IDENTIFICATION AND CHARACTERISATION OF PROTEINS THAT INTERACT WITH THE DROSOPHILA TRANSCRIPTION FACTOR MIRROR

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ABSTRACT

Mirror is a homeodomain transcription factor in *Drosophila melanogaster*. The *mirror* gene is part of the Iroquois complex (Iro-C) which also contains *araucan* and *caupolican*. The Iro-C genes have important roles in the development and patterning of the eye and wing imaginal discs as well as the oocyte and embryo. The Iroquois gene products are the founding members of the IRO family of proteins which are characterised by a highly conserved homeodomain of the TALE class (three amino acid loop extension). IRO proteins have been identified in organisms from *C. elegans* to humans and seem to have conserved roles in developmental patterning.

To discover more about how Mirror functions as a transcription factor, the yeast-two-hybrid system was used to screen a *Drosophila* embryonic cDNA library for proteins that interact with Mirror. Several putative interactors were identified including two known transcription factors and a chromatin-remodelling factor as well as a number of previously uncharacterised gene products. A subset of the confirmed yeast-two-hybrid interactors have been investigated for biological relevance by comparing their expression patterns and mutant phenotypes to those of *mirror* as well as through genetic interactions.

Two of the putative Mirror-binding proteins have been studied in detail, the novel forkhead associated (FHA) domain containing protein CG1135 and chromodomain helicase/ATPase DNA-binding protein 1 (CHD1). A P element insertion allele of CG1135 was shown to interact genetically with *mirror* alleles. Characterisation of the CG1135 phenotype revealed a putative role in cell proliferation or survival. The function of CHD1 in *Drosophila* is not known. In order to characterise the role of CHD1 and investigate any interactions with Mirror, a putative dominant negative version was generated and analysed. Studies of the localisation of CHD1 on polytene chromosomes indicate that CHD1 may be associated with transcriptional elongation.
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CHAPTER 1

Introduction

Mirror is a homeodomain containing transcription factor in *Drosophila melanogaster*. The Mirror homeodomain is a member of a class of atypical homeodomains called the TALE (three amino acid loop extension) superclass. Section 1.1 introduces the homeodomain (HD) and the TALE class of HD containing proteins. Mirror is part of the Iroquois complex (Iro-C) in *Drosophila*, which constitutes the founding members of a large family proteins with highly conserved homeodomain sequences. Members of the IRO family of proteins have been found in organisms ranging from *C. elegans* to humans. Section 1.2 introduces the *Drosophila* Iroquois genes and proteins as well as the rest of the IRO family. The Iroquois locus was first identified due to mutations which cause bristle defects in the developing notum. It is now known that the Iro-C genes have many roles during the development of the wing and notum. Section 1.3 describes the expression patterns of the Iro-C genes in the wing disc and what is known about the role of Mirror and the Iro-C in notum and wing development. Mirror was first described as having a role in eye development and later the other Iro-C genes were also found to be essential for proper growth and patterning of the eye. Section 1.4 contains an introduction to eye development and planar polarity in the *Drosophila* eye and the role of the Iro-C genes. Mirror is expressed in the oocyte and in the embryo in dynamic patterns. The role of mirror in oocyte development and during embryogenesis is discussed in section 1.5. In addition to protein sequence homology within the homeodomain, the vertebrate IRO
proteins also have similar functions to the *Drosophila* Iro-C. Section 1.6 outlines some of the functions of vertebrate IRO proteins. Although quite a lot is known about the role of the Iro-C genes in *Drosophila* development, there is very little known about how Mirror regulates gene expression what its targets are. To find out more about how Mirror functions as a transcription factor, the aim of this project was to identify proteins that interact with Mirror. Section 1.7 outlines the aim of the project and some preliminary results.

### 1.1 Homeodomain transcription factors

#### 1.1.1 Introduction to the homeodomain

The homeodomain or homeobox was named after the first genes discovered to contain this domain, the homeotic genes (Gehring *et al.* 1994; McGinnis *et al.* 1984). Homeotic genes are so called because mutations in these genes lead to developmental abnormalities characterised by transformation of one type of structure into the likeness of another type of structure. The classic examples of such transformations include transformation of antenna into leg caused by mutations in the *Antennapedia* gene and transformation of haltere into wing caused by *Ultrabithorax* mutations. The homeotic or Hox genes have since been found to be an ancient genetic mechanism for patterning of the anterior/posterior axis in all bilateral animals (McGinnis and Krumlauf 1992). The homeodomain has also been found in an immense variety of other proteins from yeast, plants and animals. All of these proteins are transcription factors that activate or repress target genes and most are involved in developmental patterning.

Classical homeodomains consist of 60 amino acids and form a helix-turn-helix type DNA-binding motif (Gehring *et al.* 1994). The structures of several homeodomains
and homeodomain-DNA complexes have been solved by NMR spectroscopy and X-ray crystallographic studies. The homeodomain generally consists of an N-terminal flexible peptide, helix I, a six amino acid loop, helix II, a three amino acid turn and helix III/IV (figure 1.1). The three dimensional fold of the domain is held together by a hydrophobic core of 11 highly conserved amino acids. Helix III/IV is the recognition helix and lies in the major groove of the DNA. Residues within helix III/IV make contacts with DNA bases and confer sequence specificity to the interaction. Residue 50 has been shown to be very important for DNA-binding specificity. The homeodomain also makes contacts with bases in the minor groove through the N-terminal flexible arm as well as with the DNA backbone through residues in the first loop and helix II or helix III/IV. The exact residues that make the various DNA contacts differ slightly from homeodomain to homeodomain.

The classical homeodomain sequences have been classified into two superclasses, the Complex Superclass and the Dispersed Superclass (Gehring et al. 1994). This classification is based mainly on the fact that the original homeodomain proteins, the Hox family, exist in chromosomal clusters or complexes. The well conserved Drosophila and vertebrate Hox genes therefore make up the Complex Superclass and all the rest of the homeodomain proteins are part of the Dispersed Superclass. However, there is a lot of divergence within the latter class of homeodomains, both within the homeodomain and with respect to additional domains found in these proteins. The Dispersed Superclass contains at least 16 subclasses or families (Gehring et al. 1994). Some of these families are characterised by their similarity within the homeodomain, such as the even skipped, engrailed, caudal and distalless classes, whereas other families are distinguished by the domains they contain in addition to the homeodomain. For example, the paired class proteins contain a Paired domain in addition to the homeodomain, the LIM class contains the LIM motif and the POU class contains the POU domain.

In addition to the two superclasses of classical homeodomains, there are also some atypical homeodomains which are made up of more than 60 amino acids (Burglin 1997;
Gehring et al. 1994). The first of these to be studied was the MAT α2 mating type gene which has three additional amino acids in the loop between helix I and II. The human LFB1 transcription factor contains an additional 21 amino acids which are found between helix II and III. Structural studies found however that the overall three-dimensional conformation of these domains is still the same as for the typical homeodomains.

