAATTC** *Eco*RI recognition site

2.3.3. Molecular methods

Standard molecular methods like miniprep, DNA digestion, alkaline phosphatase treatment, phenol-chloroform extraction of DNA, ligation, preparation and transformation of competent cells, western blotting were performed according to (Sambrook and Russel, 2001) and will not be further described. Only additional information about some protocols less commonly used are given in this section.

Western blotting

Polyacrylamid gels (usually 10%) were run at 150V and transferred overnight at 50mA onto a nitrocellulose membrane. Transfer was verified by Ponceau red staining. The samples were boiled for 6 minutes in Laemmli buffer.

Concentration of primary antibodies used:

Mouse monoclonal EFEMP1 (Marmorstein et al., 2002) 1:2000

The secondary antibody for detection of the signal was used at a dilution of 1:2000 (HRP-coupled mouse antibody from DAKO A/S) and the signal was detected using a chemoluminescence kit (Amersham).

Pull-down Assay

Pull-down assays are used to detect protein-protein interactions. One of the putative binding proteins is synthesized as a GST-fusion protein. Such a fusion protein can easily be purified due to the high affinity of GST to glutathione Sepharose. A putative binding protein is radiolabelled and tested whether it co-purifies with the GST-fusion protein.

Protein synthesis in *E.coli* and affinity purification of the fusion protein was done according the GST gene fusion system manual from Pharmacia.

10 µl (1µg) from *E.coli* synthetisized GST-EFEMP1 (for constructs see above) fusion proteins were incubated together with 1-5µl *in vitro* translated (TNT Coupled Transcription/Translation System, Promega), S^{35} marked extracellular part of Human Notch-1 for one hour at RT.

20 µl glutathione Sepharose 4B (Pharmacia) beads were pre-blocked with 50 µg Bovine serum albumin (BSA) for 15 min at room temperature (RT) followed by washing twice with 1ml phosphate-buffered saline (PBS) +0.01% NP40. 500 µl PBS + 0.01% NP40 (Nonidet P-40) were added.

The incubation mixture was added to the pre blocked beads. The mixture was incubated on a rotating wheel at RT for 45 min to 60 min.
The beads that bound the GST fusion protein (EFEMP1, DSL, EFEMP1-DSL, DSL+2EGF) and eventually the Human Notch-1 extracellular part were washed 3 times with 1ml IPP150 (10mM Tris-Cl pH 8.0, 150mM NaCl, 0.1%NP40).
The washed beads suspended in 24 µl protein loading buffer (Laemmli buffer) were boiled for 5 minutes and loaded into a 10% SDS gel.
To detect a 'pull down' of S^{35} marked HN-1 extracellular part the SDS gel was exposed overnight to an X-ray film (BioMax MS-1 Film, Kodak).

Production of EGF10-13 from *Drosophila* Notch

EGF repeats are intramolecular linked by 3 disulfide bridges which only form in oxidizing environment. *In vivo*, the establishment of such bonds is assisted by disulfide-isomerases.
Drosophila Notch EGF10-13 comprises 4 EGF repeats and therefore 12 disulfide bridges. To make sure that these repeats are folded properly we made use of a signal peptide that directs EGF10-13 to the periplasmatic space. Furthermore we co-expressed a disulfide-isomerase to the periplasmatic space and added N-Acetylcysteine as a Redox buffer to the LB medium.

EGF10-13_His@Cterm:
The EGF10-13 repeats of Drosophila Notch were amplified by PCR. The primers were designed in a way that the PCR product had a *Stu*I site at the 5' and a 6xHis tag followed by a *Hind*III site at the 3' end. This PCR product was cloned *Stu*I-*Hind*III into pRBI-DsbC (Maskos et al., 2003) resulting in pEGF10-13, 6xHis@C'-DsbC. That way, EGF10-13 are cloned downstream of a signal peptide (OmpA) that directs the protein to the periplasmatic space. Furthermore EGF10-13 is cloned within a polycystronic unit that co-expresses a disulfide isomerase to the periplasmatic space.
Protien charakteristics: pI 4.64, MW 18kD

Purification of recombinant EGF10-13 with 6xHis at its carboxyterminus:
E. Coli JM83 cells carrying the plasmid pEGF10-13, 6xHis@C'-DsbC were grown in LB medium containing ampicillin (100 µg/mL) at 37°C, over night (ON). 3L LB medium containing ampicillin (100 µg/mL) was infected with 60ml from the ON

culture and incubated at 26°C to an OD600 of 1.0 (4-5 hours incubation). IPTG and N-Acetylcysteine were added to final concentrations of 1mM and 5mM, respectively (0.82 g/L of solid N-acetylcysteine was added) and the culture was incubated for another 16 hours at 26°C.

The culture was cooled down on ice and centrifuged at 7K (SLA-3000) for 10 min. The cells were resuspended in 300ml 20%sucrose, 30mM Tris-HCl pH 7.9. EDTA was added to 1mM final conc. and the cells were incubated 5-10 minutes at room temperature (not longer!).

The cells were centrifuged (7K for 10 min in SLA-3000) and the supernatant was removed. The cells were resuspended in ice-cold 5mM $MgSO_4$ and incubated for 10 min at 4°C on a magnetic stirrer.

The cells were centrifuged (7K for 10 min SLA-3000) and the cold osmotic shock fluid was collected in the supernatant.

To adjust the cold osmotic shock fluid for the Ni-NTA purification, the following chemicals were added: Imidazole to 5mM, NaCl to 500mM, Tris-HCl pH 7.9 to 20 mM, NP-40 to 0.1%, $CaCl_2$ to 2mM.

500 µl Ni-NTA superflow (Qiagen) was equilibrated with binding buffer. The 300ml cold osmotic shock fluid was loaded on the Ni-NTA with 100ml per hour. The Ni-NTA was washed twice with 10ml wash buffer. 6 elution fractions of 1.5ml were collected and analysed on a 15%SDS page.

The fractions containing protein were pooled and dialyzed against storage buffer.

To confirm that the produced protein is EGF10-13 a MALDI (Matrix-assisted Laser Desorption/Ionization) was performed.

Yield: 50-100µg for 3l

In a 25l fermenter preparation the yield was also only around 100µg for the entire 25l culture!

Binding buffer		Wash buffer	
5mM	Imidazole	40mM	Imidazole
500mM	NaCl	500mM	NaCl
20mM	Tris HCl pH 7.9	20mM	Tris HCl pH 7.9
2mM	$CaCl_2$	0.1%	NP-40

Elute buffer		Storage buffer	
1M	Imidazole	100mM	NaCl
500mM	NaCl	50mM	Tris HCl pH 7.9
20mM	Tris HCl pH 7.9	0.01%	NP-40
0.1%	NP-40	10%	Glycerol
2mM	$CaCl_2$	2mM	$CaCl_2$

Protease inhibitors: For 300 ml, 100µl Leupeptin (2mg/ml in H_20), 175µl pepstatin (2mg/ml in Ethanol) and 2,5ml PMSF (0,1M in Ethanol) was added.

2.3.4. Histology

The histological methods used are standard procedures and therefore won't be further discussed, except for the references given. *In situ* hybridization on discs was performed as described in protocol 82 (Ashburner, 1989). Antibody staining of discs was performed according to (Halder et al., 1998a).

The dilution of the antibodies was as follows:

Monoclonal mouse anti-EFEMP1 (Marmorstein et al., 2002)	1:2000
Secondary fluorescent antibodies were diluted	1:500.

Secondary HRP-coupled antibodies were diluted 1:500 and detected by DAB (diamino-benzidin).

2.3.5. Cell Culture Methods

Baculovirus system

Protein was synthesized according to the BAC-TO-BAC Baculovirus Expression System Manual from GibcoBRL.

500ml SF900 medium with 0.5 $x10^6$ SF9 cells were infected with the baculovirus and incubated for 4 days. The 6xHis tagged EFEMP1 protein was purified with Ni-NTA (Qiagen) according to the pET System Manual from Novagen.

Maintenance of COS cells

COS cells were split 1:10 every 3-4 days.

The cells are grown in DMEM media. The recipe is as follows:

For 500 ml

440 ml	DMEM w/o L-glutamine

50 ml	Fetal Bovine Serum, 30 min heat inactivated at 65°C
5 ml	200 mM stock solution of L-Glutamine (Gibco-BRL # 25030-081)
5 ml	100X stock of Penicillin-Streptomycin (Gibco-BRL #15070-063)

Notch activity assay

We used a cell culture assay to detect Notch activity (kindly provided by S. Artavanis-Tsakonas). The core element of this *in vitro* assay is a plasmid which contains six Su(H) binding sites in front of a minimal promotor that drives the expression of a luciferase gene. Upon binding of a ligand, the Notch receptor gets cleaved, N^{IC} enters the nucleus and binds to Su(H). This interaction converts Su(H) from a repressor into a transcriptional activator and drives the expression of the luciferase gene. The luciferase acts on its substrate and leads to a measurable light formation.

The night before transfection the cells were plated in a 24-well tissue culture plate at $5x10^4$ cells per well. For the ligand expressing cells, $25x10^4$ cells were plated in a 6-well tissue culture plate.

Cells are transfected using Lipofectamine Plus reagent (Gibco # 10964-013). For each well, 0.5 µg of TP1-Luc (Luc = Luciferase) and 0.003 µg of CMV-renLuc are transfected (the plasmids were kindly provided by S. Artavanis-Tsakonas). The CMV-renLuc plasmid is used to normalize the firefly luciferase values coming from the TP1-Luc plasmid. In addition, 0.5 µg to 1µg of pcDNA3-HN-1 were co-transfected into the cells. The ligand expressing cells were transfected with 2.5 µg to 5 µg of pcDNA3-EFEMP1 (pcDNA3 from Invitrogen).

- In a polypropylene tube DNA (TP1-Luc + CMV-renLuc + pcDNA3-HN-1), PLUS reagent, and OPTIMEM (GibcoBRL # 31985-070) were mixed. 0.5 µg TP1-Luc, 0.003 µg CMV-renLuc, 4 µl of PLUS reagent, and 25 µl of OPTIMEM for each well were used. The well mixed DNA and PLUS reagent were incubated at room temperature for 15 minutes.
- In a separate tube, 1µl Lipofectamine and 25 µl OPTIMEM were mixed and incubated for 15 minutes.
- During this incubation, the cells were washed one time in 1 ml of OPTIMEM and afterwards covered with 200µls of OPTIMEM.

- To the 200 µl of OPTIMEM, 50 µl of the Lipofectamine PLUS/DNA reagent was mixed into each well.
- Cells were incubated in a tissue culture incubator for 3 hours. After 3 hours, the transfection reagent was replaced with 1 ml of normal DMEM media.

The day following transfection nothing was done with the cells, giving them a day to recover.

The second day following transfection, the transfected cells (pcDNA3-HN-1 and reporter constructs) were co-cultured with $1x10^5$ ligand-expressing (pcDNA3-EFEMP1) cells or with $1x10^5$ Delta expressing cells as positive control. The DMEM media was simply aspirated from the transfected cells and the ligand-expressing cells were added.

Transfected cells were co-cultured with ligand-expressing cells for 24-30 hours.

After the co-culture is complete, cells are harvested for measurements of luciferase activity. Luciferase activity is measured using the Dual-Luciferase Reporter Assay System (Promega #E1960). All reagents and a manual are provided in this kit.

2.4. Results

2.4.1. In silico structure comparison between EFEMP1 and known Notch ligands

All Notch ligands known so far are single pass transmembrane proteins containing between two (Lag-2, the *C. elegans* Notch ligand) and 16 EGF repeats in their extracellular domain (Lissemore and Starmer, 1999). Furthermore, they share a DSL domain adjacent to the EGF repeats. Comparison of the DSL domains from different Notch ligands of different phyla revealed a consensus of eleven amino acids (Lissemore and Starmer, 1999). The DSL domain is crucial for the physical interaction with the EGF repeats number 11 and 12 from the Notch receptor (Rebay et al., 1991; Shimizu et al., 1999).

EFEMP1 is a protein of the extracellular matrix that is secreted from the RPE (Marmorstein et al., 2002) of the human eye and thus does not have a transmembrane domain. Like the known Notch ligands it comprises several EGF repeats. EFEMP1 has 5 EGF repeats and an additional atypical EGF domain. (Human Delta1 has 8 EGF repeats (Swiss-Prot: O00548), *Drosophila* Delta has 9 EGF repeats (Swiss-Prot: P10041)).

By comparison of its sequence with the DSL consensus, we found that the atypical EGF repeat of EFEMP1 is related to the DSL consensus sequence. In this putative DSL domain 8 out of the 11 consensus amino acids are present (Figure 4.1)

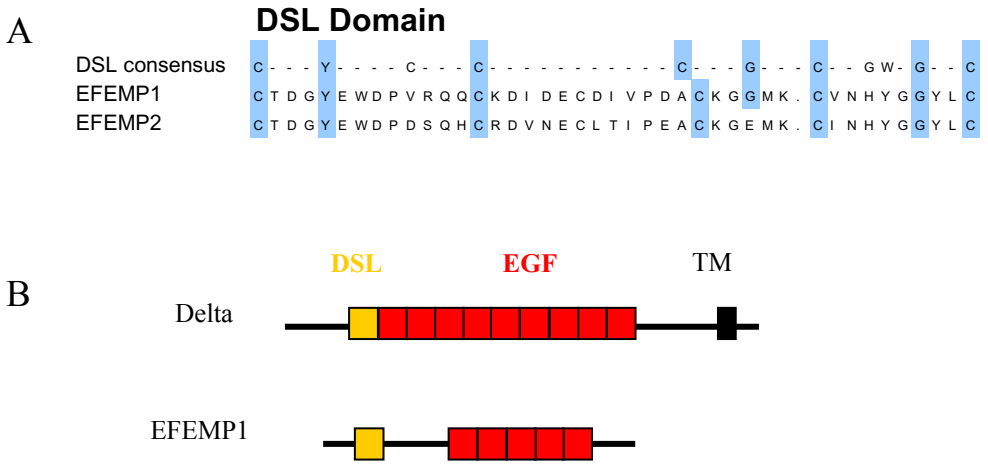


Figure 4.1 Putative DSL domain of EFEMP1 and comparison of EFEMP1 with Delta
A Close to the aminoterminal end of EFEMP1 a putative DSL domain was found that fits with eight out of eleven amino acids to the consensus sequence of DSL domains.
Red amino acids show the DSL consensus (these 11 amino acids and the distance between them are conserved in almost all Notch ligands described so far (Lissemore and Starmer, 1999)).
Blue boxed amino acids indicate matches between the DSL consensus and the putative DSL domain of EFEMP1 and EFEMP2. Dash = any amino acid; point = gap;
DSL consensus from (Lissemore and Starmer, 1999); EFEMP1 from NCBI accession NP_4096, aa29-70; EFEMP2 from NCBI accession AAF65188 aa39-80.
B Comparison of EFEMP1 with Delta (a *Drosophila* Notch ligand) shows that they share a similar structure. TM = Transmembrane domain.

2.4.2. Overexpression of EFEMP1 / EFEMP1^{Arg345Trp} in *Drosophila*

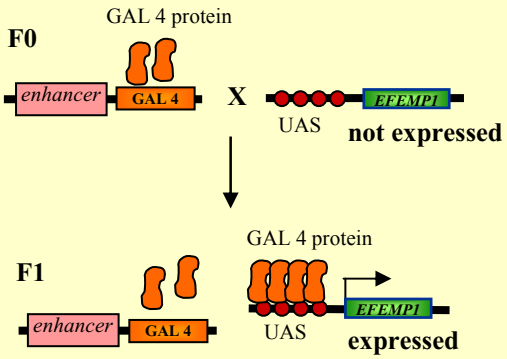
Modulation of Notch receptor activity has tremendous effects on the development of the fly. In eye development for example, expression of a constitutively activated form of the Notch receptor (N^{act}) leads to a strong overproliferation of the compound eye (Kurata et al., 2000). Expression of the *Drosophila* Notch ligands Delta and Serrate during eye development leads to overgrowth and reduction of the eyes, respectively (Figure 4.3 C,D). The *Drosophila* eye is in general a good system to detect slight alterations of molecular interactions because morphological abnormalities are easily observable in the highly repetitive, precisely patterned compound eye; Small changes in one ommatidium are amplified over the whole lattice of the compound eye.

Upon expression of EFEMP1 during eye development we would therefore expect a deranged compound eye if any signaling is disturbed or even a Notch specific alteration of the eye phenotype in the case that EFEMP1 is a Notch ligand.

As first experiment we tested whether targeted expression (Box 4.1) (Brand and Perrimon, 1993) of EFEMP1 in the fly results in any visible change of phenotype. Therefore, cDNA of human EFEMP1, EFEMP1^{Arg345Trp} (the mutation that causes ML) and EFEMP2 (a human paralog of EFEMP1) was cloned downstream of UAS (upstream activation sequences) in a p-element vector and flies were transformed with these constructs. These flies were crossed to flies expressing Gal4 under the control of different enhancers (Figure 4.2 A). In this way EFEMP1, EFEMP1^{Arg345Trp} and EFEMP2 were expressed in the fly during different developmental stages and in different tissues. To show that EFEMP1 is expressed at the RNA and protein level, RNA *in situ* hybridisation and antibody staining against EFEMP1 was performed on imaginal discs of third instar larvae (Figure 4.2 B,C)

With these overexpression experiments we could not observe any change of the fly morphology.

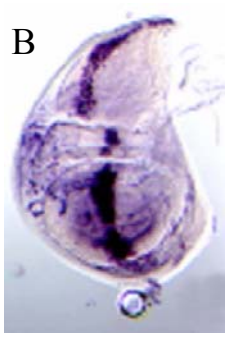
Targeted gene expression
Brand and Perrimon, 1993



Box 4.1 Targeted gene expression
F0: Two different parental fly strains:
One fly line has the Gal4 transcription factor from yeast under control of a genomic enhancer. In these flies, Gal4 is expressed in the tissue and the developmental stage specific to the expression pattern of the enhancer. As a yeast transcription factor, Gal4 alone has no effect on these flies.
A second fly line carries a gene of interest (like EFEMP1) under the control of the yeast promotor/enhancer UAS that remains silent in these flies.
F1: Crossing these lines together results in flies that express Gal4 in a specific spatial and temporal pattern which in turn binds to UAS and activates the transcription of the transgene.

A

eyeless-GAL4	X	UAS-EFEMP1
dpp-GAL4		UAS-EFEMP1Arg345Trp
dpp-GAL4, UAS-Gal4		UAS-EFEMP2
GMR-GAL4		
so-GAL4		
Elav-Gal4		
Rhodopsin-1-Gal4		



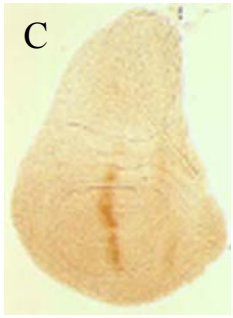


Figure 4.2 Expression of EFEMP1 in the fly
A Flies that drive Gal4 under the control of different enhancers were crossed to flies that are transgenic for EFEMP1, EFEMP1^{Arg345Trp} and EFEMP2 under the control of UAS.
Enhancers:

dpp	expressed in leg, wing, antennae and eye discs
eyeless, so	expressed in eye discs (eye specification, early in eye development)
GMR	expressed in all cells of the differentiated eye
ELAV	expressed in all differentiated neurons, including photoreceptor cells
Rhodopsin-1	expressed in outer photoreceptor cells (R1-R6) (still expressed in the adult)

B *in situ* hybridisation with EFEMP1 RNA. Shown is a wing imaginal disc where EFEMP1 is expressed with the enhancer of the dpp gene along the anterior/posterior compartment boundary.
C antibody staining of the same expression pattern like in B. This pattern is highly specific for the dpp enhancer and thereby confirms that EFEMP1 is expressed at both, the RNA and protein level.

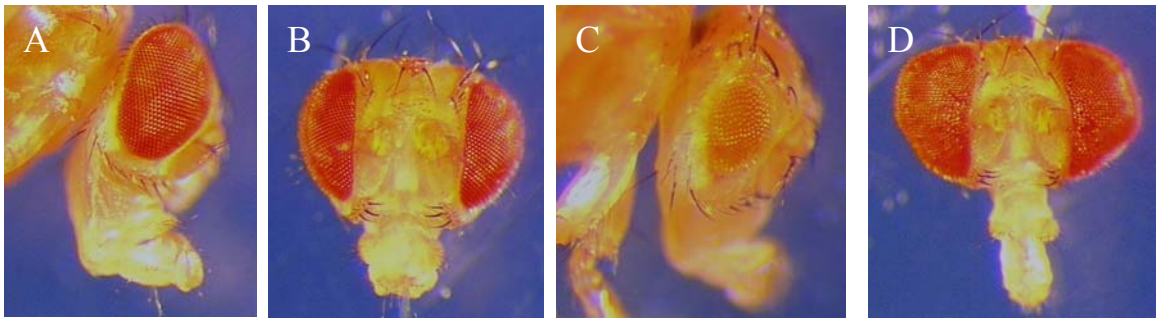


Figure 4.3 wildtype and altered compound eyes
A view onto the normal compound eye of *Drosophila*
B top view of a the head of a wildtype *Drosophila*
C expression of Serrate with an eye specific enhancer (*ey*-Gal4) leads to a reduction of the eye size.
D expression of Delta with an eye specific enhancer leads to overproliverated compound eyes with an increased number of ommatidia.

2.4.3. Expression of EFEMP1 together with human Notch-1 and a chimeric Notch

EFEMP1 is a human protein. Thus, even though *Drosophila* Notch and Human Notch-1 share almost 60% amino acid identity and have the same protein domains, it could be that the lack of phenotypic effects is due to a lack of interaction between the human EFEMP1 and the *Drosophila* Notch due to minor differences in their amino acid sequences. To test this hypothesis, EFEMP1 was expressed together with the Human Notch-1 receptor in *Drosophila*. Expression of Human Notch-1 alone during eye development behaves like a dominant negative form of Notch and disrupts eye development. It is likely that this phenotype is due to a competition of *Drosophila* Notch and Human Notch-1 for the ligands where the Human Notch-1 is able to bind the ligand but is not processed properly resulting in an inadequate activation of the Notch pathway. By expressing EFEMP1 together with Human Notch-1 we expected to suppress this phenotype by increasing the level of ligands. However we did not see any change of the Human Notch-1 caused phenotype by coexpressing EFEMP1 and Notch-1 during eye development.

One could still argue that even though EFEMP1 now would interact with the extracellular part of the human Notch-1, the intracellular part of human Notch-1 does not transduce the signal properly to the nucleus of *Drosophila* cells. Therefore, a chimeric Notch was constructed that consists of the extracellular part of human Notch-1 and the intracellular and transmembrane part of *Drosophila* Notch (Figure 4.4). But still, overexpression of EFEMP1 together with this chimeric Notch did not result in an EFEMP1 specific phenotype.

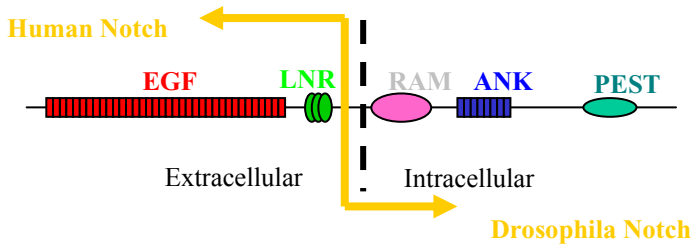


Figure 4.4 Chimeric Notch
The extracellular, human part of this chimeric Notch should be able to interact normal with the human protein EFEMP1, whereas the intracellular part from *Drosophila* should signal properly to the nucleus.

2.4.4. Expression of a mammalian protein in *Drosophila*

Notch and its ligands have several EGF repeats which are intramolecularly linked by disulfide-bonds which only form extracellular under oxidative conditions. Furthermore, Notch signaling is dependent on enzymes that modify these EGF repeats like the glycosyltransferase fringe (Bruckner et al., 2000) and the O-fucosyltransferase OFUT1 (Okajima and Irvine, 2002). Taken together, it seems crucial for Notch and its ligands that they are secreted, folded and modified properly to get their full activity.

As a first test whether the mammalian EFEMP1 is normally secreted in flies we looked at signal peptides of different mammalian and fly proteins and compared them to EFEMP1 (Table 4.1). From the literature we know that signal peptides do not have a well-defined consensus sequence, but share some features: they range in length from 13 to 36 residues, the aminoterminal part contains at least one positively charged residue and they bear a highly hydrophobic stretch, typically 10 to 15 residues long (Stryer, 1999). The signal peptide of EFEMP1 fulfills these criteria and there seem no distinctions between insect and mammalian signal peptides. In addition, Invitrogen writes in one of their protocols that all mammalian secretion signals they have tested functioned properly in insect cells (Invitrogen, 2002).

EFEMP1	M L K A L F L T M L T L A L V K S
EFEMP2	M L P C A S C L P G S L L L W A L L L L L L G S A
Delta 1 Human	M G S R C A L A L A V L S A L L C
Notch1 Human	M P P L L A P L L C L A L L P A L A
Delta Drosophila	M H W I K C L L T A F I C F T V I V
Drosophila Glue Protein	M K L L V V A V I A C M L I G F A

Table 4.1 Signal peptides
Amino-terminal signal sequences of some eukaryotic secretory and plasma membrane proteins. Most of them share a hydrophobic core (yellow) and a basic residue (blue).

For our *in vitro* assays that are described below we synthesized EFEMP1 in different ways. For the *pull-down* assay we produced EFEMP1 in bacteria and purified the protein from the lysed cells. For the cell culture assay we synthesized EFEMP1 with the baculovirus system from which we purified EFEMP1 from the supernatant of these insect cells. Thus we have EFEMP1 produced by bacteria and therefore without properly formed EGF repeats and EFEMP1 produced by a eukaryotic cell system where we expect the EGF repeats to be folded correctly. When we run these two

different proteins on either a SDS gel under reducing conditions or on a SDS gel under non-reducing conditions we see that they behave differently. EFEMP1 from bacteria behaves the same in both reducing and non-reducing conditions (Figure 4.5 B lane 2,3 and 5,6). EFEMP1 from the eukaryotic system migrates different in these two diverse conditions. In reducing conditions, only one band with the expected molecular weight is visible on a western blot (Figure 4.5 A lane 1-4 and B lane 1). In non-reducing conditions, two bands with an apparently higher molecular weight appear on the western blot (Figure 4.5 A lane 5-8 and B lane 4). Although this is not a proof for well formed EGF repeats it is what one would expect if this domain is properly folded.

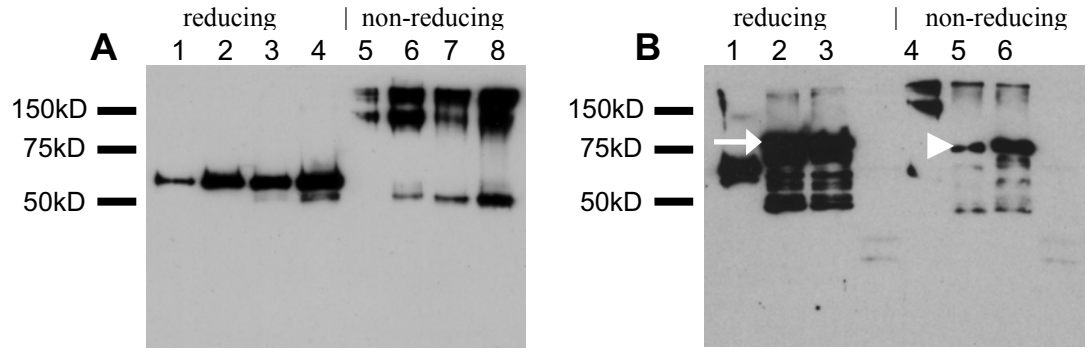


Figure 4.5 EFEMP1 under reducing or non-reducing conditions, produced from insect cells or bacteria

A Different fractions of EFEMP1 produced with the baculovirus system (insect cell system)
lanes A 1-4 (and **B 1**) different fractions of EFEMP1 under reducing conditions, where disulfide bonds are broken; only one band with the expected molecular weight appears.
lanes A 5-8 (and **B 4**) the same fractions under non-reducing conditions (without β–mercaptoethanol in the loading buffer) where disulfide bonds remain stable; additional bands with apparently higher molecular weight appear probably due to different migration properties of the stably folded EGF domains.

B lanes 2,3 and 5,6 EFEMP1 produced in bacteria
lane B1 the same protein like in A1/A5 under reducing conditions.
lane B4 the same protein like in A1/A5 under non-reducing conditions.
lanes B 2, 3 EFEMP1 produced in bacteria under reducing conditions (arrow)
lanes B 5, 6 the same proteins like in lanes B 2, 3 under non-reducing conditions (arrowhead) (without β–mercaptoethanol in the loading buffer).
EFEMP1 produced in bacteria migrates similar in reducing and non-reducing conditions, indicating that in bacteria EGF repeats are not intramolecular linked by disulfide bonds.
The different molecular weight of the protein that was produced in bacteria is due to a GST domain that was fused to EFEMP1 for purification. In addition, several bands with lower molecular weight appear in lane 2,3 and 5,6 that are due to degradation of the protein.

Finally we made confocal pictures of imaginal discs where the human EFEMP1 is expressed (Figure 4.6). These pictures do not allow us to precisely assign the protein to the extracellular matrix but at least the antibody staining is clearly visible in the space between the nuclei where we would expect a secreted protein. We made similar

pictures of the expression pattern of EFEMP1^{Arg345Trp} (not shown) but we could not observe a different localization of the mutated protein as it was observed in cell cultures (Marmorstein et al., 2002). These pictures are not sensitive enough to clearly distinguish between a localization of the protein in the cytoplasm and the extracellular matrix.

Taken together we have no reason to believe that the human EFEMP1 is not processed normally when we overexpress it in the fly or with any other insect cell system.

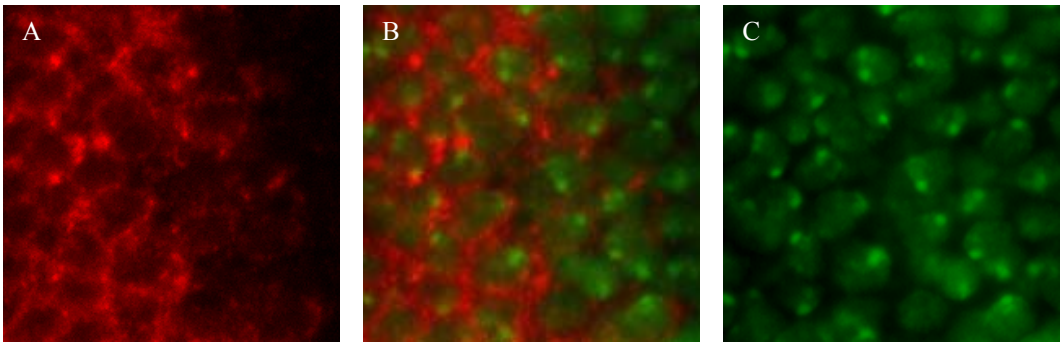


Figure 4.6 Immuno-localization of EFEMP1 in fly imaginal discs
A-C confocal picture of the same sector of a wing imaginal disc.
A Antibody staining against EFEMP1. **B** fusion of the pictures A and C. EFEMP1 is localized between the nuclei, presumably to the extracellular matrix. **C** staining of the nuclei with SYTOX Green.

2.4.5. Notch activity measurement using a cell culture assay

To further test whether EFEMP1 is able to activate the Notch receptor, a cell culture assay was used (kindly provided by S. Artavanis-Tsakonas). The core element of this *in vitro* assay is a plasmid which contains six Su(H) binding sites in front of a minimal promotor that drives the expression of a luciferase gene. Upon binding of a ligand, the Notch receptor gets cleaved, N^{IC} enters the nucleus and binds to Su(H). This interaction converts Su(H) from a repressor into a transcriptional activator and drives the expression of the luciferase gene. The luciferase acts on its substrate and leads to a measurable light formation (Figure 4.7)

cDNA of EFEMP1 was cloned into the pcDNA3 vector downstream of a constitutively active eukaryotic promoter (CMV). This construct was cotransformed with the reporter plasmid to COS cells and the cells were tested for luciferase upregulation.

With this assay, a constitutively activated Notch receptor led to a more than sixty fold upregulation of the luciferase activity whereas there was no detectable increase of the light emission due to EFEMP1 (Figure 4.8). Therefore, EFEMP1 is not able to activate the Human Notch-1 receptor in this assay. (The apparently activation of the reporter in lane 5 of figure 4.8 is due to a lower transfection efficiency by adding an additional plasmid to the transfection mixture and not due to an enhanced activation of the system.)

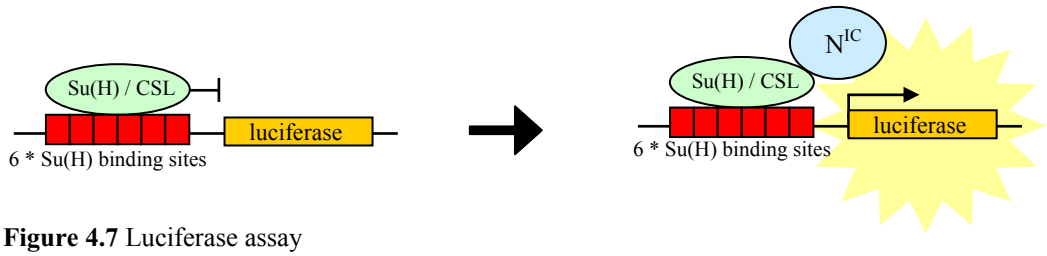


Figure 4.7 Luciferase assay
COS cells were transfected with a reporter plasmid carrying six Su(H) binding sites upstream of a minimal promotor in front of a luciferase gene. Without activation of the Notch receptor, Su(H) acts as transcriptional repressor. Upon activation of the Notch cascade by a ligand, N^{IC} enters the nucleus, binds to Su(H) and converts this transcriptional repressor into an activator. As a result, the luciferase gene gets transcribed, leading to a measurable light formation by acting on the luciferin substrate.

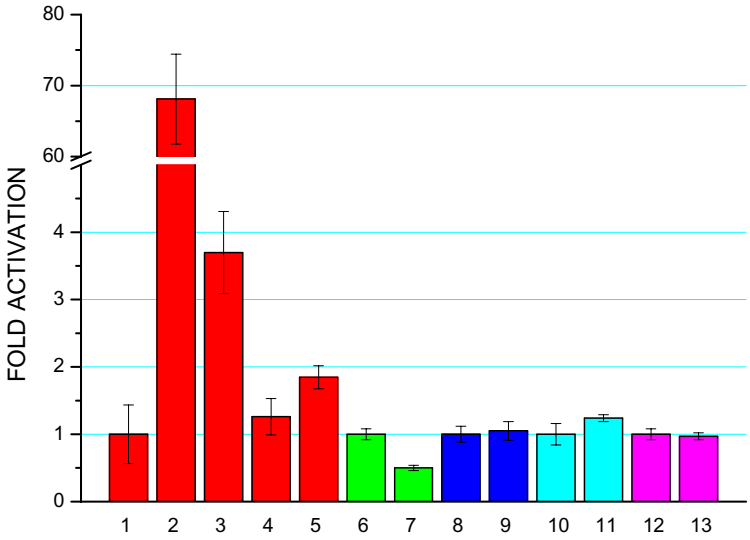


Figure 4.8 Cell culture assay to detect Notch activity

COS cells that were transfected with the Notch activity reporter plasmid and a plasmid that constitutively expresses a luciferase gene to normalize the measurements are referred to as the reporter cells in the text below. After transfection, the cells were incubated 24 hours until luciferase activity was measured.
1 Basal activation. The luciferase activity of the reporter cells alone was measured. This result was used to normalize the measurements 1-5.
2 Notch activated. The reporter cells were co-transfected with a constitutively active form of Notch (consisting of only the intracellular part of Notch) leading to a more than 60 fold activation of the system.
3 Delta. Stably Delta expressing cells were incubated with the reporter cells leading to a 3,5 fold activation.
4 EFEMP1 expressing cells (pcDNA3 EFEMP1) were incubated with the reporter cells
5 pcDNA3-EFEMP1 was co-transfected to the reporter cells
6 Negative control. The empty pEGFP/N3 vector was co-transfected with the reporter cells
7 pEGFP/N3 EFEMP1 was co-transfected to the reporter cells (normalized with **6**)
8 Negative control. pEGFP/N3 (without insert) expressing cells were co-incubated with the reporter cells
9 pEGFP/N3-EFEMP1 expressing cells were co-incubated with the reporter cells (normalized with **8**)

10-13 Protein that was synthesized with the baculovirus system was added to the reporter cells.
10 Negative control 100 µl elution with CSF protein, a nuclear protein that served as negative control, was added to the reporter cells. This protein was produced and purified with the same procedure like EFEMP1 **11** 0.5µg EFEMP1 protein (100 µl of the elution) was added to the reporter cells (normalized with **10**).
12 Negative control 50 µl elution with CSF protein was added to the reporter cells.
13 0.25µg EFEMP1protein (50 µl of the elution) was added to the reporter cells (normalized with **12**)

2.4.6. In vivo assay to detect Notch activity

To test whether we can detect an activation of the Notch receptor *in vivo*, we used flies that express *LacZ* under control of Su(H) in cells where Notch is activated (kindly provided by S. Bray). To enhance the activity of Su(H), three binding sites for the Grainyhead transcriptionfactor (Grh) that is ubiquitously active in imaginal discs was cloned in front of the two Su(H) binding sites. In cells where Notch is silent, the activity of the Grainyhead promotor is blocked by the repressing properties of Su(H). Conversely, if Notch gets activated and N^{IC} enters the nucleus, Su(H) acts together with Grh and N^{IC} as an activator of transcription leading to a strong *LacZ* expression (Figure 4.9) (Furriols and Bray, 2001).