1.1.2 The TALE class of homeodomains

The Mirror homeodomain is part of the TALE (three amino acid loop extension) superclass of atypical homeodomains (Bertolino et al. 1995). The yeast MATα2 homeodomain mentioned above was one of the original members of this class in which three extra amino acids are found in the loop between helix I and II. The TALE superclass now consists of four subclasses in animals: PBC, MEIS, TGIF and IRO; two subclasses in plants: KNOX and BEL; and two in yeast and fungi: M-ATYP and CUP (Burglin 1997). Figure 1.1 shows a comparison of a classical homeodomain with representatives of three classes of TALE homeodomains (including Mirror). In addition to the extra amino acids, the loop between helix I and II in the TALE homeodomains is much more conserved than in typical homeodomains. Residues within helix I which are well conserved in typical homeodomains are however not so well conserved within the TALE class. Importantly, residue 50 in helix III is most often a non-polar amino acid such as isoleucine, glycine or alanine. In typical homeodomains this residue which is important for the DNA binding specificity of the domain, is often a polar residue such as glutamine, cysteine, lysine, histidine or serine. The variations in the types of residues present at this critical position was thought to indicate that TALE class homeodomains interact slightly differently with DNA (Burglin 1997).
### Figure 1.1A Homeodomain sequences

<table>
<thead>
<tr>
<th></th>
<th>Helix I</th>
<th>Loop</th>
<th>Helix II</th>
<th>Helix III/IV or III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antp (classical)</td>
<td>FGKQERKRGSQTYERYQTLELEKEFHFN----AVLTRRRRIEIAHALCLTERQIKIFQFRMRKKKKNKTKEFG</td>
<td>1</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>Hth (MEIS)</td>
<td>DASGKKNQKKRGIQPKVAEILLRAWLFQHLYTHPSEPDIQQKLDDTGLTILQVWNSNFNARRRIVQHMDQSNRA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exd (PBC)</td>
<td>RSRFLOARRKREPSKQASEI1NEYFYSRSLPMWPBEEKEELARKGFTVSQVSNWFGKIRKYYKNIGK QEA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mirror (IRO)</td>
<td>SYGMDLNARGKNAATRETTSTKLAMNLKEKKPNPTKEKIMLA1ITKLRTQSTMFANARRRKKEKEMTKEPR</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Figure 1.1B Structure of the Ubx-Exd-DNA complex

![Figure 1.1B](image)

**Figure 1.1A.** Comparison of the homeodomain (HD) sequences of Antennapedia (Antp, classical HD), Homothorax (Hth, MEIS subclass), Extradenticle (Exd, PBC subclass) and Mirror (IRO subclass). Positions of the three helices of the domain are indicated. Residues conserved between the Mirror HD and the other HDs are indicated in red. Boxed residues are conserved between three or more HDs. Residue 50 is in green. **Figure 1.1B.** From Passner et al., 1999. Model representing the Ubx-Exd-DNA ternary structure. Helix III from both Ubx and Exd lie in the major groove and make specific contacts with DNA bases. The YPWM motif of Ubx (N-terminal to the HD) makes contacts with the Exd homeodomain.
Chapter 1

One of the most interesting features of TALE class homeodomain containing proteins is that they interact with typical homeodomain proteins and with each other (Mann and Chan 1996). Biochemical and genetic studies on the mating type genes in yeast found that the atypical MATα2 homeodomain protein interacts with the typical MATα1 homeodomain in mating type switching. The most detailed studies have been performed on the interaction between the PBC class, MEIS class and the Hox proteins. Mutations in the Drosophila gene extradenticle (exd) which is part of the PBC family, was identified as causing developmental transformations similar to those caused by Hox gene mutations (Rauskolb and Wieschaus 1994). It was found that exd controlled the expression of some Hox gene targets without affecting the expression of Hox genes themselves. Therefore it was proposed that Exd was a Hox cofactor. This was later confirmed by studies showing that Exd and Hox bind cooperatively to DNA (Mann and Chan 1996). The mammalian homologues of Exd, PBX1,2 and 3, also bind to DNA cooperatively with mammalian HOX proteins. Using deletion constructs, it was found that the interaction between PBC and HOX proteins depends mainly on the two homeodomains and residues C-terminal to the PBC homeodomain as well as a conserved hexapeptide N-terminal to the HOX homeodomain (Lu and Kamps 1996). Mutagenesis studies also suggested that the loop between helix I and II may be involved in the interaction. The solution of the crystal structure of the UBX-EXD-DNA ternary complex confirmed that the main interactions between the two proteins depend on the conserved YPWM motif N-terminal to the Hox homeodomain which interacts with a hydrophobic pocket in the EXD homeodomain (Passner et al. 1999). Figure 1.1B shows a model of the EXD-UBX-DNA complex.

The function of the interaction of PBC proteins with HOX proteins has been proposed to be two-fold: PBC proteins increase the DNA selectivity of HOX proteins and can change HOX proteins from repressors to activators (Mann and Affolter 1998). The HOX proteins all contain homeodomains with very similar DNA-binding activities in vitro and a long-standing question was how specificity is achieved for different target
genes *in vivo*. The discovery that PBC and HOX proteins bind cooperatively to bipartite DNA sequences suggested that PBC proteins could be cofactors that help HOX proteins to select their targets. PBC proteins can form heterodimers with different HOX proteins and this affects the preferred DNA sequence bound by the complex (Courey 2001). In addition to affecting the DNA binding specificity of HOX proteins, PBC family members have also been shown to affect how HOX proteins affect transcription of target genes. In *Drosophila* it was found that Exd is required to cooperate with the Hox protein Deformed (Dfd) to autoactivate *dfd* expression but not to repress transcription (Pinsonneault *et al.* 1997). It is thought that the N-terminal hexapeptide of the HOX homeodomain may have an inhibitory effect on transcription which is relieved by the interaction with PBC proteins (Chan *et al.* 1996). In addition to interacting with HOX proteins, members of the PBC family also interact with the MEIS class of TALE homeodomain proteins. The *Drosophila* member of this class is *homothorax*, (*hth*) was found to give similar phenotypes to *exc* when mutated and genetic experiments suggested that *hth* was necessary for *exd* function (Rieckhof *et al.* 1997). It was found that the presence of Hth was required for the nuclear localisation of Exd. Studies of this interaction in cell culture found that in the absence of Hth, Exd is exported from the nucleus and the interaction between Hth and Exd is necessary for the nuclear localisation of both proteins (Berthelsen *et al.* 1999). Hth is highly homologous to its mammalian counterparts the Meis proteins and Prep1. Both Meis1 and Prep 1 can interact with Exd and cause nuclear localisation in vivo (Berthelsen *et al.* 1999; Rieckhof *et al.* 1997). Mammalian PBX proteins have been shown to interact with the Meis proteins and bind DNA cooperatively at a combination site. A PBX-Prep1 heterodimer is part of the UEF3 (urokinase enhancer factor-3) transcription factor. The interaction between PBC and MEIS proteins does not depend on DNA and is mediated by conserved motifs in the N-terminal parts of both proteins, the PBCA domain of PBX/Exd and the HM/HR domain of Meis/Prep1/Hth (Mann and Affolter 1998). As PBC interacts with HOX and MEIS via
different domains it raises the possibility of the formation of trimeric complexes. HOX-PBC-MEIS complexes have been identified in vivo. In *Drosophila*, Hth was found to bind to DNA together with a Exd-Labial (Lab) heterodimer and formation of this complex was found to be essential for the activity of the lab48/95 enhancer in vivo (Ryoo et al. 1999). A complex of Prepl, Pbx1, and Hoxb1 was found to be important for the regulation of rhombomere r4-specific expression of Hoxb1 and Hoxb2 in mouse (Ferretti et al. 2000).