In imaginal discs of third instar larvae, activation of the Notch pathway occurs in a specific pattern although Notch is expressed in all cells of the disc at this stage (Fehon et al., 1991). Therefore we expected an alteration of the endogenous Notch activity pattern by expressing a Notch ligand at ectopic positions.

Expression of EFEMP1 in these flies did not result in any variation of the Notch activity pattern (Figure 4.10 B,E).

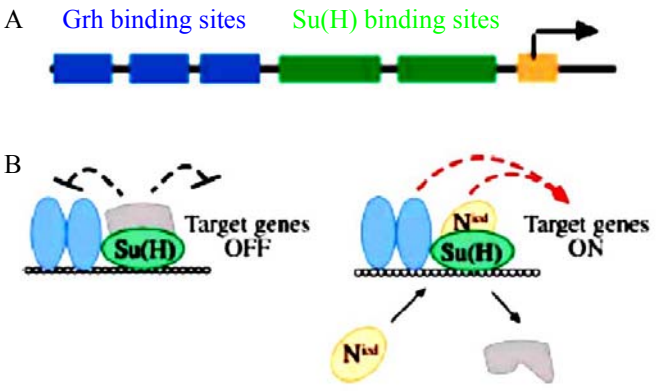


Figure 4.9 Reporter construct
A schematic view of the construct that is integrated into the genome of flies which were used to detect Notch activation. Binding sites for Grh were cloned upstream of Su(H) binding sites that are in front of a minimal promotor which drives LacZ expression.
B On its own, Su(H) acts as a transcriptional repressor and blocks the activity of Grh. Together with N^{IC}, Su(H) turns into a transcriptional activator which is enhanced by the action of Grh.

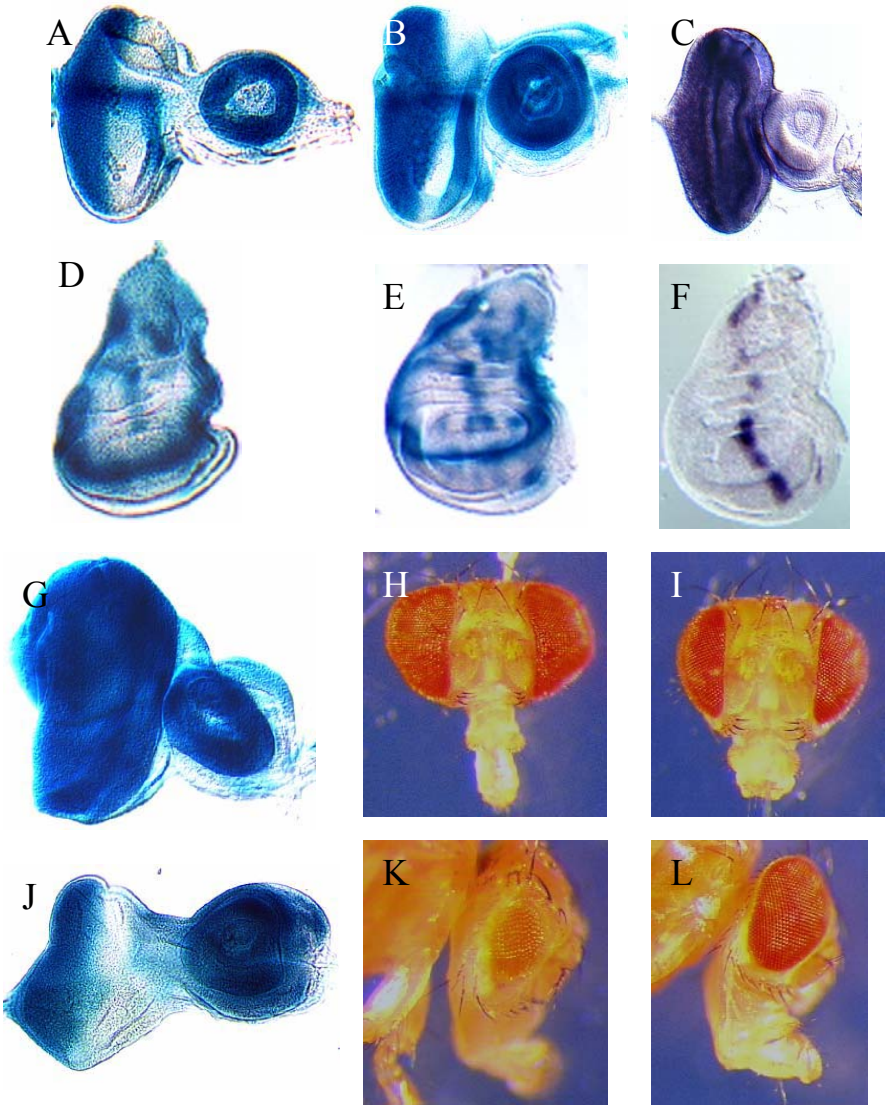


Figure 4.10 Notch activity reporter flies
A wt eye-antennal disc. LacZ expression (blue) reflects the normal Notch activity pattern, which is along the dorsal/ventral boundary and posterior to the MF.
B eye-antennal disc of a fly in which GMR-Gal4 drives UAS-EFEMP1 expression shows no alteration of the endogenous Notch activity pattern.
C *in situ* with EFEMP1-RNA on an eye-antennal disc from the same flies like in B shows EFEMP1 expression all over the eye disc.
D wt wing disc. LacZ expression reflects the normal Notch activity pattern
E wing disc of a fly where EFEMP1 is expressed shows no alteration of the endogenous notch activity pattern.
F *in situ* with EFEMP1-RNA on a wing disc from the same flies like in E shows EFEMP1 expression reflecting the pattern of the driver that was used to express EFEMP1 (dpp-Gal4).
G expression of the Notch ligand Delta in the entire eye disc expands Notch activity all over the eye disc.
H Ectopic activation of the Notch pathway with Delta in the entire eye disc leads to over-proliferated eyes when compared to wt eyes (in **I**)
J expression of the Notch ligand Serrate disrupts Notch activity at the dorsal ventral boundary leading to smaller eyes (in **K**) when compared to wt eyes (in **L**)
For all discs: posterior is to the left, dorsal is top

2.4.7. Expression of a non-secreted version of EFEMP1

In the human eye, EFEMP1 is secreted from the RPE and is located to the extracellular matrix surrounding the outer segments of the photoreceptor cells (Marmorstein et al., 2002). The mutated form, EFEMP1^{Arg345Trp}, is not properly secreted and accumulates in and underneath the RPE which could be the mechanism that provokes ML (Marmorstein et al., 2002).

Cleaving off the signal peptide, which is crucial for secretion of EFEMP1 to the extracellular matrix, should lead to a protein that remains intracellular. Assuming that such a non-secreted protein mimics the behavior of EFEMP1^{Arg345Trp} but in an enhanced way, where all of the protein remains intracellular, a non-secreted version of EFEMP1 (EFEMP1-sp) was created. A question that could be addressed with such a protein using the fly as a model system is if intracellular accumulation of EFEMP1 accounts for the retinal degeneration.

To test whether EFEMP1-sp is expressed, RNA *in situ* hybridization and antibody staining were performed. Whereas mRNA of EFEMP1-sp could be detected (Figure 4.11) it was not possible to detect EFEMP1-sp at protein level. EFEMP1-sp protein therefore is either immediately degraded, not translated or expressed at such low levels that it is not detectable by antibody staining.

Nevertheless, in 15 different transgenic lines that were screened for mutant phenotypes caused by expression of EFEMP1-sp with different drivers (eyeless-Gal4, dpp-Gal4), not a single abnormal compound eye was observed.

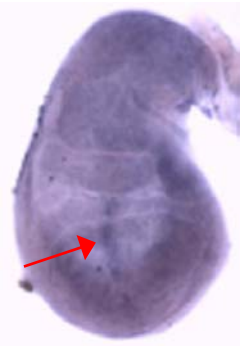


Figure 4.11 expression of EFEMP1-sp

In situ hybridization against EFEMP1-RNA on a wing disc where EFEMP1-sp is expressed under the control of the enhancer of the dpp gene. Along the anterior/posterior boundary a stripe is visible (arrow), reflecting the expression pattern of EFEMP1-sp with the dpp enhancer. posterior is to the left, dorsal is top.

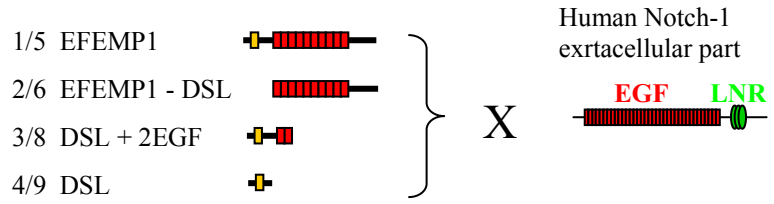
2.4.8. Pull-down assay

So far we tested whether EFEMP1 can activate the Notch receptor. We then wanted to analyze if EFEMP1 can bind *in vitro* to the extracellular part of the human Notch-1 receptor and therefore used a *pull-down* assay.

Four different EFEMP1 constructs were cloned. The whole EFEMP1, EFEMP1 without DSL domain (EFEMP1-DSL), the DSL domain with two EGF repeats (DSL + 2EGF) and the DSL domain alone.

These EFEMP1 constructs were fused downstream of a GST coding sequence and produced from *E. coli*. The extracellular part of Human Notch-1 was expressed from a reticulocytelysate and marked with S^{35} methionin (S^{35} HN-1^{EC}).

With these constructs we were not able to *pull-down* the extracellular part of Human Notch-1 (Figure 4.12).



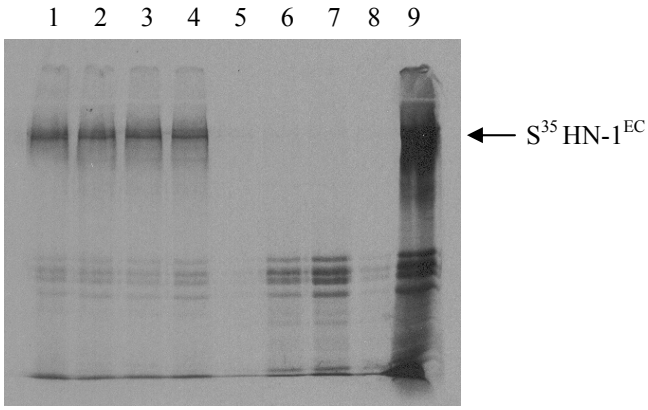


Figure 4.12 *Pull-down* experiment

1-4 Supernatant(SN) after incubation of S^{35} HN-1^{EC} with different EFEMP1 constructs and sepharose beads. The S^{35} HN-1^{EC} is still strong detectable in the SN.

5-8 *pull down:* no S^{35} HN-1^{EC} was co purified with the EFEMP1 constructs that are bound to the sepharose beads due to their fusion with the GST protein. The different EFEMP1 constructs do not interact with HN-1^{EC} in this assay.

9 "input" of *in vitro* synthesized S^{35} HN-1^{EC}.

2.4.9. EFEMP1 in aging flies

Malattia Leventinese has an onset of about 40 years and to observe the retinal degeneration an ophthalmoscope is needed. In our experiments we so far searched for dominant phenotypes of the ectopically expressed protein during eye development of *Drosophila*. In a next experiment we wanted to know whether over-expression of EFEMP1 and EFEMP1^{Arg345Trp} shows an age-related phenotype or a mild phenotype with macroscopically normal looking eyes, but degenerated photoreceptor cells.

To look for degenerated photoreceptor cells (neurons) in adult and aged flies, we used an assay that has previously been used to detect photoreceptor degeneration in flies that served as model systems for Huntington's disease (Steffan et al., 2001).

If over-expression of EFEMP1 and EFEMP1^{Arg345Trp} causes retinal degeneration in aging flies, we would expect a similar phenotype that we see in flies with mutations in the rhodopsin-1 gene. In these flies, the outer photoreceptor cells, where rhodopsin-1 is usually expressed, degenerate (Figure 4.13 C).

To express EFEMP1 and EFEMP1^{Arg345Trp} in the fly retina we used enhancers that drive expression to the outer photoreceptor cells (Rh1-Gal4) (Figure 4.13 A) or to all cells in the retina (GMR-Gal4) of adult flies. The photoreceptor cells were observed by light microscopy (Franceschini and Kirschfeld, 1971).

In an observation period of more than two months, by a live expectation of around three months, we did not observe degeneration of the photoreceptor cells in the eye of aged *Drosophila* (due to the fact that all of these transgenic flies looked normal, only the wt picture is shown (Figure 4.13 B)).

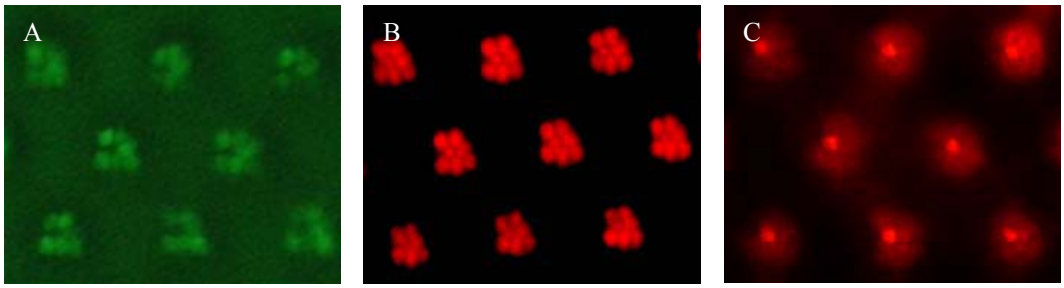


Figure 4.13 Photoreceptor cells of ageing flies
Visible in A to C is the light-gathering parts of the photoreceptor cells, the rhabdomeres.
A Green fluorescence protein (GFP) was expressed with the promotor from the rhodopsin-1 gene to test the driver. Rhodopsin-1 is expressed in the six outer photoreceptor cells (R1-R6) that are green in this picture.
B Representative picture of the observe ability of the photoreceptor cells. The picture shows 9 ommatidia in which the rhabdomeres of 7 normal Photoreceptor cells are visible.
C One week old flies with a mutation in the rhodopsin-1 gene which is specifically expressed in the photoreceptor cells R1-R6. In these flies, the outer photoreceptor cells (R1-R6) are degenerated.

2.4.10. Exposure of flies to constant light

In a next essay we tested whether overexpression of EFEMP1 and EFEMP1^{Arg345Trp} in a constant light environment leads to retinal degeneration.

The idea for this experiment came out of recent work that was done with the *Drosophila crumbs* gene.

Drosophila Crumbs is a single pass transmembrane protein. Mutations in the human homologue of *crumbs*, CRB1, are associated with retinitis pigmentosa which is a disease with a progredient loss of vision (den Hollander et al., 1999).

Drosophila and human Crumbs are similar in structure and are expressed at homologous locations in the human and fly retina (Pellikka et al., 2002). In *Drosophila*, the human *crumbs* can partially substitute for the *Drosophila* gene (den Hollander et al., 2001).

From experiments with *Drosophila* it is known, that flies which have clones of photoreceptor cells lacking the extracellular part of Crumbs are sensitive to light exposure. These flies get photoreceptor degeneration after one week of exposure to constant light (Johnson et al., 2002).

Since the extracellular part of Crumbs comprises several EGF repeats, Crumbs could belong to a functionally similar group like EFEMP1.

Therefore we tested whether overexpression of EFEMP1 or the mutated form of EFEMP1 that leads to ML could cause retinal degeneration in aging flies that were kept in constant light. (The flies were illuminated from both sides with two 8 Watt neon lights.)

During an observation period of two months, flies that express EFEMP1 or EFEMP1^{Arg345Trp} in this constant light environment did not show any signs of retinal degeneration.

2.4.11. *Drosophila* Notch EGF10-13 as template for Aptamers

The idea that Notch signalling is involved in AMD was reinforced by an article that showed Notch signalling involvement in muscle regeneration (Conboy et al., 2003). In this work, the authors showed that in aged mice, the repair mechanism of harmed muscle cells is impaired and they attribute this to a decline of *delta* expression in aged

animals. This is in good agreement with our working hypothesis that a decline of Notch signalling is involved in AMD.

As EFEMP1 has not been proven to be a Notch agonist, we were interested to find another soluble molecule that could be used to activate the Notch receptor. Due to their smallness end ease of production we considered making aptamers as a reasonable approach.

Aptamers are known since 1990. At that time, two articles introduced the procedure of making aptamers (Ellington and Szostak, 1990; Tuerk and Gold, 1990). In both papers similar methods are described how a single stranded RNA (or DNA) molecule that specifically binds to a target is selected. The procedure was called SELEX (systematic evolution of ligands by exponential enrichment) and the single stranded RNA (or DNA) molecules were called aptamers (aptus = "to fit"). The SELEX procedure is depicted in (Fig. 4.14).

Compared to antibodies, aptamers have several advantages. First, it is an *in vitro* process and therefore aptamers can be raised against substances that are toxic for animals or that have only weak immunogenic properties. Second, the selection conditions can be varied (different buffers or temperatures can be used). Third, there is little batch to batch variation and finally, they can be chemically crosslinked. For example: F or NH_2 groups can be attached to increase the stability against nucleases or biotin or fluorescin can be linked for histochemistry purposes (for review see(Jayasena, 1999)).

For our project to use aptamers as soluble Notch agonists for therapeutic reasons, aptamers have two important advantages: First, they are small (at most 40 nucleotides, single strand) and therefore well diffusible. Second, they consist of DNA or RNA and have less immunogenic potential than antibodies.