No interactions between members of the IRO family and HOX proteins or other TALE proteins have been reported. It is interesting to note however that within the TALE superclass, the IRO family, although quite divergent, is most closely related to the PBC class (Burglin 1997). It is therefore possible that the IRO proteins could interact with other homeodomains in a similar way to how the PBC proteins interact with HOX homeodomains. As the similarity between IRO and PBC proteins is limited to the homeodomain, the interaction involving other domains such as the interaction with MEIS proteins would not be expected to be conserved.

1.2 Introduction to the Iroquois family of proteins

1.2.1 The IRO protein family

The three founding members of the IRO family are the gene products of the Iroquois complex (see below) in *Drosophila*: Mirror, Araucan (Ara) and Caupolican (Caup) (Cavodeassi et al. 2001). The similarity between the Mirror, Ara and Caup proteins is illustrated in figure 1.2. The homeodomains of the three proteins are nearly identical (figure 1.3A). In addition, there are several other regions of homology including an N-terminal EGF-like motif, an acidic domain which follows the homeodomain, and a novel motif called the IRO-box (Burglin 1997) (figure 1.3B). The Ara and Caup proteins
share a higher degree of homology (41% overall identity), whereas the Mirror protein is more divergent (27% overall identity with Ara).

Many IRO family proteins have been identified in other organisms. So far one IRO protein has been identified in *C. elegans*, three in *Xenopus*, three in zebrafish, five in chick and six in both mouse and humans (Gomez-Skarmeta and Modolell 2002). The *Xenopus* and zebrafish IRO proteins are abbreviated Xiro and Ziro, whereas the chick, mouse and human IRO proteins are abbreviated Irx (or IRX). The IRO proteins are numbered according to which of the other IRO proteins they are homologous to. For example, the Xiro4 protein is most homologous to the chick, mouse and human Irx4 proteins. Some examples of vertebrate IRO proteins are illustrated in figure 1.2. The homology between the *Drosophila* and vertebrate homologues is limited to the homeodomain and the IRO-box (figure 1.3). However, most IRO proteins contain a region of acidic residues following the homeodomain, even if the exact composition of this acidic domain is not conserved. In addition, the distance between the homeodomain and the IRO-box is similar in all IRO proteins despite differences in the total number of amino acids. The vertebrate IRO proteins are much more similar to each other than to the *Drosophila* proteins.
Figure 1.2 Schematic diagrams of the *Drosophila* IRO proteins (Mirror, Ara and Caup) as well as some vertebrate representatives (c=chick, m=mouse, h=human, X=Xenopus, Z=zebrafish). The IRO proteins are highly homologous within the homeodomain (HD) and the Iro Box (I). In addition all IRO proteins contain an acidic domain (A). The *Drosophila* proteins contain an EGF-like domain (N), proline rich (P) and glutamine rich (Q) motifs. The *Drosophila* proteins are more similar to each other than to the vertebrate proteins.
Figure 1.3. Comparison of the homeodomain and Iro box from IRO proteins

A

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<tr>
<th></th>
<th>helix I</th>
<th>helix II</th>
<th>helix III</th>
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<td></td>
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B

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<tr>
<td>Mirror</td>
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<tr>
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Figure 1.3 A shows the comparison of homeodomains from various IRO proteins and B shows a comparison of Iro box sequences (h= human, m=mouse, X= Xenupus, c= chick, Z= zebrafish).
1.2.2 Genomic organisation of the Iro genes

As with the original homeobox genes, the *Drosophila* Iroquois genes *araucan*, *caupolican* and *mirror* are part of a gene complex (Gomez-Skarmeta *et al.* 1996; McNeill *et al.* 1997). The three genes are referred to collectively as the Iroquois complex or the Iro-C. The name Iroquois was given to the locus as the first mutations analysed lead to loss of all lateral bristles on the fly notum, leaving a band of bristles in the centre of the notum resembling the hairstyle of the Iroquois American Indians (Leyns *et al.* 1996). The three Iro-C genes are found within a ~140 kb region on 3L (Netter *et al.* 1998). The distance between the *ara* and *caup* genes is less than 25kb whereas *mirror* is found over 70kb downstream of *caup*. This organisation reflects the degree of similarity between the gene products as well as the similarity in expression patterns. As will be discussed in the following sections, the expression patterns of *ara* and *caup* are essentially identical. The *mirror* expression pattern overlaps with *ara* and *caup* in many cases, but *mirror* is also uniquely expressed in some areas during development, especially during early stages of embryonic development. In addition, *ara* and *caup* are also uniquely expressed for example in parts of the wing disc. The close proximity of the genes and the similarity in their expression patterns indicate that *ara* and *caup* are controlled by the same enhancer elements (Cavodeassi *et al.* 2001). As *mirror* expression overlaps with *ara* and *caup* in many areas, part of these enhancers probably also act on *mirror*.

In addition to the protein sequence homology, the genomic organisation of the Iroquois genes also seems to be conserved between species (Gomez-Skarmeta and Modolell 2002). The six *Irx* genes in mouse and humans are found in two genomic clusters of three genes each, just like the *Drosophila* genes (Ogura *et al.* 2001; Peters *et al.* 2000). The mammalian clusters consist of *Irx1*, *Irx2* and *Irx4* in one complex (cluster A) and *Irx3*, *Irx5* and *Irx6* in the other complex (cluster B). The two groups were probably created by a duplication of an ancestral cluster as the members of each cluster are most
similar to the Irx gene in the same position in the other cluster (ie Irx1~Irx3, Irx2~Irx5 and Irx4~Irx6). Although fewer Iro genes have been found in chick and zebrafish, mapping of existing genes indicated that they are also found in two clusters (Ogura et al. 2001; Wang et al. 2001).

The presence of Iro-complexes in other organisms could be taken to suggest that all the Iro genes are derived from a common three gene ancestral cluster, but this is probably not the case (Gomez-Skarmeta and Modolell 2002). As the vertebrate IRO proteins are more related to each other than the Drosophila versions, it seems that the duplications within the Iro gene clusters happened independently in the Drosophila and vertebrate lineages. In addition, the orientation of transcription of genes within the clusters varies from vertebrates to Drosophila. It is still intriguing however, that both Drosophila and vertebrate complexes happen to contain three genes.

As mentioned above, the close proximity of the Iro-C genes in Drosophila allows them to be controlled by shared regulatory elements leading to overlapping expression. Iro genes in several vertebrate species have been shown to have overlapping expression patterns (Cavodeassi et al. 2001). Therefore, shared enhancers may also exist for vertebrate Iro clusters and this may be one reason why the gene complexes have persisted in both Drosophila and vertebrates. It is interesting to note that the expression patterns of the first two genes in the mouse clusters are almost identical whereas in many tissues the third gene has a more divergent expression pattern (Houweling et al. 2001). This is reminiscent of the situation in the Drosophila Iro complex. Even more intriguing is the fact both Drosophila and vertebrate Iro genes can be positively regulated by Wnt signalling and negatively regulated by TGFβ signalling (see sections below) hinting that parts of the regulatory mechanisms as well as genomic organisation could be conserved.
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1.2.3 Shared functions within the IRO protein family

Vertebrate and *Drosophila* IRO proteins have been proposed to have similar functions during development (Cavodeassi *et al.* 2001). The *Drosophila* proteins seem to have three main functions: specification of large territories by acting as selector genes, establishment of organising centres at borders of Iro-C expression and subdivision or further specification of distinct structures. So far the roles of vertebrate IRO proteins seem to indicate that they act in similar ways during vertebrate development. IRO proteins are certainly involved in the specification of large territories during *Xenopus* and *Zebrafish* development and probably also in chick and mouse. They are also important in subdivision of territories to specify distinct identities. Although borders of IRO protein expression have been suggested to have organising activities in vertebrates, it is not clear if they function in the same way as the *Drosophila* organising centres. An important function of IRO proteins in flies is the generation of domains with different cell affinities. It is not known if the vertebrate IRO proteins have the same function, but hints from over-expression experiments in chick (see section 1.6) suggest that they may. So in addition to homology within the homeodomain and conserved genomic organisation, it seems that the IRO proteins have similar functions in *Drosophila* and vertebrates.

The existence of several proteins with very similar sequences suggests that they may function redundantly. High degrees of similarity between Mirror, Ara and Caup are restricted to specific regions. However, the fact that the DNA binding domain is nearly identical and that they are expressed in similar patterns, indicates that they may have overlapping and/or redundant functions (Cavodeassi *et al.* 2001). Mutations which only affect *ara* or *caup* do not exist so it is not known if they can substitute for each other. Alleles of *mirror* have similar, but milder, phenotypes to the *Iro* alleles that affect the whole Iro-C. In addition, ectopic expression of any of the three Iro-C proteins produces the same phenotypes. IRO proteins in vertebrates may also act redundantly. The only loss
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of function study of an individual Irx gene showed that other Irx genes could substitute for Irx4 during heart development. Experiments in zebrafish using morpholinos also uncovered redundant functions of Ziro1 and Ziro7. The role of the *Drosophila* IRO proteins will be presented in detail in sections 1.3-1.5 and vertebrate IRO proteins are discussed in section 1.6.

1.3 The role of Mirror and the IroC in notum and wing development

1.3.1 Development of the *Drosophila* wing and notum

The wing imaginal disc gives rise to the adult wing as well as the dorsal mesothorax or notum and the ventral thorax or pleura (Garcia-Bellido *et al.* 1973). Like all imaginal discs, it is a single layer epithelium and develops from about 20 cells in the embryo into a highly folded structure of about 75,000 cells in the late third instar. As the disc develops into two very different structures, wing and notum, an early patterning event involves the subdivision of the disc into notum and wing territories (Klein 2001). It has recently been found that the early subdivision of the wing disc is accomplished by activation of the epidermal growth factor receptor (EGFR) in the notum part of the disc and the expression of *wingless* (*wg*) in the wing part of the disc (Klein 2001). The EGFR is activated by the ligand Vein, and this represses expression of *wg* in the notum region. In addition, *wg* represses *vein* in the wing region leading to the establishment of reciprocal expression domains. Wingless activates the expression of wing specific genes such as *nubbin* (*nub*), *vestigial* (*vg*) and *scalloped* (*sd*) to specify the wing pouch (the part of the disc that forms the wing blade and margin) (Ng *et al.* 1996).

Once the wing and notum domains have been delineated, the wing region is divided into the wing blade at the centre and the wing hinge at the periphery of the disc.
The hinge region can be distinguished by the restricted expression of the transcription factors *homothorax* (*hth*) and *teashirt* (*tsh*), which are both required for proper development of the hinge (Klein 2001). The expression of these genes suppresses the formation of the wing pouch by repressing *vg* expression. In addition, *vg* suppresses the expression of *hth*, leading to the subdivision of the wing field into wing blade and wing hinge regions. Both the notum and wing regions of the disc are then further patterned by subdivision into smaller territories.

The patterning of the wing pouch of the disc is quite well understood and involves another set of subdivisions of the wing disc, namely the dorsal and ventral (D/V) and anterior and posterior (A/P) compartments. The A and P compartments are determined in the embryonic anlagen of the wing disc (Cohen 1993), whereas the D and V compartments are established through the activation of EGFR signalling in the notum (Zecca and Struhl 2002a; Zecca and Struhl 2002b). At the boundaries between the D/V and A/P compartments signalling centres are established which control the growth and patterning of the wing (Cohen 1996). The dorsal compartment is characterised by the expression of the transcription factor Apterous. Apterous controls the expression of the Notch ligand Serrate and the regulator Fringe and this leads to the activation of Notch signalling at the D/V boundary. Notch signalling induces the expression of *vg* and later *wg*, which act to pattern the wing field and the wing margin. The posterior compartment is characterised by the expression of the transcription factor *engrailed* and the secreted protein Hedgehog (Hh) (Brook 2000). Hh acts on adjacent anterior cells through the transcription factor *cubitus interruptus* (*ci*) and induces the expression of *decapentaplegic* (*dpp*) at the A/P boundary. Dpp then acts as a long-range morphogen to control growth and patterning of the wing blade. The wing blade is further divided into even smaller territories to delimit where the wing veins and sensory organs will form. This further subdivision is achieved by the interaction between signalling pathways at the D/V and A/P boundaries (Cohen 1996).
Less is known about the further patterning of the wing hinge region and the notum. The wing hinge is further subdivided into proximal, intermediate and distal domains, but the mechanisms involved are not well understood (Klein 2001). The notum is further divided into medial and lateral domains (Calleja et al. 1996). In addition, the specification of the adult sensory organs, the macrochaete, requires the pre-patterning of the notum into areas where pro-neural clusters will form (Calleja et al. 2002). The Iro-C genes are involved in several steps during the patterning of the wing disc. First of all, they are important for specifying the notum during early disc development. The Iro-C is also important for the patterning of the wing hinge region, and the development of the alula. In addition, they are involved in the subdivision of the notum into medial and lateral domains as well as the pre-patterning of the sensory organs in the lateral notum. Finally, *araucan* and *caupolican* are involved in patterning of a subset of wing veins.