Due to their advantageous properties medical research became interested in aptamers. One aptamer that is in clinical trials is an aptamer against vascular endothelial growth factor (vEGF). Aptamers against vEGF help to prevent choroidal neovascularization, an undesirable vascular outgrowth secondary to AMD and a leading cause of blindness (Kim et al., 1993; TheEyetechStudyGroup, 2003).

For the production of the aptamers we collaborate with Larry Gold from Somalogic. Our part of the collaboration is the production of the protein and the functional assays

of the aptamers. We would like to test the aptamers we get from Somalogic for their ability to work as agonist/antagonist on the Notch receptor in cell culture and on imaginal discs.

The starting point was the production of the extracellular part of Notch. Notch, as described previously in this work, has a large extracellular domain including 36 EGF repeats. Each EGF domain is intramolecular linked by 3 disulfide bridges in a distinct pattern: C1-3, C2-4, C5-6. EGF repeats do not form in a reducing environment, thus a standard protein production in bacteria would not produce a proper folded protein.

After using various systems with different parts of the extracellular domain of Notch, we ended up producing EGF10-13 in little quantities with bacteria under special conditions. To assure that these bacteria fold the protein properly, a signal peptide was attached to the protein so that it is secreted to the periplasmatic space. In addition, a disulfide isomerase was co-secreted and the growth medium was supplied with L-Acetylcysteine as a redox buffer (see materials and methods). Under these conditions oxidative protein folding should work properly (Maskos et al., 2003).

At the moment we have 0.5 mg of EGF10-13 protein which is the required amount for the SELEX procedure and we are ready to ship the protein for aptamer production.

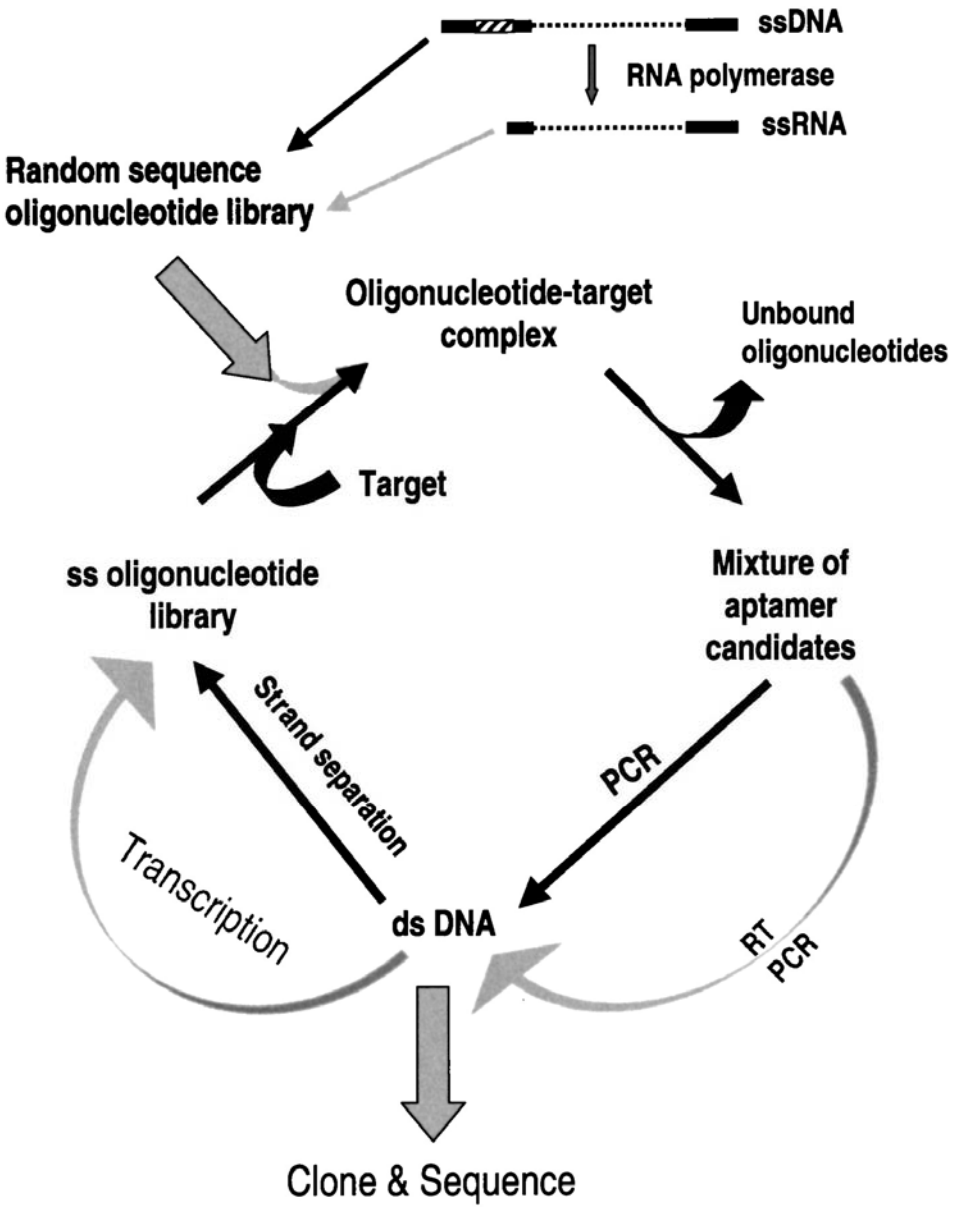


Figure 4.14 The SELEX procedure
The SELEX procedure starts with a library of random sequences of single stranded RNA or DNA molecules with a length between 20 and 40 nucleotides. Theoretically, a library comprises around 10^{24} different sequences.
Such a library is incubated with the target of interest at a given temperature in a specific buffer. The bound population is then eluted, amplified by PCR and, after strand separation, again incubated with the target. After 8 to 15 cycles, affinity saturation is achieved and the selected aptamers can easily be sequenced and synthesized.
Picture from (Jayasena, 1999).

2.5. Discussion

During the last years much has been revealed of the complex genetic network and its intriguing interplay that controls eye development. Strikingly, many genes and their interactions that are involved in eye development apparently are conserved throughout the animal kingdom (reviewed by (Treisman, 1999; Kumar, 2001). Mutations in the transcription factor *Pax-6* - a master control gene of eye development (Gehring and Ikeo, 1999) - are responsible for *Aniridia* in humans and *Small eye* in mice (Hill et al., 1991). Mutations in the *Drosophila Pax-6* gene *eyeless* are responsible for an eyeless phenotype (Quiring et al., 1994a). Furthermore, the mouse *Pax-6* protein is able to induce ectopic eyes in *Drosophila*, which shows the high degree of functional conservation of the *Pax-6* gene between species (Halder et al., 1995). Conversely the *Drosophila eyeless* is able to induce ectopic eye structures when ectopically expressed in *Xenopus* embryos (Onuma et al., 2002). In the ribbonworm *Lineus Pax-6* is not only important for eye development but also for maintenance and regeneration of the adult eyes (M.Tarpin, pers. Comm.). Notch, which lies genetically upstream of *Pax-6 (Kurata et al., 2000; Kumar and Moses, 2001)*, is crucial for eye development in *Drosophila* and vertebrates and Notch-1 as well as *Pax-6* are expressed in the retina of adult mice and humans (F. Hafezi, pers. Comm.).

So far neither in vertebrates nor in *Drosophila* much is known about the molecular mechanisms that are necessary for the maintenance and eventually the regeneration of the retina.

We hypothesize that the Notch pathway and the transcription factor *Pax-6* are both involved in maintenance and regeneration of the adult *Drosophila* and vertebrate retina. Thus having a soluble Notch ligand that could activate Notch from the outside of the cell would be a promising tool to activate the receptor, increase the transcription of *Pax-6* and thereby prevent retinal degeneration.

Recently a single point mutation in the human protein EFEMP1 has been identified that causes the retinal dystrophy Malattia Leventinese (Stone et al., 1999). Due to the similar phenotype of ML and the frequent age-related macular degeneration, ML appears to be an early onset form of AMD (Marmorstein et al., 2002). The structural similarities between EFEMP1 and the so far known Notch ligands makes us believe that this human protein might be a soluble Notch ligand and that the disease is a result of an inappropriate activation of the Notch pathway in the retina.

In our work we wanted to test whether EFEMP1 is indeed a Notch ligand and if not, whether the fly is an appropriate tool to study the molecular function of this human protein.

In silico structure comparison of EFEMP1 with Notch ligands

All Notch ligands known so far that are able to activate Notch share several properties. First they all are single pass transmembrane proteins. EFEMP1 is a protein of the extracellular matrix secreted from the RPE and does not comprise a transmembrane domain. Different studies could not clarify whether attachment to the membrane is crucial for a ligand to be able to activate the Notch receptor. Truncated, soluble forms of Serrate and Delta have been shown to act as antagonists of Notch signaling in *Drosophila* (Fleming et al., 1997; Hukriede and Fleming, 1997; Hukriede et al., 1997; Sun and Artavanis-Tsakonas, 1997; Qi et al., 1999). Other secreted forms of either Delta1 or Jagged1 have been used as activators or repressors of Notch signaling in mammalian cell types (Li et al., 1998; Varnum-Finney et al., 1998; Wang et al., 1998; Qi et al., 1999; Sestan et al., 1999; Morrison et al., 2000; Ohishi et al., 2000). Recent *in vitro* studies suggest that the ability of Delta to activate Notch is related to its multimerization (Hicks et al., 2002).

Taken together, further investigations are necessary to answer the question if soluble proteins are able to activate the Notch receptor and how soluble Notch agonist in general look like.

A second property which all Notch ligands share is an array of tandem repeated EGF domains. EGFs are cysteine rich domains that are intramolecularly linked by disulphide bridges and are involved in protein-protein interactions. EGF repeats are common in extracellular matrix proteins and are present in the fibulins, in crumbs and other proteins mostly involved in cell adhesion and cell to cell signaling.

The third and most important feature common among Notch ligands is their DSL domain that is located near the aminoterminal end through which the ligand interacts with Notch (Rebay et al., 1991; Fitzgerald and Greenwald, 1995; Shimizu et al., 1999). The name DSL represents Delta, Serrate and Lag-2, the *Drosophila* and *C. elegans* Notch ligands in which this domain was described first. By comparison the DSL domains of all so far known Notch ligands from different species a consensus sequence of 11 amino acids was found (Lissemore and Starmer, 1999). When we analyzed the

sequence of EFEMP1, we found a putative DSL domain that shows identity to 8 amino acids of the consensus sequence. From the amino acid sequence it is difficult to predict whether this is a true DSL domain. It could also act as a further EGF repeat since DSL domains are related to EGF repeats due to their high content of cysteines. However, as an EGF repeat this DSL domain of EFEMP1 would be most closely related to DSL domains among all EGF repeats.

In conclusion, EFEMP1 is a protein of the extracellular matrix secreted from the RPE with high similarities to Notch ligands.

Overexpression experiments

Upon over-expression of a Notch ligand in *Drosophila* we expected phenotypes like overgrown eyes or truncated wings and legs depending on the stage and tissue where the protein is expressed. In all our experiments where we over-expressed EFEMP1 and EFEMP1^{Arg345Trp} either alone or together with human Notch-1 or a chimeric Notch in different developmental stages and tissues we did not see any EFEMP1/ EFEMP1^{Arg345Trp} specific phenotype. Further over-expression experiments using the putative fly homologue of EFEMP1 did also fail to produce a mutant phenotype in the fly. By interpreting these results it has to be kept in mind, that most of the *Drosophila* genes do not exhibit any change of morphology upon over-expression.

Another reason for the lack of mutant phenotypes could be the fact that the mammalian protein might not be secreted to the extracellular matrix in insect cells and therefore can not interact with its receptor. However, for the following reasons we do not think that this is the case: First, when running EFEMP1 that was purified out of the supernatant of an insect cell system on a SDS gel in non-reducing conditions, we see slow migrating bands as we would expect it from a protein, that has properly formed EGF domains.

Second, signal peptides of insect and mammalian proteins look very similar and all mammalian secretion signals that were tested by Invitrogen functioned properly in insect cells (Invitrogen, 2002).

Third, antibody staining for EFEMP1 upon expression in imaginal discs shows an accumulation of the protein that is compatible with a proper secretion of the protein to the extracellular matrix.

Out of these reasons we assume that EFEMP1 is processed normally in the fly but that the protein does not provoke a dominant phenotype upon over-expression.

Assays to detect interaction with the Notch receptor

Posttranslational modifications like glycosylation, phosphorylation and others are very common in vertebrate proteins. In addition, tertiary structures formed by extracellular matrix proteins are different from that of intracellular proteins due to their oxidizing environment (eg. disulfide bonds). In our *pull-down* experiment the proteins were synthesized by bacteria and thus probably not correctly processed. Therefore we can not exclude that EFEMP1 does not interact with Notch in the assay since no correctly folded EFEMP1 was produced in bacteria and hence the protein lacked appropriate efficacy. Still it is a fast and easy method to detect protein-protein interactions based on their primary structure.

In a second *in vitro* experiment the proteins were synthesized by vertebrate cells. Hence we expected all modifications to occur properly. A luciferase reaction was used to detect Notch activation: activation of the receptor by a ligand results in a measurable light formation in this assay.

Unfortunately, EFEMP1 also did not induce this system. Therefore our results suggest that EFEMP1 is not an agonist of the Notch pathway, at least not as a monomer or a homo-multimer.

In an *in vivo* assay we finally tried to detect alterations of the Notch pathway. Here activation of Notch becomes visible as a strong blue staining on imaginal discs. Due to the lacZ reporter, this is a sensitive method to detect activation or repression of the signaling cascade.

When we tested EFEMP1 we could not observe any effect on the receptor with this assay. Still, as a human protein it is conceivable that although the high sequence similarity between human and *Drosophila* Notch, EFEMP1 is not able to interact with *Drosophila* Notch. Therefore, we still have to test the putative EFEMP1 homologous protein of *Drosophila* within this assay.

Conclusion and outlook

With the so far used methods we could not get any evidence that EFEMP1 is indeed a Notch ligand despite its high sequence homology to known Notch ligands. Our over-expression experiments show that EFEMP1 and EFEMP1^{Arg345Trp} do not have a dominant effect - neither on eye development nor on the retina of aging flies.

The work presented so far is limited with respect to the fact that we only tested for gain-of-function properties of EFEMP1. Another means to elucidate the function of a gene is to study its loss-of-function. Up until very recently we did not have any null mutation of the putative *Drosophila* homologous gene of EFEMP1 and making a knock-out is difficult and time consuming in flies. A method that became established for flies is the conditional knock-out of genes by RNA interference (RNAi) (Kalidas and Smith, 2002). At the moment we are using this method to block protein synthesis of the *Drosophila* homologue of EFEMP1. If the conditional knock-down of this gene results in a mutant phenotype we will be able to find out which mechanism is disrupted and can furthermore try to rescue this phenotype by expressing human EFEMP1 combined to the RNAi against the fly gene to proof that EFEMP1 can functionally replace its fly homologue.

In situ hybridization shows that the putative *Drosophila* EFEMP1-homologue is expressed in the developing eye. Using antibodies we are about to detect where the protein is located on the cellular level in the developing and adult eye of the fly. If this *Drosophila* gene similar to EFEMP1 finally turns out to be homologous to EFEMP1 not only regarding its structure but also exhibits a similar function, we expect it to be expressed and secreted to the extracellular matrix surrounding the photoreceptor cells of the compound eye.

If we are able to elucidate the function of this gene in *Drosophila,* we can draw conclusions on the function of EFEMP1 and probably get more ideas of what kind of experiments will be appropriate to elucidate the pathomechanism of Malattia Leventinese and consequently AMD.

3. Molecular genetic analysis of a *sine oculis* enhancer

3.1. Summary

The *sine oculis* (*so*) gene is important for the development of the entire visual system of *Drosophila* melanogaster. *so* shows homology to the vertebrate *Six*-gene family that is characterized by genes encoding a *Six* domain and a Six-type homeodomain. Like *twin of eyeless* (*toy*), *eyeless* (*ey*), *eyes absent* (*eya*), and *dachshund* (*dac*), *so* belongs to a network of genes that by complex interactions render the genetic background of eye development.

An eye specific enhancer fragment of the *so* gene (so10) has previously been shown to be directly regulated by *ey* and *toy* during compound eye development. It is sufficient to rescue the compound eye by driving *so* in a *so*-mutant background but only in combination with the adjacent sequences (so9), so10 re-establishes both, the eye- and the ocelliless phenotype of so^1 mutant flies.

We studied the regulation of the so9 enhancer further and found that a fragment as small as 27bp (soAE) is sufficient for so9 mediated expression. In addition, we show that SO itself binds and regulates soAE. The resulting auto-regulatory loop is important for ocellar development.

By systematic analysis of the DNA-protein interactions between soAE and SO we identified the most important nucleotides for this interaction. Using the emerging consensus for SO-DNA binding we performed a genome wide search for putative *so*-target sites and have thereby been able to identify *ey* and *hedgehog* (*hh*) as putative targets of *so*. Our results, by providing an additional example, strengthen the general assumption that feedback loops among the genes of the retinal determination network are crucial for proper development of eyes and ocelli.

3.2. Introduction

The *Drosophila* visual system consists of two compound eyes and three ocelli, which are simple eyes located on the adult vertex (Stark et al., 1989). Both types of optical organs develop from a small number of cells that are set aside during development in the early embryo. These cells form the eye part of the eye-antennal imaginal disc and proliferate during the larval stages. Finally, the compound eye emerges from the central part of the eye imaginal disc whereas the ocelli develop from the anterior-medial region. The compound eye in *Drosophila* consists of a precisely organized array of approximately 750 ommatidia, each containing eight photoreceptor neurons and twelve accessory cells. The ommatidia begin to form in the early 3rd instar larva, when the morphogenetic furrow (MF), a wave of pattern formation marked by an indentation, moves across the eye disc from posterior to anterior (reviewed by Wolff and Ready, 1993). Although committed to retinal fate, cells anterior to the furrow are still undifferentiated, whereas cells posterior to it are sequentially recruited into ommatidial clusters undergoing retinal differentiation (reviewed by Treisman and Heberlein, 1998).