### 1.3.2 The Iro-C are required for the specification of the lateral notum

The Iro-C genes have a role in the early subdivision of the wing disc (Calleja et al. 2000; Diez del Corral et al. 1999). In second instar discs the Iro-C is expressed in the proximal wing disc which defines the notum territory (Diez del Corral et al. 1999) (figure 1.4). The early expression of Iro-C genes is induced by activation of the EGFR by the ligand Vein (Zecca and Stuhl 2002a; Zecca and Struhl 2002b). EGFR activity is required to maintain Iro-C expression in this territory. Ectopic activation of the EGFR signalling pathway through expression of activated forms of EGRF and Ras can induce Iro-C expression in other parts of the wing disc. As described above, the activation of EGFR is restricted by *wg* expression in the distal wing disc, but *wg* is not required to restrict Iro-C expression (Cavodeassi et al. 2002). Instead, it seems that Dpp, which is expressed at the A/P compartment boundary is responsible for delimiting Iro-C expression to the notum.
region. In the third instar, Iro-C expression is repressed in the proximal most part of the
disc which will become the medial notum (figure 1.4). Dpp signalling seems to be
involved in this repression as well, which is mediated by the *pannier* transcription factor.

The establishment of the early Iro-C expression domain is essential for notum
specification (Diez del Corral *et al.* 1999). Clones of cells homozygous for an Iro-C
deletion in the notum leads to a change in cell fate from notum to wing hinge. In addition,
clones of the *mirror* allele *mir*\(^{E48}\), also causes malformations in the lateral notum that
contain ectopic hinge structures. When clones were induced during the first and second
instar, they were always associated with malformations, whereas clones induced later in
development, only caused malformations of the lateral notum. This corresponds to the
restriction of Iro-C expression to the lateral notum during the third instar stage. Although
the Iro-C genes are required for notum specification, they can not induce a notum fate in
other regions of the disc. Ectopic expression of any of the Iro-C proteins in the wing
pouch leads to removal of the dorsal hinge territory and malformations of the wing, but
not the development of notum structures.
Figure 1.4 The Iro-C in the wing imaginal disc

Figure 1.4. A is taken from Diez del Corral et al, 1999 and shows the expression of Caup in the notum part of the second instar wing discs (antibody staining in red). B shows the expression of the mirror-laZ enhancer trap in the third instar wing disc (H. McNeill). C shows a fly of the genotype mirr<sup>p</sup>/mirr<sup>p2</sup> (I. Dahlsveen). Note the lack of some of the lateral bristles and the held-out wings.
The restricted expression of the Iro-C in the notum may help to establish a border between notum and wing regions which is maintained through differences in cell affinities. Although the border between notum and wing regions in the disc is not a cell lineage border like the compartment boundaries, a difference in cell affinity between the two populations of cells would keep them separate. Several lines of evidence suggest that cells that express Iro-C genes have different cell affinities to surrounding cells. Clones of cells that lack or have reduced Iro-C function have smooth borders and look more rounded that clones of wild type tissue (Diez del Corral et al. 1999; Yang et al. 1999). Clones that ectopically express any of the Iro-C proteins in areas where they are not normally expressed, are also round and have smooth borders (Calleja et al. 2000; Zecca and Struhl 2002a). In the lateral notum part of the wing disc, Iro-C clones are surrounded by a visible fold and in the adult present as invaginating cuticle vesicles. This suggests that the apposition of Iro-C expressing and non-expressing cells can lead to changes in morphology possibly due to cell adhesion differences. The border between the notum and wing hinge regions of the third instar disc is marked by a fold, and the Iro-C expression domain ends directly adjacent to this fold (Diez del Corral et al. 1999). Clones of Iro-C deletions that are generated close to the notum-hinge fold cause the fold to be re-routed around the non-expressing cells. The above results suggest that the Iro-C are important for keeping the notum and hinge regions of the disc separate by creating regions of different cell affinities.

1.3.3 The Iro-C is important for the development of the wing hinge region

As well as the role in early subdivision of the wing disc, the Iro-C is also important for the development of the wing hinge region. Many of the Iro-C and mirror alleles available are regulatory mutants that exhibit lower levels of Iro-C or mirror expression (Kehl et al. 1998; Leyns et al. 1996). Some combinations of these alleles are viable and
display varying degrees of the characteristic Iroquois phenotype of missing lateral bristles. In addition, some of these flies have held-out wings which lack several hinge structures (figure 1.4). These flies also lack the alula, a balancing organ associated with the hinge. Mirror and the other Iro-C proteins are expressed in the area of the wing disc which give rise to the alula. Loss of mirror expression from this region leads to loss of the adult structure, indicating that the Iro proteins have a direct role in the specification of the alula (Kehl et al. 1998). On the other hand, the Iro-C genes are not expressed in the wing hinge region of the disc, so their role in the development of this region is indirect.

The border of the Iro-C expression between the notum and the wing region may establish a signalling centre that patterns the proximal hinge region and notum (Diez del Corral et al. 1999), (Klein 2001). This would explain how Iro-C mutations can affect a region in which the Iro-C genes are not expressed. Clones of cells lacking Iro-C function have non-autonomous effects in the notum. In the adult, Iro-C clones caused changes in surrounding wild type tissue and recruited wild type cells to form ectopic hinge structures (Diez del Corral et al. 1999). Non-autonomous effects could also be observed in the disc. Iro-C clones in the notum cause the induction of the hinge region marker l(2)09261 and derepression of tsh in neighbouring wild type cells. These non-autonomous effects indicate that the Iro-C expression border can affect neighbouring tissues. As will be described below, the Iro-C is involved in establishing a signalling centre in the eye disc by creating a fringe expression border (similar to the D/V compartment boundary generated by apterous). It is not know if Notch signalling is involved in any signalling events at the notum-hinge border in the wing disc.

1.3.4 The Iro-C in the specification of mechanosensory organs and wing veins

The Iro-C is required for the development of specific adult structures within the notum and wing (Gomez-Skarmeta et al. 1996; Kehl et al. 1998; Leyns et al. 1996). As
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described above, the Iro-C genes were named after a phenotype which caused the loss of all lateral bristles on the notum (Leyns et al. 1996). The bristles, which are mechanosensory organs, appear in 11 fixed positions in each hemi-notum (figure 1.5). They are made up of four cells which form the four components of the bristle; the sensory neuron, the sheath cell and the bristle shaft and socket. These four cells originate from a single precursor cell, the sensory mother cell (SMC). Each SMC develops from a proneural cluster in the wing disc. These clusters are marked by the presence of the proneural genes achaete (ac) and scute (sc). In order to generate the specific pattern of bristles in the adult, the expression of ac and sc is regulated by a network of positional clues which all feed into an array of enhancer elements which are specific for a subset of the proneural clusters (Calleja et al. 2002). The pre-pattern of transcription factors that make up the positional clues are probably controlled by overlapping signalling pathways such as the Wg, Hh and Dpp pathways.

The Iro-C genes are important for the development of 8 of the 11 macrochaetae in each hemi-notum as combinations of some Iro alleles leads to loss or reduced levels of these bristles (Leyns et al. 1996). Other combinations of Iro-C alleles however, affect a subset of these bristles to varying degrees. Hypomorphic alleles of mirror lead to the loss of a specific subset of bristles which are indicated in figure 1.5 (Kehl et al. 1998), (I. Dahlsveen unpublished). In addition to interactions within the Iro-C complex, the Iro-C genes also interact genetically with other genes to affect the bristle pattern. Interactions between Iro alleles and components of the Hh and Dpp signalling pathways have been observed to affect specific bristles suggesting that the Iro-C either interacts with or is downstream of these pathways in bristle specification (Fujise et al. 2001; Leyns et al. 1996).
Figure 1.5 Bristles in the lateral notum affected by Iro-C mutations

Figure 1.5 Drawing (from FlyBase) and picture of the notum indicating the positions of the eight lateral bristles affected by Iro-C mutations (PS, presutural; aNP, anterior notopleural; pNP, posterior notopleural; aSA, anterior supraalar; pSA, posterior supraalar; pPA, posterior postalar; aSC, anterior scutellar). Bristles affected by mirror mutations are shown in red.
The loss of lateral macrochaetae in Iro-C mutants is due to loss of the corresponding proneural clusters and SMCs in the wing disc (Kehl et al. 1998; Leyns et al. 1996) (Gomez-Skarmeta et al. 1996). Reduction in Iro-C expression leads to reduced ac-sc expression in the lateral notum. Only proneural clusters that lie within the Iro-C expression domain are affected, indicating that the Iro-C controls ac-sc expression autonomously. Ara and Caup have been suggested to control the expression of ac-sc directly. DNAse protection assays using Ara protein found that it bound to DNA within an enhancer upstream of sc. Moreover, lacZ expression controlled by this enhancer was affected by Ara and Caup expression whereas a mutated enhancer showed no effect on lacZ expression. Although it has not been demonstrated, it is likely that Mirror also controls proneural formation by controlling ac-sc expression.

In addition determining the positions of the bristles, the Iro-C are also important for defining their lateral identity (Grillenzoni et al. 1998). The sensory neurons of bristles in the medial and lateral notum send axons that follow different paths towards the CNS. Axons of medial neurons cross the CNS midline, whereas the axons from lateral neurons do not. If mechanosenrory organs are induced in a Iro mutant background (by ectopic expression of sc), the neurons innervating these bristles adopt a medial fate even though they are found in the lateral notum. The specification of lateral identity in bristles may be a result of the proposed general role of the Iro-C in specifying lateral domains in various parts of the body (Calleja et al. 2000).

Araucan and Caupolican are important for the development of wing veins and are expressed in the regions of the wing pouch that give rise to veins L1, L3 and L5 (Gomez-Skarmeta et al. 1996). The expression of Ara and Caup in the wing pouch has been shown to be under the control of signals emanating from both the A/P and D/V compartment boundaries (Gomez-Skarmeta and Modolell 1996). High levels of Hh signalling, as well as the presence of Dpp, are required to induce ara and caup expression in the L3 territory. ara and caup expression is negatively regulated by Wg at the D/V boundary where there is
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a gap in the expression. Engrailed and an as yet unidentified factor delimit the posterior and anterior borders of the L3 expression pattern respectively.

Araucan and Caupolican are important for the formation of both vein material and sensilla in the adult wing. Mitotic clones that lack Iro-C function lead to loss of vein material from vein L5, L3 and L1 as well as loss of the L3 campaniform sensilla, sensilla of the anterior cross vein and the twin sensilla of the wing margin (TSM). The loss of sensory organs in these areas, as in the notum, were found to be due to the loss of ac-sc expression. Ectopic expression of Ara leads to the accumulation of ectopic vein material. The areas of the wing veins are delimited by the expression of rhomboid. In the disc, the ectopic Ara expression caused an expansion of the rhomboid (rho) expression domain indicating that the Iro-C may control the specification of wing veins through controlling the expression of rho.

1.4 The role of Mirror and the IroC in eye development

1.4.1 Introduction to Drosophila eye development

The *Drosophila* adult eye is made up of about 800 units called ommatidia, which contain eight photoreceptor cells, called R1-8, as well as cone cells and pigment cells (Wolff and Ready 1993, figure 1.6). *Drosophila* eyes develop from two eye-antennal imaginal discs which give rise to most of the adult head. The eye part of the disc forms the eye and most of the head capsule and the antennal part forms the antenna, rostral membrane and maxillary palpus (Cavodeassi et al. 2000). The cells that make up the eye-antennal disc are specified during embryogenesis and the disc develops as a monolayer epithelium during larval stages (Wolff and Ready 1993). The eye and antennal parts of the disc appear morphologically different early in development, but eye or antennal fate is not pre-determined and depends on a number of signalling cascades and restricted expression
of transcription factors. Until recently it was thought that the specification of the eye field was completely under the control of the so-called “master control” genes: *twin of eyeless* (*toy*), *eyeless* (*ey*), *eyes absent* (*eya*), *sine oculis* (*so*), *dachshund* (*dac*), and *eye gone* (*eyg*) (Treisman and Heberlein 1998). The absence of any of these genes leads to reduction or loss of the eye and ectopic expression can induce ectopic eyes in other imaginal discs (except *so*). However, ectopic eyes can only be induced in specific parts of other imaginal discs suggesting that the presence (or absence) of other factors is necessary to allow eye development. The seven genes encode nuclear factors and form a complex regulatory network which involves both regulation of each others expression and direct protein-protein interactions. The Pax6 homologues *toy* and *ey* seem to be at the top of the hierarchy as expression of these genes occurs first in the eye-antennal disc primordium. However, specification of eye versus antennal fate apparently occurs much later in development.