Determination of the eye primordium requires several nuclear proteins that are known to act as transcriptional regulators. The *Drosophila Pax6*-gene *eyeless* (*ey)* was the first gene shown to display the capacity to induce ectopic eye morphogenesis upon ectopic expression (Halder et al., 1995). A second Drosophila *Pax6*-gene, *twin of eyeless* (*toy*), like *ey* encodes a protein with two DNA-binding domains (Czerny et al., 1999). Further genes in early eye determination are *eye gone* (*eyg*) which also shows homology to *Pax6* (Jun et al., 1998; Chao et al., 2004; Dominguez et al., 2004), *sine oculis* (*so)*, a homeobox gene (Cheyette et al., 1994), *eyes absent* (*eya)* and *dachshund* (*dac)* both encoding nuclear proteins (Bonini et al., 1993; Mardon et al., 1994). Analyses of the expression patterns of these genes combined with genetic approaches have revealed a complex genetic regulation network during compound eye development. *toy* is the first of the mentioned genes to be expressed during embryogenesis and activates *ey* in the eye primordium (Czerny et al., 1999). *so* is required later for the development of the entire visual system, including the compound eyes, the ocelli, the optic lobe of the brain and the larval photoreceptors designated as Bolwig's organ (Cheyette et al., 1994; Serikaku and O'Tousa, 1994; Pignoni et al., 1997). *eya* expression comes up later in the compound eyes and can be found in the

ocelli-specifying region in third instar eye imaginal discs. Recently, eya has been shown to have protein phosphatase activity (Li et al., 2003; Tootle et al., 2003). *so* and *eya* are both required for compound eye and ocelli formation, since the according mutants lack both visual systems (Zimmerman et al., 2000). *so*, *eya* and *dac* have been shown to be regulated by *ey* (Halder et al., 1998a; Niimi et al., 1999; Zimmerman et al., 2000). SO and DAC have been proposed to function as cofactors for EYA, and genetic studies in Drosophila have demonstrated synergistic interactions between *so*, *eya* and *dac* during eye development (Chen et al., 1997; Pignoni et al., 1997). The respective protein complexes feed back on *ey* expression and *eya* and *dac*, like *ey* and *toy* are capable of inducing ectopic eye morphogenesis (Bonini et al., 1993; Bonini et al., 1997; Pignoni et al., 1997).

Although much knowledge has been gathered during the last years about the complex genetic network that orchestrates eye development, only a small number of observed regulatory interactions have been analyzed down to the level of DNA-protein interactions. Analysis of further components controlling expression patterns of genes involved in early eye development should therefore provide important details on the genetic hierarchy that mediates eye specification and may help to identify direct targets of the known eye specification genes.

Among the already described direct interactions *toy* has been shown to induce *ey* expression by an eye-specific enhancer in embryonic eye precursor cells, but not during larval stages in the later emerging eye imaginal disc (Czerny et al., 1999). *ey* on the other hand together with *toy* directly regulates *so* expression by an eye specific enhancer which is deleted in the so^{1} allele (Niimi et al., 1999; Punzo et al., 2002). Further *ey*-regulated, eye specific enhancers could be identified by genomic deletions within the *eya* gene-locus (Zimmerman et al., 2000).

In this study, we address the regulatory potential of a previously described *so*7 enhancer fragment during ocelli morphogenesis. So7 represents the DNA-fragment which is deleted in the so^{1} mutation and contains the *ey* and *toy* regulated enhancer element so10. We show that a 27bp fragment within so7, soAE, is sufficient do expand expression of a reporter gene to the ocellar region when fused to the so10 enhancer. Furthermore we show that soAE is a direct target of *so* in compound eye and ocelli development and that the autoregulatory feedback of *so* on its own expression is needed for the ocelli specific expression of *so*.

By analyzing the sequence specificity of SO-binding in more detail, we were able to identify the nucleotides specifically important for SO-soAE interaction. Using the thereby emerging *cis*-regulatory signature for *so*-dependent regulation we performed a genome-wide search for further potential *so*-target genes. Sequences that fit our selection criteria were identified in the *ey* and *hedgehog* (*hh*) loci. We show that both of these genes contain eye specific enhancers that are directly regulated by *so*. Our results emphasize the importance of autoregulatory feedback loops in morphogenesis and development.

3.3. Results

3.3.1. Defining a minimal eye/ocelli specific enhancer of the so gene

A 1.6 kb enhancer fragment (so7, Fig.1A) spanning the entire genomic region deleted in so^1 is able to recapitulate the expression pattern of *so* in 3rd instar eye imaginal discs when driving a *lacZ* reporter gene (Fig 1B). Furthermore, so7 is able to completely rescue the eyeless and partially the ocelliless phenotype of so^1 mutant flies when driving the *so* gene (Punzo et al., 2002).

So10 (400bp) and so9 (1,2 kb) (Fig. 1A) are subfragments of so7. so10 mediates expression in the compound eye part of 3rd instar eye-antennal imaginal discs and contains the previously described *ey* and *toy* specific binding sites (Fig. 1C). These include five binding sites bound by *toy*. Three of these are also binding sites for *ey* and are important for compound eye development whereas the two *toy* specific sites are required for ocelli development (Niimi et al., 1999; Punzo et al., 2002). Consistent with its expression pattern, so10 is able to rescue the eyeless phenotype but not the ocelliless phenotype of so^1 mutant flies (Punzo et al., 2002).

so9 mediated expression appears at the posterior margin of the eye disc (Fig. 1D). When combined with so10, so9 provides additional transcriptional input to expand the expression to the ocellar region.

Trans-acting factors that bind the *cis*-regulatory so9 element and cooperate with *toy* to confer expression in the ocellar region were unknown when this work was started. In order to locate the binding sites of such additional transcription factors we first aimed at the isolation of the smallest version of so9 that still would be able to drive expression of a *LacZ* reporter to the ocellar region of eye imaginal discs when combined with so10 (Fig. 1A).

Our search resulted in the identification of a fragment as small as 27bp (Fig1A Fragment 21) that will be referred to as soAE in the following (*sine oculis* autoregulatory element).

The expression pattern mediated by a combined so10-soAE-element was indistinguishable from expression mediated by so7 (Fig. 1A Nr. 14), whereas soAE alone resembled the expression pattern of so9 (Fig. 1A Nr. 21). Therefore soAE contains all regulatory elements that are sufficient for so9 mediated expression. Further evidence for the functional relevance of this sequence came form the comparison of

D.melanogaster and the genomes of other *Drosophila* species in which the soAE sequence shows a high degree of conservation between 6 different species (see appendix).

3.3.2. *sine oculis* is able to recognize its own enhancer

In soAE three sequence motifs can be found, that are reminiscent of well known transcription-factor binding-sites. These are a motif related to the *Pax6*-consensus-binding site (Epstein et al., 1994), a TAAT-motif that is a hallmark of most homeodomain recognition sequences and a GATA-motif. We mutated these sites, respectively, and tested the according fragments (so10-mutPAX, so10-mutHD, so10-mutGATA) for the resulting expression patterns.

so10-mutPAX mediated expression was indistinguishable from the so10-soAE expression pattern (Fig. 2C). Conversely, mutating the putative homeodomain binding site (so10-mutHD) or the GATA sequence (so10-mutGATA) resulted in loss of reporter gene expression in the ocellar region (Fig. 2A, 2B).

We then oligomerized (4 times) soAE, to boost its expression pattern. As a result, an expression signal became apparent posterior and in front of the MF (Fig. 2E) as well as in the optic lobe (data not shown). However, 4xsoAE was not able to drive expression in the ocellar region. Additional copies of soAE did not lead to a further strengthened expression. Expression of 10xsoAE, for example, appears blotchy and weaker in the eye disc than expression of 4xsoAE (Fig. 2F).

Since the expression pattern of 4xsoAE is reminiscent of *so*-expression in the eye disc, we hypothesized *so* itself to be the soAE regulating factor. Both expression patterns show signal in the optic lobe as well as posterior, within and in a few cells in front of the MF. The only difference is the ocellar expression of *so*, which cannot be seen using the 4xsoAE reporter construct.

The idea that *so* itself is the soAE binding factor was further supported by a previous work, where Hazbun et al. showed that SO binds to (C/T)GATA *in vitro* (Hazbun et al., 1997), a motif that is present in soAE (Fig.4C nt. 7-11).

To experimentally determine whether the expression pattern of the mutated fragments correlates with the ability of these fragments to bind SO *in vitro* we performed EMSA experiments. SO protein was able to shift radiolabeled mutPAX but failed to bind to mutHD and mutGATA DNA-fragments (Fig. 2H).

These results in combination with our *in vivo* data strongly suggest that *so* itself is responsible for the ocellar specific expression of so10-soAE.

3.3.3. so10-soAE-*LacZ* and so7-*LacZ* are not expressed in the ocellar region of *so*^{2} mutant flies

To further test this hypothesis, we moved on to a genetic approach. *so*^{2} is a hypomorphic allele that originated as a spontaneous partial reversion of *so*^{1} (Lindsley and Zimm, 1992). Different from *so*^{1} adult flies which completely lack compound eyes and ocelli, *so*^{2} flies develop compound eyes that range from normal appearance to slightly reduced shapes but still lack ocelli in total. In *so*^{2} /*so*^{1} flies, eyes are of intermediate size (Heitzler et al., 1993). Due to the common origin and the genetic interaction of these two alleles, we tested if there is a mutation in *so*^{2} flies that affects the genomic so9/so10 sequences. Using PCR on genomic *so*^{2}-DNA, we found a deletion of 1.2kb that indeed affects so7. We further confirmed this result by southern blot (data not shown). The deletions of *so*^{1} and *so*^{2} partially overlap (Fig. 1A) and in *so*^{2}, 4 of the 5 previously described *Pax6*-binding sites are missing (Punzo et al., 2002). In fact, both binding sites solely recognized by TOY are deleted. According to Punzo et al, these *toy* specific binding sites within the so10 enhancer fragment are required for ocelli development. The sequence representing so9, that contains the soAE fragment, appeared not to be affected by the *so*^{2} deletion.

In a next step we took advantage of *so*^{2} mutant flies to test whether the *cis*-regulatory potential of soAE depends on SO protein *in vivo*. Therefore we analyzed so10-soAE mediated expression in the ocellar region in *so*^{2} mutant flies. As expected, so10-soAE-*LacZ* expression is lost in the ocellar region of *so*^{2} mutant flies (Fig. 3D) supporting the idea of *so* being required for the ocelli specific expression of so10-soAE further. The absence of reporter gene expression can not be explained by a loss of ocelli specific precursor cells as *eya* expression, which represents a marker for this specified cell population, is detectable in *so*^{2} mutant flies in the prospective ocellar region (Fig. 3E).

Taken together, *toy* and *so* binding to so10 and soAE, respectively, seem to cooperatively drive *so*-expression in the ocellar region of 3rd instar eye discs.

To further examine the hypothesis that *so*-autoregulation is important for ocelli development and as a control, we tested so7-*LacZ* expression in *so*^{2} mutant flies. so7

was shown to be sufficient to completely rescue the eyeless and to partially rescue the ocelliless phenotype of *so*^{1} mutants when driving *so* (Punzo et al., 2002). Like so10-soAE-*LacZ*, so7-*LacZ* expression is lost in the ocellar region of *so*^{2} mutant flies (data not shown). To do the reverse experiment we mutated the SO binding site of so7 and looked at the expression pattern in a wt fly (Fig. 1A nr.22 and Fig. 1E so7mut). So7mut-*LacZ* expression is hardly detectable in the ocellar region and resembles expression of fragments nr7 and nr11 (Fig. 1A,E). These data strongly suggests that feedback of *so* on its own enhancer is needed for ocelli development.

3.3.4. 4xsoAE is not expressed in *so*^{3} clones

To assess whether soAE is a target of *so* also in the compound eye part of the eye disc, we tested the expression of the 4xsoAE reporter construct in cells homozygous for *so*^{3}, a null allele of *so* (Cheyette et al., 1994). *so*^{3} mutant cells, however, tend to overproliferate, fail to differentiate into neurons and subsequently die (Pignoni et al., 1997). Hence, to be able to analyze reporter gene activity in non-dying cells within *so*^{3} clones we tested them for *eya* expression. *eya* expression is considered to mark the healthy, differentiating cells that normally would express *so* and *eya* together, but lack *so* due to the *so*^{3} mutation, for the following reasons: First, *so* and *eya* both are targets of *ey* and show the same expression pattern in 3rd instar eye discs (Halder et al., 1998b; Niimi et al., 1999; Bui et al., 2000b). Both are expressed in a few cells anterior to the MF, within the MF, and in the differentiating photoreceptors posterior to the MF (Curtiss and Mlodzik, 2000). Second, SO and EYA proteins form a complex that works as a transcriptional activator when the proteins are coexpressed. (Pignoni et al., 1997; Silver et al., 2003). Third, *so*^{1} mutant eye discs still express *eya* whereas in *eya*^{1} mutants, expression of *so* is lost (Halder et al., 1998b). Finally, *so* can be induced by *eya* in 3rd instar eye imaginal discs (Curtiss and Mlodzik, 2000). For these reasons we assume *eya*-positive-cells of 3rd instar eye discs to also express *so* during normal development. Therefore only the reporter gene expression of *eya*-expressing cells within the clones was considered in our assay. In fact, in *eya*-expressing cells within *so*^{3} clones, expression of the 4xsoAE reporter construct is lost (Fig.3H,I). This strongly suggests, that SO protein in general is needed to activate the soAE element in the eye-field.

3.3.5. 4xsoAE-*LacZ* is induced in ectopic eyes and in cell culture

To further analyze whether soAE is a general *in vivo* target of SO we tested reporter gene activity as a result of ectopic eye induction. *so* on its own is not able to induce ectopic eyes. In contrast, *eya* alone, synergistically strengthened by *so*, is sufficient to induce ectopic eye development on antennae, wings and legs (Pignoni et al., 1997).

We induced ectopic eye development by combining a *dpp*-GAL4 driver with UAS:*so*, UAS:*eya* or both of them and tested whether the reporter construct 4xsoAE-*LacZ* was induced ectopically. As expected, ectopic *so* alone does not result in reporter gene activity wheras *eya* alone or *eya* combined with *so* in a synergistic manner is able to activate the reporter construct in wing discs (Fig. 3A-C).

In another *in vitro* approach, we took advantage of *Drosophila* S2 cells to address whether SO and EYA proteins work as a complex on soAE to induce transcription. Consistent with the *in vivo* data, our *in vitro* results using S2-cells show that SO, which has DNA binding properties but lacks a transactivation domain, on its own is not able to activate soAE mediated *LacZ* expression (Fig. 3J). Likewise, EYA which contains a transactivation domain but lacks DNA binding properties also fails to induce transcription in S2 cells when alone (Fig. 3J). Only when coexpressed, SO and EYA cooperatively work as transcriptional activators on soAE (Fig. 3J).

Interestingly, both SO and EYA mediate weak transactivation when the oligomerized mutated sites mutHD and mutGATA are used (Fig. 3J), despite the fact that these sites do not show any expression pattern *in vivo* (Fig. 2A,B).

3.3.6. Defining a consensus sequence for SO - DNA interaction

To date, there is only one direct target of *so* described in *Drosophila*, which is the Runx class transcription factor *lozenge* (*lz*) (Yan et al., 2003). Consistent with a previous *in vitro* study that addressed the DNA specificity of the SO homeodomain (Hazbun et al., 1997), the authors show that the sequence (C/T)GATA plays a crucial role in SO-DNA interaction. Another study reports of SO together with EYA being able to transactivate by a AREC3/Six4 binding site in cell culture. This motif, however, diverges from the C/TGATA-motif (for sequence see Fig. 4C) (Silver et al., 2003).

Our identified soAE fragment harbors CGATA and therefore is consistent with the SO binding consensus of the *lz* promotor. In our experiments, however, also mutations upstream of this GATA core motif (Fig 4C nt. 8-11) were not only able to abolish

expression of the reporter construct *in vivo*, but they also impaired the capability of SO to shift DNA fragments in EMSA. This observation made us believe that additional sequences upstream of the GATA motif are as well necessary for SO binding to its target DNA-sequence.

Therefore we decided to elucidate the sequence specificity of SO-DNA-binding by analyzing a systematic series of point mutations for their effect on protein-DNA complex formation (Fig. 4A)

These *in vitro* experiments revealed a stretch of 13 nucleotides to be important for protein-DNA interaction of SO. There are three nucleotides G, A, A at positions 1, 4, 9, respectively (Fig. 4A lanes 9, 12, 17 and Fig. 4C nt. 1, 4, 9), that appear to be most important for the interaction. These nucleotides show the strongest effects upon mutation and are missing in constructs so10-mutHD and so10-mutGATA as well. These nucleotides are also found in the AREC3/*Six4* binding site. Therefore, there is strong evidence for these nucleotides to be especially important for soAE-mediated reporter gene expression *in vivo* (Fig. 2A,B).

3.3.7. Genome-wide search for potential *sine oculis* target genes

Combining our *in vitro* data on the autoregulatory element with the known *so*-target-sequence of *lz* and the AREC3/*Six4* binding site, we defined the consensus sequence: GTAANYNGANAYC/G necessary for SO-protein binding to DNA. This consensus sequence was taken as a basis for scanning the *Drosophila* genome for similar sites (see experimental procedures). 1632 putative *so* targets emerged from this survey. Out of the affected genes several candidates are already known to be involved in eye development. In the following we will describe two of these genes that we picked for further analysis: *ey* and *hh*.

3.3.8. *eyeless* is a direct target of *so*

The first soAE similar element that caught our attention was located within the previously described eye specific enhancer of the *ey* gene (Czerny et al., 1999; Hauck et al., 1999). A positive feedback loop already has been postulated due to the fact that *ey* is induced in ectopic eye development upon coexpression of *so* and *eya* (Pignoni et al., 1997). Furthermore, the ability of *so* and *eya* to induce ectopic eyes is lost in ey^2 mutants (Pignoni et al., 1997). In ey^2 mutant flies, the previously mentioned eye specific enhancer of *ey* is disrupted by insertion of a transposable element (Quiring et

al., 1994b) (see also Fig. 5A). These experiments genetically show that *so* and *eya* are able to feedback on *ey* and that this feedback loop relies on the eye specific enhancer of the *ey* gene. A direct interaction of *so*, *eya* and the *ey*-enhancer, however, has not been shown yet.

The fact that the potential *so* target site within the eye specific enhancer is perfectly conserved between *D. melanogaster* and *D. pseudoobscura* and two other *Drosophila* species (see Appendix), encouraged us to perform more assays to obtain evidence for a direct interaction.

First we showed that oligos containing this sequence are strong competitors for the binding of SO to soAE in EMSA, whereas this competing potential is lost when the GAT core (Fig. 4C nt. 8-10) of the sequence is mutated (Fig. 4B, *eyeless* and *eyeless* mut).