The EGFR and Notch signalling pathways seem to be upstream of the master control genes for eye development in specifying the difference between eye and antennal fates (Kumar 2001 figure 1.7). Activation of the EGFR is required for the subdivision of the wing disc into notum and wing regions and it seems that it is also important for subdivision of the eye-antennal disc. Hyperactivation of the EGFR as well as expression of some of the downstream components of the pathway, leads to the transformation of eye into antenna. In addition, inactivation of the Notch signalling pathway also induces this transformation. The transformation to antennal fate is accompanied by the loss of expression of *toy*, *ey*, *eya*, *os*, and *eyg* suggesting that EGFR and Notch signalling repress and activate these genes respectively. The critical time for specification of eye versus antennal fates seems to be during the second larval instar. This is also the time at which the expression of all the “master control” begin to overlap at the posterior portion of the eye disc (Kumar and Moses 2001).
Figure 1.6 The *Drosophila* adult eye

Figure 1.6 Figure courtesy of Helen McNeill. A) Scanning electron microscopy picture of the Drosophila adult eye showing the external structure of the ~800 ommatidia and the inter-ommatidial bristles. B) Section through adult eye (dorsal is up) showing the trapezoid arrangement of the rhabdomeres. The ommatidia in the dorsal half of the eye have opposing polarity and chirality compared to those in the ventral half. C) Schematic of an individual dorsal ommatidium indicating the positions of each of the photoreceptors.
Investigations into the specification of antennal fate has been mostly focused on the specification of antenna versus leg and not the distinction between the antennal and eye fates. Early patterning events in antennal and leg discs are similar involving the establishment of reciprocally exclusive domains of Wg and Dpp expression (Theisen et al. 1996). Accumulation of both Wg and Dpp activates the expression of distalless (dll) in both discs. However, the presence of homothorax (hth) in the antennal disc ensures the specification of antennal versus leg fates (Dong et al. 2000). The results described above suggest that activation of the EGFR pathway as well as the absence of Notch signalling is necessary to specify antennal versus eye fate. This could involve the regulation of one or more of the genes mentioned above (figure 1.7).

Early patterning of the eye disc involves establishment of the dorso-ventral and anterior-posterior axis (Treisman and Heberlein 1998. figure 1.7). During early development, possibly before the second larval instar, the eye disc is divided into dorsal and ventral compartments. There has been some debate concerning the characterisation of the dorsal and ventral regions in the eye disc as true compartments. Although there is a general restriction for clones to cross the D/V border, progeny from a single cell can give rise to ommatidia in both the dorsal and ventral halves of the eye. However, the discovery of the restricted expression of factors in the dorsal and ventral halves of the disc indicates that they are indeed true compartments. As with the wing disc, growth and patterning of the eye disc depends on the establishment of a signalling centre at the boundary of the D and V compartments. In the eye disc the D/V compartment boundary is also marked by a boundary of Fringe expression (Cho and Choi 1998; Dominguez and de Celis 1998). Fringe is a glycosyltransferase and is expressed in the ventral half of the eye. Fringe affects the interactions of the Notch receptor with its ligands Delta and Serrate and this causes activation of Notch signalling in a narrow band around the midline. The Iro-C is essential for the D/V patterning of the eye and this will be discussed in detail below.
Figure 1.7 Models for early and late patterning of the eye disc

**A)** From Kumar and Moses 2001, EGFR and Notch signaling pathways are suggested to be upstream of the “master control” genes for eye development in the specification of eye versus antennal fate. Notch activation induces eye development and represses antennal development and EGFR activation represses eye development and induces antennal development.

**B)** From Cho and Choi, 1998. The third instar eye disc is patterned in the anterior-posterior and the dorso-ventral axis. Wg and Dpp divide the disc into anterior and posterior regions. Mirror and the Iro-C are involved in setting up the D/V boundary which becomes a signaling center. The intersection of Notch activity at the midline with the posterior margin leads to localised activation of Hh and the differentiation of photoreceptors.
The eye disc is also patterned in the anterior-posterior axis although there is no A/P compartment boundary (Treisman and Heberlein 1998). As mentioned above, the posterior part of the disc is where differentiation of the photoreceptors of the eye will begin. This part of the disc is marked by the expression of the eye specification genes and by Dpp expression at the margin. The most anterior parts of the eye disc will give rise to the dorsal and ventral head capsule (Pichaud and Casares 2000). In the third instar, this part of the disc is marked by the expression of Wg and Homothorax. Both Wg and Hh are upstream of the Iro-C in D/V patterning (see below) and the establishment of mutually exclusive Wg and Dpp domains is important for A/P patterning. (Treisman and Heberlein 1998, figure 1.7).

The differentiation of photoreceptors is marked by an indentation called the morphogenetic furrow (MF) which moves across the disc from posterior to anterior (Treisman and Heberlein 1998, figure 1.8). The MF is induced at the posterior margin of the disc where it is intercepted by the D/V boundary. The initiation of differentiation is dependent on the presence of Dpp and Notch signalling and is marked by the localised upregulation of Hh. The progression of the furrow is dependent on continuous Hh signalling. As the furrow moves across the disc, the differentiating photoreceptors express Hh which induces Dpp in cells within the furrow. Neuronal differentiation is a prerequisite for Hh expression ensuring that the movement of the furrow is linked to the progression of photoreceptor specification. Hh and Dpp signalling control the rate of furrow progression by inducing proneural genes just ahead of the furrow and antineural genes in a region more anterior to the furrow where signalling activity is lower. In addition to the morphological changes occurring in the MF, the progression of the furrow is also marked by synchronisation of the cell cycle (Baker 2001). In response to Dpp signalling, cells just anterior to the furrow are arrested in G1. The differentiation of photoreceptors is thereby also coordinated with regulation of the cell cycle.
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Figure 1.8 The morphogenetic furrow and photoreceptor development

Figure 1.8 Figure courtesy of Helen McNeill. Top panel: The morphogenetic furrow is initiated at the posterior margin and moves anteriorly. Photoreceptor differentiation starts within the furrow with the specification of the R8 cell. Bottom panel: The development and rotation of photoreceptor clusters. The five cell preclusters consist of the R8 (yellow), the R3/4 pair (green) and the R2/5 pair (blue). As the preclusters emerge from the morphogenetic furrow (black bar) they start to rotate in the opposite direction on each side of the midline (arrow). After completing mitosis, the R1/6 (brown) and R7 (red) photoreceptors are recruited to the cluster.
The first photoreceptor to be specified is the R8 cell which is responsible for recruitment of the rest of the cells to the photoreceptor cluster (Frankfort and Mardon 2002). Specification of the R8 cell depends on the proneural gene *atona1 (ato)*. High levels of Hh and Dpp signalling from the furrow induces the expression of the proneural gene *ato* in a stripe just ahead of the MF. Expression of *atona1* is first restricted to small groups of proneural cells within the furrow and is further refined to single cells through lateral inhibition by the Notch pathway. This leads to the regular spacing of R8 cells which are required for the induction of the rest of the cells in each ommatidium. Recruitment of cells is dependent on cell-cell interactions and EGFR signalling from the R8 cell via the ligand Spitz (Freeman 1997). The first cells to be recruited are the R2-R5 pair and the R3-R4 pair which with the R8 cell makes up the pre-cluster. Cell in the pre-cluster stay arrested in G1 and start neuronal differentiation. The rest of the cells undergo a last mitosis, called the second mitotic wave. Further cells are then recruited starting with the R1 and R6 pair. Once the photoreceptor cells are specified, they start differentiating and express markers that can be used to identify each cell in the developing cluster. EGFR activation is also required for the recruitment of the four cone cells.