We then compared the expression pattern of different mutated versions of a 160bp fragment, comprising the eye specific *ey*-enhancer, driving a *LacZ* reporter (sequences shown in Fig. 5A). The wt enhancer mediates expression posterior to the MF, most prominent at the posterior margin (Fig. 5B) (see also Hauck et al. Fig. 4D) (Hauck et al., 1999). By mutating the *Pax6* sites, expression in the eye disc is reduced to the posterior margin (Fig. 5C) (see also Hauck et al. Fig. 4F) (Hauck et al., 1999). Mutating both, the *so* site and the *Pax6* sites further reduces expression in the eye disc (Fig. 5C). The fact, that we still see weak expression at the posterior margin in some of the transgenic lines harboring B4M-SOmut-*LacZ* might be explained by residual activity of the mutated sites or another yet unknown *cis*-acting element within this *ey*-enhancer. Nevertheless, the genetic and *in vitro* data suggest that *so* directly binds the eye specific enhancer of the *ey* gene.

3.3.9. *hh* is a direct target of *so*

hh is a secreted signaling protein that plays an important role in patterning the *Drosophila* eye field. Many lines of evidence suggest that *hh* signaling is required for the initiation and the propagation of the MF. Accordingly, *hh* is expressed at the posterior margin of the eye imaginal disc prior to photoreceptor differentiation and in all cells posterior to the MF during its progression (Borod and Heberlein, 1998). Loss of *hh* function blocks initiation of the MF and impedes its progression (Borod and Heberlein, 1998). Posterior margin clones of a null allele of *smoothened* (*smo*), the cell-autonomous receptor of *hh* signaling, lack differentiated photoreceptors

(Greenwood and Struhl, 1999; Curtiss and Mlodzik, 2000). Conversely, ectopic *hh* expression anterior to the MF gives rise to a progressing ectopic MF (Heberlein et al., 1995; Pignoni and Zipursky, 1997).

so and *eya* also have been shown to be required for initiation and propagation of the MF (Pignoni et al., 1997) and both are expressed at the posterior margin prior to initiation and later in front of the MF (Bonini et al., 1993; Serikaku and O'Tousa, 1994). Furthermore, ectopic MFs are found in ectopic eyes induced by *so* together with *eya* (Pignoni et al., 1997). These data suggest, that a feedback loop between *hh* and *so*/*eya* might influence the proper initiation and propagation of the MF. Consistent with that, *hh* fulfills our criteria to be a putative SO target. Both sites found within the *hh* locus show almost perfect conservation between 7 *Drosophila* species (see Appendix) and are able to compete for SO binding in EMSA (Fig. 4B, *hh* first, *hh* second). In addition, we found these sites to be located within an area that is deleted in the *bar-3* allele. *bar-3* is a weak *hh* allele affecting adult flies. The according deletion can be found in the first intron of the *hh* gene (Mohler, 1988; Lee et al., 1992). The predominant phenotype of *bar-3* is a reduction of eye facets. Therefore the deletion leading to the *bar-3* allele may affect an eye specific enhancer of *hh* (Renfranz and Benzer, 1989). This idea is supported by the observation of Kango-Singh et al. that in *bar-3* mutant flies (hh^1), targeted expression of *ey* fails to induce ectopic eyes (Kango-Singh et al., 2003).

We chose to clone 1.4kb out of the *bar-3* deletion that are encompassing the two *so* sites and again ligated this fragment to the *lacZ* reporter gene. Expression of the resulting *bar-3-LacZ* construct is found exclusively in the eye disc in cells posterior to the MF (Fig. 6B) in perfect agreement with the observation of Lee et al. that *hh* is expressed in differentiating photoreceptor cells (Lee et al., 1992). In a next step we mutated the two SO binding sites by replacement of GAG with CCC and GAT with CCC in the first and the second SO binding site, respectively (Fig. 6A *hh bar-3* SOmut). The resulting construct has lost its capability to induce *LacZ* expression. Only in a few of several observed transgenic lines, weak expression in the same pattern like the wt construct is detectable (Fig. 6C). This residual activity is likely due to a weak interaction of SO with the mutated binding sites similar to what we see in our cell cultutre assays (Fig. 3J). When we test a construct in which the first SO binding site is

deleted and the second is mutated this residual expression is lost (Fig 6A *hh bar-3* Δ5', 6D).

These results show that the two SO binding sites within the first intron of the *hh* gene are functional *in vivo* and sufficient to mediate expression reminiscent of the known *hh* expression pattern in a late 3rd instar eye imaginal disc. This strongly suggests that *hh* is directly regulated by *so*.

3.4. Discussion

3.4.1. *so* autoregulation is essential for ocelli development

so gene activity is crucial for proper development of the entire visual system of *Drosophila melanogaster*, including the larval visual system (Bolwig's organ), the optic lobe, the compound eye and the ocelli. Starting from research that was done on an eye specific enhancer of *so* and identified *ey* and *toy* to regulate *so*-expression, but considering that these factors alone are not sufficient to establish the natural *so*-expression in the ocelli-region, we tried to find additional factors that regulate *so*-expression in ocelli development.

We were able to show that *so* itself, in cooperation with *eya*, forms an autoregulatory feedback-loop. Since auto-regulation can never initiate expression of a gene, the initiation of *so*-expression in the ocelli region must be triggered by other means. Most probably, a dynamic expression pattern of *so* initiated and regulated by *ey* and *toy* can explain, how *so*-activity emerges in a cluster of cells, that later in development gives rise to the ocelli. One first hint is given by the expression dynamics mediated by the so10-enhancer-element: so10, which is activated by *ey* and *toy* in the compound eye field, mediates expression in early 3rd instar larvae all over the eye disc (Punzo et al., 2002). Later in development, however, expression mediated by so10 is lost in the ocellar region. Therefore, initiation of *so* expression in the early 3rd instar stage most probably is mediated by *ey* and *toy* throughout the eye disc including the ocellar precursors. Later, after this first induction by the activity of the so10 enhancer, *so* in the ocelli region can maintain its own expression - cooperatively with *toy* and *eya* - regulating itself.

Due to this consideration, one would expect *so* to be expressed all over the eye disc in 3rd instar stages, which is, however, not the case. The potential of *so* to also mediate gene-repression might explain this observation. It was previously proposed that *so* may function as both, a transcriptional activator and a repressor dependent on the context-specific expression levels of particular cofactors (Silver et al., 2003). In a complex with EYA, SO works as transcriptional activator. In contrast, SO is expected to function as a transcriptional repressor when interacting with the transcriptional corepressor *groucho* (gro) (Silver et al., 2003). Therefore, *eya* and *gro* seem to dominate the quality of a *so*-autoregulatory feedback-loop. This explanation is supported by the fact, that EYA protein can only be found in the ocellar region itself,

no EYA is present in the proximity of these cells. This idea is further strengthened by the fact that *eya*^{4} mutants show an eyeless and ocelliless phenotype (Zimmerman et al., 2000) whereas *gro*^{1}/*gro*^{1} flies have enlarged and partially fused ocelli (Price et al., 1997). Therefore, to elucidate the mechanisms that control *so* expression in ocelli, additional studies on *eya* and *gro* are required.

Nevertheless, *so*/*eya* and *toy* are unlikely to be the only transcription factors that bind to the ocellar specific enhancer of *so*, since the enhancer of the *ey* gene contains binding sites for both factors but *ey* cannot be found to be expressed in the ocellar region. Further studies will need to identify the entire transcriptional machinery that regulates *so* and consequently ask for *eya* regulation to understand the expression pattern of *so* completely.

Amazingly, 4xsoAE mediated expression in the compound eye and in the ocellar area is different despite the fact that cells of both tissues express *so* and *eya*: 4xsoAE-*LacZ* is solely induced in the compound eye. Possibly a co-repressor like *gro* prevents expression driven by 4xsoAE in the ocellar region. When soAE is combined with so10, this co-repressor might be displaced by the formation of a protein complex of SO, EYA and TOY and probably other factors. Therefore it will be interesting to investigate whether such a complex is formed indeed on so7 and so10-soAE.

3.4.2. *so* directly regulates *ey* in eye development

Positioned high up in the hierarchy of the retinal determination network, *ey* is a potent inducer of ectopic eyes and is able to directly induce *so* and *eya*. Like *ey*, *so* and *eya* are able to induce ectopic eyes when coexpressed, *so* alone fails to induce ectopic eyes. To accomplish this induction, *eya* and *so* need to feed back on *ey*, obviously by binding to the eye specific enhancer of *ey*. In an ectopic situation, the feedback of *so*/*eya* on *ey* is strong enough to induce *ey* for ectopic eye formation.

The function of this feedback-loop in normal eye development remains to be elucidated. *so* and *eya* are both expressed posterior to the furrow and are important for neuronal development (Pignoni et al., 1997). Nevertheless, *ey* expression was not detected posterior to the MF. The activity of the *so* site in the *ey* gene might therefore be suppressed by other factors or by *so* itself in this region. As coexpression of *ey*, *so* and *eya* only is eminent in a few cells in front of the MF and within the MF, a possible role for this feedack-loop might be to boost *ey* expression in front of and within the

furrow that consequently leads to a strengthening of *so* and *eya* expression in just a few cell rows.

For proper eye development, a well balanced expression level of the genes belonging to the retinal determination network is crucial. Loss of function mutations as well as overexpression of the eye specification genes *ey*, *eya*, *so* or *dac* during eye development impedes proper determination of the organ and results in reduction of the eye size (Halder et al., 1998a; Curtiss and Mlodzik, 2000). Therefore we further hypothesize a feedback-loop of *so* on *ey* to be also important for the fine tuning of *ey* expression during normal eye development. Due to its ability to activate as well as to repress the expression of genes, *so* is a potent regulator in this context.

3.4.3. Linking the genetic cascade to signal transduction: *so* and *hh*

dpp signaling plays an important role in the complex regulatory network of eye development. In *dpp* mutant eye discs, *eya*, *so* and *dac* signal is absent (Chen et al., 1999), whereas *dpp* is able to initiate ectopic expression of *so* and *dac* when expressed at the anterior margin of the eye disc (Chanut and Heberlein, 1997; Pignoni and Zipursky, 1997). Conversely, *dpp* expression is patchy in eye discs of *eya* and *so* loss-of-function mutants, suggesting that *eya* and *so* are required for either initiation or maintenance of *dpp* at the posterior disc-margin prior to MF initiation (Pignoni et al., 1997; Hazelett et al., 1998).

hh is required for *dpp* expression at the posterior margin prior to MF initiation (Borod and Heberlein, 1998) and *dpp* expression is induced by *hh* in the MF (Heberlein et al., 1993) supporting the assumption that *dpp* is downstream of *hh* signaling. Since *dpp* alone is not able to rescue posterior margin clones of *hh*, there have to be more eye-relevant target genes of *hh* signaling during 3rd instar larval development. *dpp* in combination with *eya* can restore photoreceptor differentiation in posterior margin clones lacking *smoothened* (*smo*) expression (*smo* is a cell-autonomous receptor of *hh* signaling). This proves that *dpp* in combination with *eya* is able to bypass the requirement of *hh* during eye development (Pappu et al., 2003). Taken together, it is evident that *hh* is necessary for proper *eya* and *dpp* expression both of which can induce *so* and it contains two *so* target sites. We therefore hypothesized that the transcriptional complex consisting of EYA and SO, like on *ey*, might feed back on *hh* as well in order to drive the furrow during late eye development. In this model the genetic cascade starts with *hh* that induces *dpp* and *eya*, moves on to *so* and by the

complex SO/EYA feeds back to *hh* in order to maintain *hh* expression as a driving force of the MF.

The impact of these *so* binding sites in the *hh* enhancer on eye development becomes evident from the fact that *bar-3* mutant flies have smaller eyes. The severity of the *bar-3* mutant phenotype probably is diminished by an additional putative SO binding site that resides outside of the area that is covered by the *bar-3* deletion (Fig. 6A SO binding motifs). If functional, this region (5' to the *bar-3* deletion) might mediate a residual *hh*-expression that overcomes the loss of the other sites to some extent. Another possible explanation for the rather weak phenotype of *bar-3* might be the assumption that the feedback of *so* on *hh* is not crucial for MF initiation but still might be of importance for the well balanced expression of *hh* during MF propagation.

3.4.4. A general theme of Six-gene target sites

so belongs to the *Six* gene family. All *Six* proteins are characterized by a *Six*-domain (SD) and a *Six*-type homeodomain (HD), both of which are essential for specific DNA binding and protein-protein interaction. Based on the amino acid sequence of their HD and SD the *Six* genes were divided into three subgroups. Each of the three *Drosophila* homologs can be assigned to one of these subgroups: *so* is mostly related to *Six1/2*, *optix* to *Six3/6* and *DSix4* to *Six4/5* (for review see Kawakami et al.) (Kawakami and Kobayashi, 1998).

Promoter analyses of the mouse *Six*-genes (*Six1*/*2*, *Six4*/5) revealed similar target sequence specificities for these mammalian counterparts of *so*. *Six2*, *Six4/AREC3* and *Six5* effectively bind to the same target sequence in a DNA fragment called ARE (Atpla1 regulatory element) that can be found in the Na,K-ATPase α1 subunit gene (Fig. 4C, ARE fragment) (Suzuki-Yagawa et al., 1992; Kawakami et al., 1996a; Kawakami et al., 1996b; Harris et al., 2000). *Six1* and *Six4* have been shown to bind to MEF3 sites in the myogenin and in the aldolase A muscle-specific (pM) promoters (Fig. 4C, MEF3 site) (Spitz et al., 1998). Recently, mammalian *Six4* has been shown to additionally bind to the transcriptional regulatory element X (TreX) within the Muscle creatine kinase (MCK) enhancer (Fig 4C, Trex) (Himeda et al., 2004).

Comparison of all these sites confirmed the three nucleotides, that we describe to be most important for SO-DNA interaction, to be present and conserved within these motifs (nt. 1, 4 and 9 in Fig. 4C).

In case of the MEF3 site which comprises 7 nucleotides that include only two of the nucleotides important for SO-DNA interaction (nt. 4 and 9 in Fig. 4C), we looked up the original publications to check for the third conserved nucleotide to be also present, and in most of the cases were able to verify its conservation. In fact, there is only one exception published in a study that describes two *Six2* target sites (Brodbeck et al., 2004). Taking into account that SO has been shown to also bind (C/G)GATA *in vitro* and that SO/EYA is able to activate a reporter by acting on the mutated oligomerized soAE sites in S2 cells, it seems that sequences differing slightly from the perfect binding sequence might still be sufficient to establish protein-DNA interaction to some extent. In case of such sequences being clustered, they might still be able to have a biological function. We therefore can not exclude that sequences differing from our consensus, even affecting one of the crucial nucleotides, might still respond to *Six*-genes.

Nevertheless, by combining the vast majority of previous studies describing protein-DNA interaction of *Six* genes and our study about SO-DNA interaction, we infer that SO, *Six1*, *Six2*, *Six4* and *Six5* have very similar properties in binding DNA. In the case of *so*, we propose that the consensus sequence GTAANYNGANAY(C/G) marks a good starting point for finding additional targets of *so* and thereby helps to get more insight into the complex genetic interactions that orchestrate the development of the visual systems of *Drosophila*.

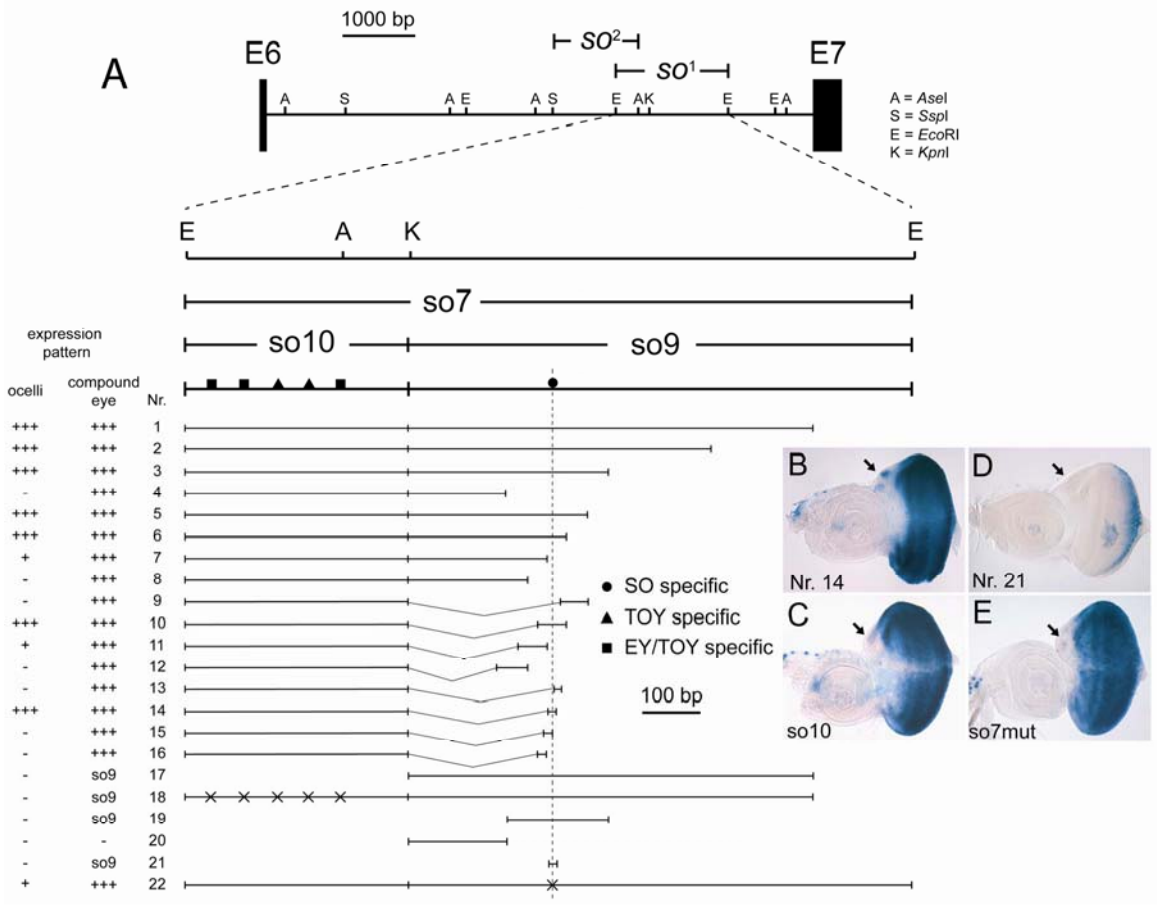


Figure 1. Defining a minimal version of so7 important for ocelli specific *so* expression
(A) Genomic map of the last intron of the *so* locus between exon6 (E6) and exon7 (E7) (black boxes). The physical mapping is indicated as A=*Ase*I, S=*Ssp*I, E=*Ecor*I, K=*Kpn*I.
An enlargement of the region deleted in so^1 shows relative positions of the enhancer fragments so7, so10 and so9. so10 contains EY/TOY and TOY specific binding sites (black boxes and black triangles, respectively). So9 harbours the SO-binding site.
The deletion map illustrates the constructs that where tested for the expression pattern they mediate (X indicates introduced mutations). 27bp fused to so10 (Nr. 14) are sufficient to resemble the so7 mediated pattern including ocelli-expression (B, referred to as +++ expression in ocelli and +++ expression in compound eye). Constructs including so10 but missing the sequence represented by Nr21 just resemble the so10-pattern and show no expression in the ocellar region (C). Constructs that are devoid of functional EY and TOY sites (Nr17, 18, 19 and 21) but include the 27bp of Nr21 show an expression pattern identical to so9 (D, illustrated by the pattern mediated by Nr.21). Fragment Nr21 is sufficient to recapitulate the expression pattern of so9. It is referred to as soAE in the following. Construct Nr.20 (no so10 and no Nr.21) does not mediate any expression at all (-).
(B-E) Arrows indicate the ocellar region. (B) Expression pattern mediated by Nr.14, similar to wt *so* expression pattern and the so7 mediated pattern. (C) so10-LacZ: resembling wt-expression despite the ocellar signal. (D) Nr.21-*LacZ*: expressing only along the posterior margin of the eye disc similar to so9-LacZ. (E) So7mut: so7 where the SO-binding site is mutated mediates only weak expression to the ocellar region (Fragment Nr. 22).

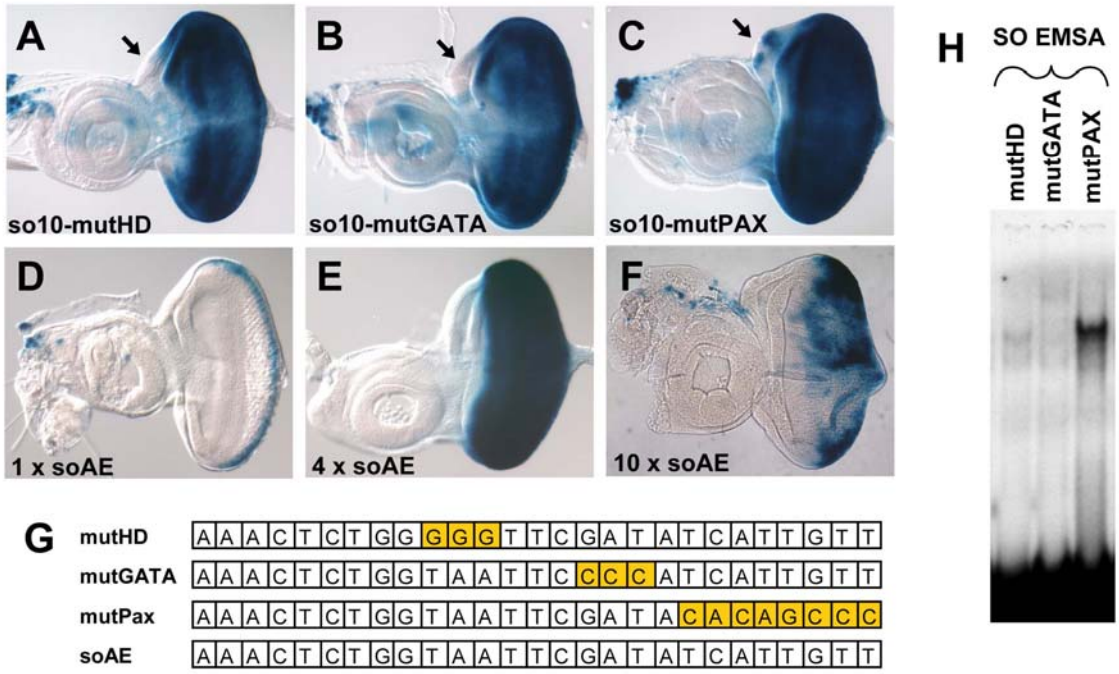


Figure 2. soAE is a direct target of *so*

soAE corresponds to fragment Nr.21 (*sine oculis* autoregulatory element). Arrows indicate the ocellar region.

(A-C) soAE harbours three putative transcription factor binding sites which have been mutated. (A) Mutating the core of the HD recognition sequence (mutHD) or (B) mutating the GATA sequence (mutGATA) abolishes expression of the *LacZ* reporter in the ocellar region. (C) so10-mutPAX in which the putative *Pax6* binding site is mutated, mediates expression indistinguishable from wt / so10-soAE-mediated expression.

(D-F) Oligomerization of soAE boosts its expression. In contrast to soAE alone, which only mediates expression in the posterior margin of the eye disc (D), 4xsoAE drives expression in all cells posterior of the MF, within the MF and in some cells in front of the MF, but not in the ocellar region (E). This expression resembles wt *so*-expression despite the ocellar region. (F) 10xsoAE does not further amplify expression intensity but results in a more blotchy type of expression pattern.

(G) Sequences of soAE and the mutated versions of it. Sequences TAA and GAT of soAE are important for the ocellar specific expression of so10-soAE-*LacZ*.

(F) Radiolabelled probes of mutHD and mutGATA are not shifted by SO in EMSA. In contrast, mutPAX is bound by SO and therefore shifted in EMSA.

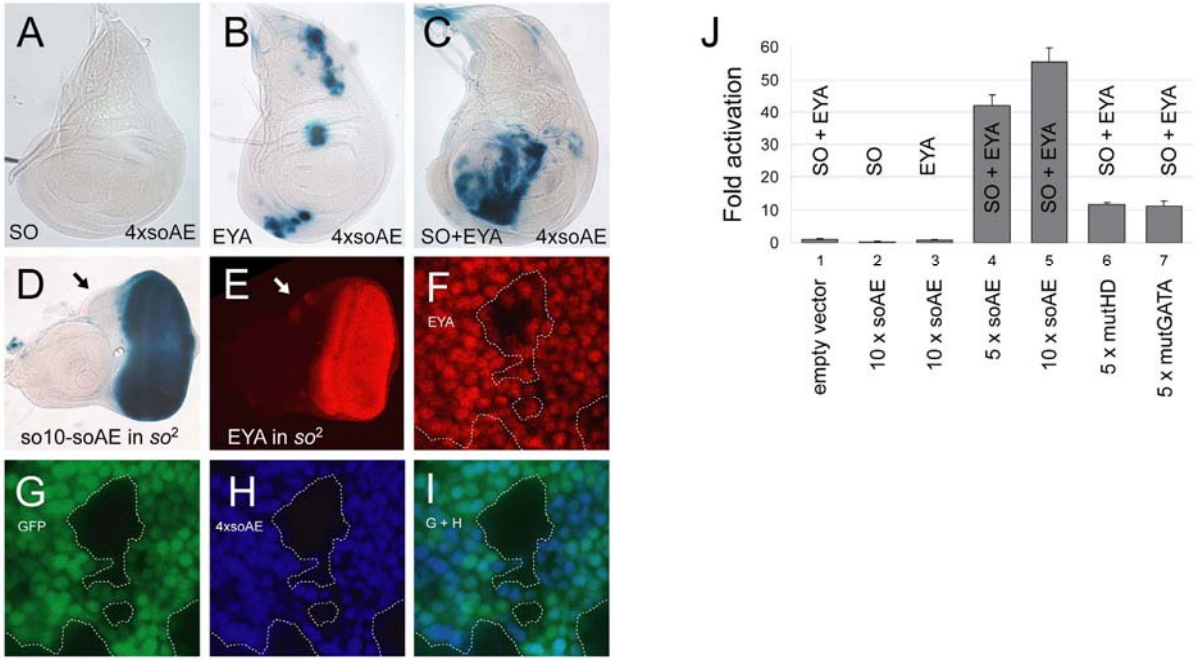


Figure 3. SO acts upon the soAE motif *in vivo* and in S2 cells.
(A-C) 4xsoAE is ectopically induced in wing discs by EYA and EYA+SO protein but not by SO protein alone. (A) *dpp*-GAL4 driving UAS-*so* does not induce 4xsoAE-*LacZ* in wing discs. (B) Ectopic expression of 4xsoAE-*LacZ* is induced in spots along the AP boundary by *dpp*-GAL4:UAS-*eya*. (C) Coexpression of *so* enhances *eya* mediated reporter gene activity.
(D) Ocelli specific expression of so10-soAE is lost in so^2 mutant flies (arrow). so^2 is a regulatory mutant that displays an ocelliless phenotype. (E) EYA protein is detectable in the ocellar region of so^2 mutant flies (arrow).
(F-I) *so* is necessary for soAE activation: In so^3 clones, 4xsoAE-*LacZ* expression is lost although EYA is present in the cells. Clones are delimited by a dashed line. (F) EYA expression is shown in red and marks the non dying cells within the clone. (G) so^3 clones are negatively marked by the absence of GFP expression (in green). (H) 4xsoAE-*LacZ* reporter gene expression (blue) is lost in $so^{-/-}$-cells. (I) overlay of G and H.
(J) β-galactosidase reporter assays in *Drosophila* S2 cells. Reporter plasmids containing the *LacZ* gene under the control of different enhancer fragments were transfected into the cells. Cotransfection of *so* or *eya* alone together with the 10xsoAE-*LacZ* reporter plasmid does not exceed basal activity (lane 2,3). In contrast, coexpression of *so*+*eya* with the 5xsoAE or 10xsoAE reporter, leads to a strong induction of β-galactosidase (lane 4,5). The mutated versions of the 5xsoAE reporter (5xmutHD, 5xmutGATA) still show some amount of induction when cotransfected with *so*+*eya* (lane 6,7). β-galactosidase values were normalized by cotransfecting 5ng of plasmid expressing luciferase as an internal standard. The results represent an average β-galactosidase activity taken from transfections done in triplicates (± standart deviation) and are illustrated as the X-fold activation over the basal activity found for the reporter plasmid alone.

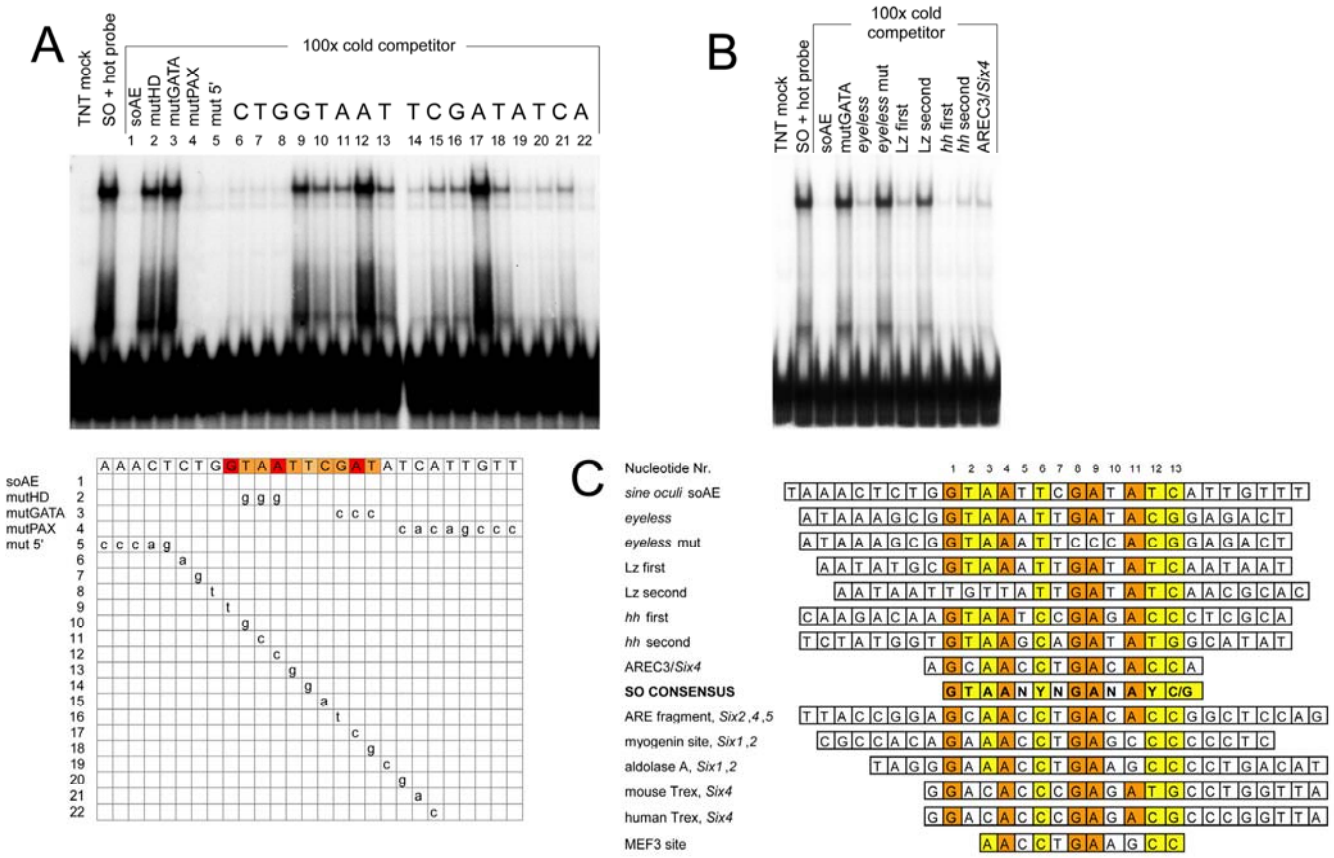


Figure 4. Identification of nucleotides important for SO-DNA (soAE) interaction.
(A) SO protein is shifted by soAE (SO + hot probe) in EMSA. Double stranded probes bearing a single point mutation (6-22) or a stretch of mutations (2-5) were used as cold competitors (100x molar excess) and compared to soAE (1) for their ability to compete for SO-binding. Nucleotides important for protein-DNA interaction are highlighted in red (very important) and orange (important) according to their competing potential. As a control, mock transfected reticulocyte lysate was incubated with p^{32} marked soAE (TNT mock). (B) DNA probes of sequences resembling soAE taken from other genes were used as cold competitors as well. A sequences out of the eye specific enhancer of the *ey* gene is a strong competitor. The fragment loses its binding property when the GAT sequence is mutated to CCC (*eyeless*, *eyeless* mut). Out of the Lozenge gene, only one of the previously described SO binding sites shows a strong competition potential in EMSA (Lz first, Lz second). Strong competing sequences are also found in the first intron of the *hh* gene (*hh* first, *hh* second). SO binding is furthermore strongly competed by the well described AREC3/*Six4* binding site.
(C) Upper half: sequences of the probes that were used as cold competitors in Fig. 4B. Based on these sequences and the results shown in (A) a consensus binding sequence for SO was proposed. Lower half: Previously described binding sites for the vertebrate *Six1*,*2*,*4*,*5*. These sequences appear to be related to the SO binding sequence.

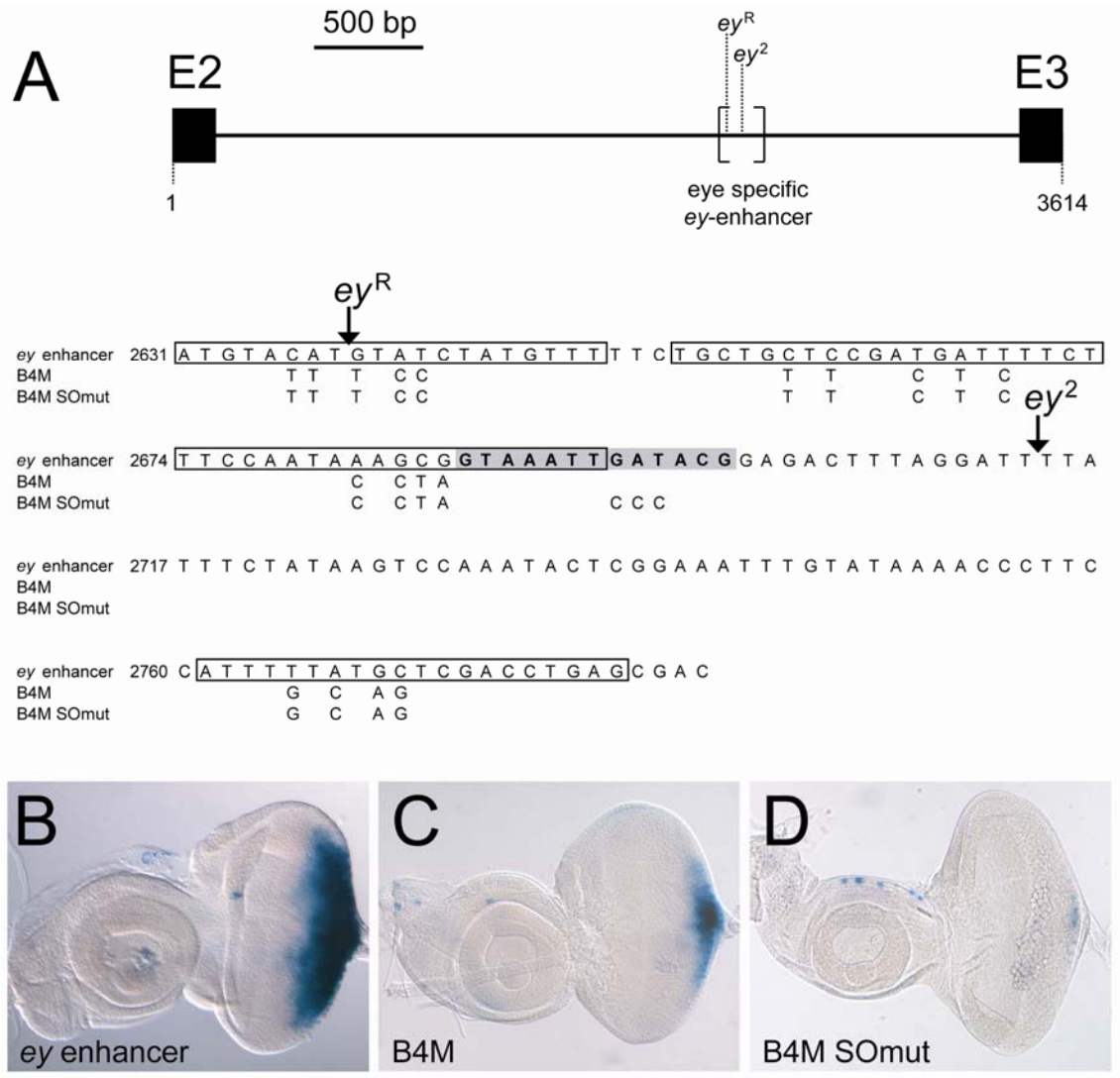


Figure 5 *ey* contains a functional SO binding site
(A) Genomic map of the *ey* locus between exon 2 (E2) and exon 3 (E3) (black boxes). The previously described eye specific *ey*-enhancer is indicated by brackets. Relative positions of transposable elements that interfere with eye development are indicated by their allelic name (ey^R, ey^2). The sequence of the eye specific *ey* enhancer is given below (*ey* enhancer). B4M: the putative *Pax6* binding sites (boxed) were mutated accordingly to Hauck et al. (Hauck et al., 1999) to get rid of *toy*-mediated signal. B4M SOmut: the putative *Pax6* binding sites and additionally the putative SO-binding site (grey shaded) were mutated for comparison to B4M.
(B) *LacZ* expression mediated by the wt *ey*-enhancer fragment in a 3rd instar eye imaginal disc.
(C) B4M-*LacZ* expression: without an influence of TOY protein, due to the mutated sites, the *so*-mediated expression is restricted to a portion of the posterior margin.
(D) B4M SOmut-*LacZ*: Mutating also the *so*-site, expression is reduced to a spot in the center of the posterior margin.

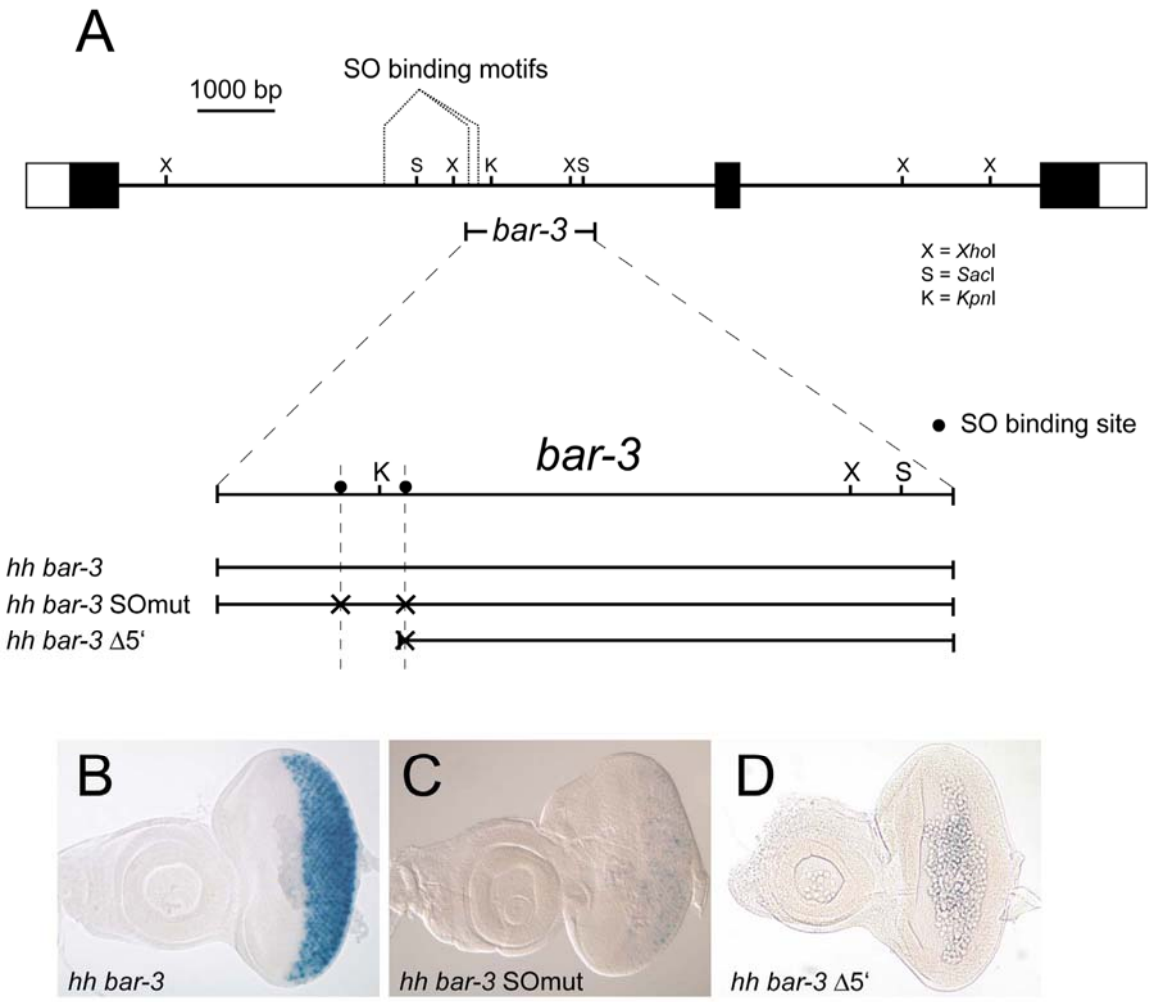


Figure 6 *hh* contains functional SO binding sites
(A) Genomic map of the *hh* locus. The physical mapping is indicated as X=*Xho*I, S=*Sac*I, K=*Kpn*I. The *bar-3* deletion is mapped according to Lee et al. (Lee et al., 1992). Two SO binding sites are located within the region deleted in *bar-3* (Sequences are given in Fig. 4C: *hh* first, *hh* second). One additional SO binding site is found upstream of the *bar-3* deletion.
(B) *hh bar-3*: *LacZ* expression posterior to the MF is mediated by 1.4kb genomic DNA from the region deleted in *bar-3*.
(C) *hh bar-3* SOmut: Expression is hardly detectable when the two SO binding sites are mutated. (*hh* first: GAG is changed to CCC, *hh* second: GAT is changed to CCC)
(D) *hh bar-3* Δ5': No expression is detected from a reporter construct containing only one mutated SO binding site.

3.5. Experimental Procedures

3.5.1. Fly strains and histology

Flies were reared on standard medium at 25°C. Lines used: UAS-*so* (Pignoni et al., 1997), UAS-*eya* (Bonini et al., 1997), so10-*LacZ* (Niimi et al., 1999), so7-*LacZ*, so9-*LacZ*, so10$^{EY+TOYmt}$-*LacZ* (Punzo et al., 2001), *dpp*^{blink}-GAL4 (Staehling-Hampton and Hoffmann, 1994), *ey*-GAL4 (Halder et al., 1998a), *FRT42D*, *so*^{3}*/CyO* (Pignoni et al., 1997). *eyFLP* (Newsome et al., 2000). *FRT42D*, *ubiquitinGFP* (Duchek et al., 2001). *so*^{2}/*so*^{2} (Bloomington Stock centre). Clones of homozygous *so*^{3} mutant cells were generated by the expression of FLP recombinase under the control of an *ey* enhancer.

Specific genotypes generated: (1) *eyFLP*; *FRT42D*, *ubiquitinGFP* (2) *so*^{2}/*so*^{2}; so10-soAE-*LacZ* (3) *so*^{2}/*so*^{2}; so7-*LacZ* (4) UAS-*so*/UAS-*so* ;UAS-*eya*/UAS-*eya*

LacZ reporter plasmids were introduced into w^{1118} by standard P-element transformation procedures. Three to ten independent transgenic lines were established for each construct and tested for expression.

Antibody staining on discs was performed according to Halder et al. (Halder et al., 1998a). Primary antibodies were anti-EyaMab10H6, 1:10 (Bonini et al., 1997), Rabbit anit-β-Galactosidase, 1:500 (Promega). Secondary antibodies used were from Jackson ImmunoResearch Laboratories: Cy5 α-rabbit (1:400), Alexa586 α-mouse (1:400).

To detect β-galactosidase activity, 3rd instar larval imaginal discs were fixed and subjected to a standard X-gal color reaction for 2 hours at 37°C.

3.5.2. Reporter Transgenes

Inserts of the reporter constructs were obtained by PCR, using so7 as a template, and subcloned into the *LacZ* pCβ vector (Niimi et al., 1999).

For the constructs: ey enhancer, B4M and B4M SOmut, the sequences given in Fig. 5 were used. A *Bam*HI and a *Kpn*I site was added at the 5' and 3'-end, respectively, and used for subcloning into the *LacZ* pCβ vector.

The *hh bar-3* sequence was obtained by PCR on genomic DNA of wt flies by using the following primer set: 5'-CTGTGCGCTCGAGTGGGCCACACAGGGTGGG-3'; rightward orientation, 5'-CGGCCCGTCTCAGATCTCGGATCTGAGATC-3' leftward orientation. Mutations were introduced by PCR. For the deletion construct *hh*

bar-3 Δ5', 5'-GGGGTACCCAAGACAAGTAATCCCCCACCCTCGC-3' was used as rightward orientaded primer (the SO site is mutated by changing GAG toCCC).

3.5.3. so^2 mutant

Genomic DNA was amplified by PCR from so^2/so^2 flies and sequenced. The sequences were confirmed on independent amplification events. Genomic DNA isolation was performed according to Bui et al (Bui et al., 2000a). Primers used for mapping the so^2 deletion were: 5'-GAAGGGCACTGCTTACTGAGAGCTCG-3', 5'-GCCCATCGAATCCGCATCTCCCCCAG-3' rightward orientation; 5'-GCGCACACTCGACAAATTTGCGATCTGGC-3' leftward orientation, Primers are located at positions 2355, 3116, 6218 respectively within the last intron. Nuclotides 3983-5181 are deleted in so^2 (the first nt of the last intron is set as 1).
Southern blotting was performed according to Sambrook et al. (Sambrook and Russel, 2001). Genomic DNA was digested using *Cla*I, *Ecor*V and *Xho*I. As probes, DIG labeled PCR products of so10 and so9 were used. so10 and so9 are described previously (Punzo et al., 2002).

3.5.4. Transfections and Reporter Gene Assays

Drosophila S2 cells were maintained in Schneider's insect medium (Invitrogen) supplemented with 10% Fetal Calf Serum and were transfected with the Effectene Transfection Reagent (Qiagen). For reporter gene assays 2 x 10^6 cells were transfected with a total of 200 ng plasmid DNA (20 ng reporter plasmid, 5 ng of a plasmid constitutively expressing firefly luciferase, the indicated amounts of expression plasmids and the parental vector pAc5.1B/V5His to bring the total amount of DNA to 200 ng). Cells were lysated 48h after transfection and lysates were assayed for β-Galactosidase and luciferase activity as described previously (Muller et al., 2003).

3.5.5. Electrophoretic Mobility Shift Assays (EMSA)

Radioactive labeled probes were generated by annealing and filling in partially overlapping oligonucleotides in the presence of (α-^{32}P)ATP. Binding reactions were carried out in 20 μl of 100 mM KCl, 20mM HEPES pH7.9, 20% glycerol, 1mM DTT, 0.3% BSA, 0.01% NP40 containing 10000 cpm probe and 1 μg dIdC. As a protein source, full-length SO protein was synthesized in reticulocyte lysates using the T7

promotor according to the manufacturer specification (Promega). For the binding reaction, 1 µl of a standard 50 µl reaction was used. After incubation for 30 min at 4°C, the reactions were analyzed by non-denaturing 6% polyacrylamide gel electrophoresis followed by autoradiography. For the cold competition experiments, the proteins were first incubated with a 100x molar excess of unlabeled doublestrand oligonucleotides for 10 min. at RT, followed by incubation with the radiolabeled probe at 4°C for 30min.

3.5.6. Computer-assisted search for SO binding sites

Putative *so*-target-genes were identified by screening the entire *Drosophila* genomic sequence with the consensus GTAANYNGANAYS using the program FLY ENHANCER (freely available at http://flyenhancer.org (Markstein et al., 2002)).
Alignements of different Drosophila species were obtained from: http://hanuman.math.berkeley.edu/genomes/drosophila.html

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3.6. Appendix

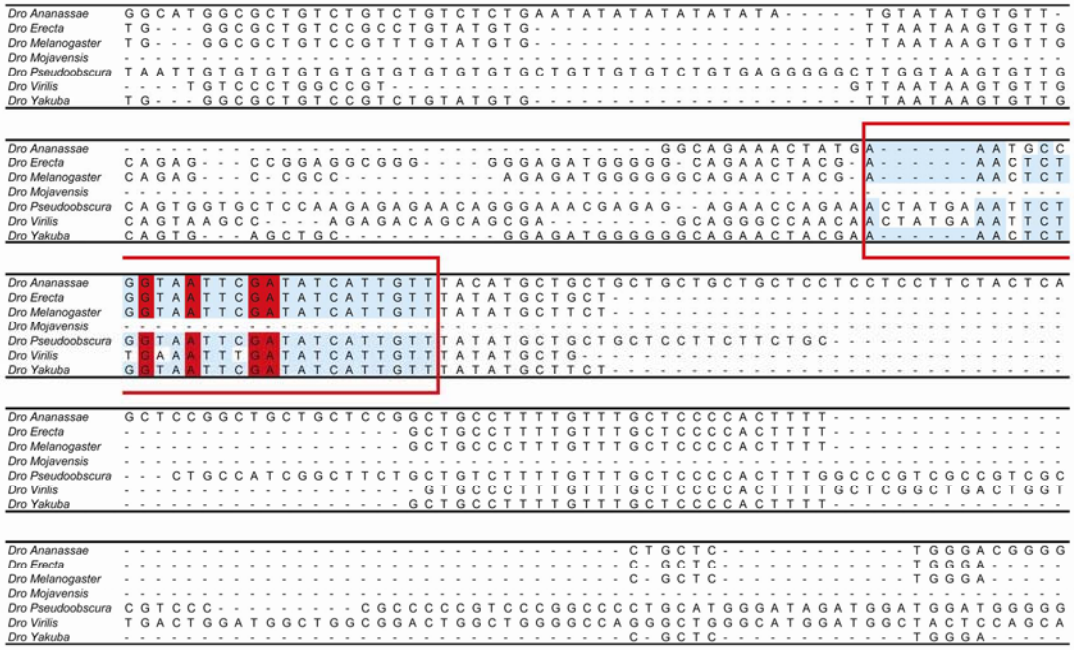

Figure A1 soAE within so7
Alignment of 7 *Drosophila* species. The sequence presented covers soAE (boxed in red) and adjacent sequences within the so7 enhancer fragment of the *so* locus. The most important nucleotides of SO-DNA interaction are shown in red.

Figure A2 Putative SO binding site of the eye specific enhancer of the *eyeless* gene.
Alignment of 7 *Drosophila* species. The most important NTs of SO-DNA interaction are shown in red.

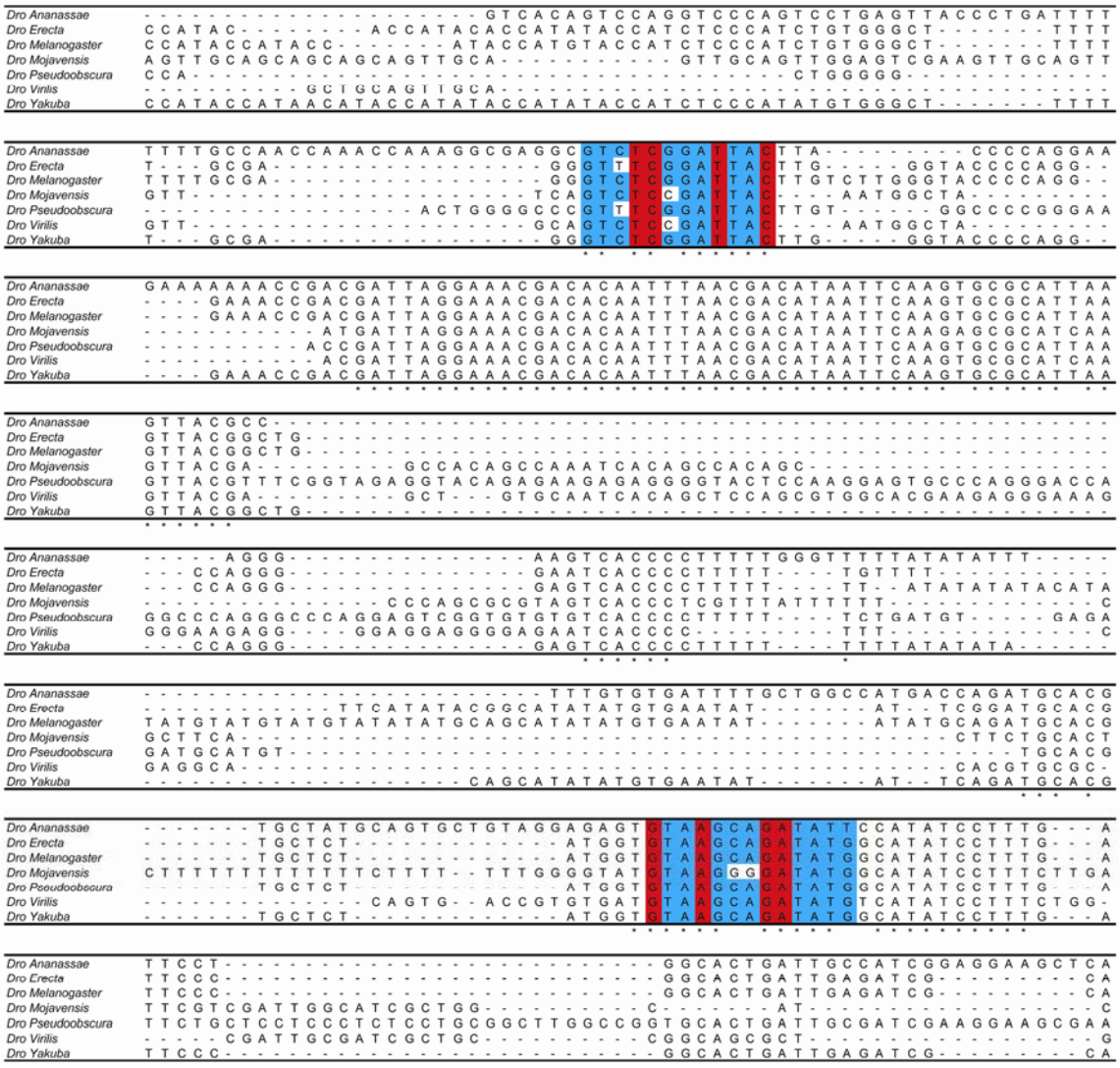

Figure A3 The two putative SO target sites within the *hh* locus
Alignment of 7 *Drosophila* species. The most important NTs of SO-DNA interaction are shown in red. The first putative SO target site is in reverse direction.

All alignments of the Appendix are based on the freely available online tool at:
http://hanuman.math.berkeley.edu/genomes/drosophila.html

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