The final stages of eye development occur during the pupal stage and involves the recruitment of pigment cells and the final differentiation of all the cell types within the ommatidium (Wolff and Ready 1993). The pigment cells are also specified in a stepwise fashion, surrounding the photoreceptors and cone cells to insulate each ommatidium. Final differentiation includes the production of rhabdomeres in the photoreceptors, secretion of lens material by the cone cells and primary pigment cells as well as production of the inter-ommatidial bristles.
1.4.2 Planar polarity in the eye

The Drosophila eye is divided into two fields of opposing polarity which meet at the equator or midline of the eye (Reifegerste and Moses 1999). The dorsal and ventral margins of the eye are referred to as the poles. The ommatidia in the dorsal and ventral halves of the eye exhibit opposite polarity and chirality. In sections of the adult eye, the polarity of the ommatidium is visualised by the positions of the rhabdomers from each photoreceptor, which are arranged in a stereotypical trapezoid shape (figure 1.6). In this arrangement the R3 photoreceptor cell is always furthest away from the equator and anterior within the cluster. This arrangement of the photoreceptors is specified as the clusters are developing behind the MF. Figure 1.8 shows a schematic diagram of the developing photoreceptor clusters in the dorsal and ventral halves of the eye. Initially, the clusters on either side of the ommatidia look symmetrical. As the clusters mature and recruit more cells, they start to rotate first to 45° and then to 90°. Clusters in the dorsal and ventral halves of the eye rotate in opposite directions.

The development of planar polarity in the eye is linked to the early subdivision of the disc into dorsal and ventral territories (Reifegerste and Moses 1999). The restriction of fringe expression to the ventral half of the eye and the activation of Notch signalling at the midline is vital for the initiation of photoreceptor differentiation and for the establishment of polarity. A current model predicts the presence of a polarising signal which forms a gradient from the midline to the poles (Axelrod and McNeill, 2002). It is hypothesised that this polarity gradient is dependent on Notch activation. The polarising signal is interpreted by the precluster such that the member of the presumptive R3/R4 pair closest to the equator is specified as the R3 cell. This directs the rotation of the cluster away from the equator and leads to the final position of the R3 cell at the top of the trapezoid.

The identity of the polarity signal is not known. However, a large number of proteins and pathways have been implicated in the interpretation of the signal. The planar
cell polarity signalling pathway through the frizzled receptor seems to be important for establishing the first asymmetries within the R3/R4 pair (Tomlinson and Struhl 1999). The cell which has the highest frizzled activity (and is closest to the source of the polarity signal) becomes the R3 cell. In addition, the Notch signalling pathway is involved reinforcing the specification of the R4 cell (Cooper and Bray 1999). High levels of Frizzled (Fz) signalling in the R3 cell leads to increased Delta expression. This in turn causes upregulation of Notch signalling in the R4 cell which is essential for the proper direction of rotation. Many proteins that contribute to the Fz and Notch signalling pathways have been shown to affect planar polarity in the eye such as Prickle-spiny legs (Pk-sple) and Barbu for Notch signalling, and Flamingo, Strabismus and Dishevelled for Fz signalling (Axelrod and McNeill, 2002). It has been proposed that RhoA and the JNK pathway may be downstream of Fz in interpreting the polarity signal. In addition, several other membrane bound and cytoplasmic proteins have been shown to affect polarity although their exact function has yet to be determined. The final step of polarity determination is the execution of the rotation which is controlled by at least two proteins, Nemo and Roulette (Choi and Benzer 1994).

1.4.3 Mirror and the IroC are expressed in the dorsal half of the developing eye disc

As mentioned at the beginning of the introduction, mirror was first discovered due to an enhancer trap which is only expressed in the dorsal half of the developing eye (McNeill et al. 1997). This expression pattern was later confirmed by in situ hybridisation and staining with Mirror antibodies (Kehl et al. 1998; McNeill et al. 1997; Yang et al. 1999). Figure 1.9 shows the expression of Mirror protein in the third instar eye disc. Mirror is also expressed in a restricted “spot” in the antennal disc. The expression of the other members of the Iro-C, Araucan and Caupolican, is also restricted to the dorsal half
of the eye disc (Dominguez and de Celis 1998). All three Iro proteins seem to show the highest levels of expression at the dorsal anterior part of the eye disc anterior to the morphogenetic furrow. Behind the furrow, the Iro-C are expressed in a subset of the differentiating photoreceptors (see below).

Expression of the Iro-C genes in the eye disc has been shown to be downstream of Wg and Hh signalling (Cavodeassi et al. 1999; Lee and Treisman 2001). Both wg and hh are expressed in the dorsal half of the eye disc at the beginning of the second larval instar. Loss of pathway components in the dorsal part of the disc leads to loss of Iro-C expression whereas activation of the pathway using shaggy mutations leads to ectopic expression. This indicates that Wg signalling is required to activate expression of the three Iro-C genes. Clones of cells in which Hh signalling is impaired also lead to loss of Iro-C expression dorsally. In addition early expression of Hh in the whole eye disc using an eyeless Gal4 driver, leads to dorsalisation of the eye field. This indicates that Hh signalling can also activate Iro-C expression. Pannier (Pnr), which is involved in the repression of Iro-C expression in the wing disc, has been proposed to be upstream of Wg signalling in the eye disc (Maurel-Zaffran and Treisman 2000). Loss of Pnr expression also leads to loss of mirror expression and ectopic expression of Pnr leads to ectopic mirror.

It has been proposed that the dorsal restriction of the Iro-C expression might be obtained through dorsal Wg and Hh signalling only (Cavodeassi et al. 1999). As high levels of these signalling molecules would only be present in the dorsal part of the eye, this could be enough to restrict Iro-C expression. However, it is difficult to see how diffusible signals alone could create an abrupt border of expression. In addition, Wg is expressed in the ventral margin of the disc in at the beginning of the third larval instar, but this does not lead to ventral Iro-C expression. Genetic interaction studies using the original Iro-C enhancer trap elements found that expression of the white gene could be derepressed in the ventral half by mutations in the Polycomb (PcG) group genes (Netter et al, 1998) The PcG
proteins are involved in the maintenance of a repressed state for many homeotic genes. This suggests that the expression of the enhancer trap was initially repressed in the ventral half of the eye and that PcG proteins are required to maintain this repression. The JAK/STAT pathway may be involved in the repression of Iro-C expression (Zeidler et al. 1999). The ligand, Unpaired (Upd) is expressed at the midline in the most posterior part of the disc. Loss of Upd leads to expansion of mirror expression. The repression of mirror by Upd may be important for establishing a very tight border of expression at the posterior margin where positioning of the midline is crucial for the beginning of photoreceptor differentiation and polarisation.

1.4.4 Mirror and the Iro-C have a dual role in dorso-ventral patterning of the eye disc

Early loss of function experiments in the eye using mirror alleles indicated that mirror was important for the formation of the equator (McNeill et al. 1997). Clones that lack mirror function lead to non-autonomous reversal of ommatidial polarity on the equatorial side of the clone. In addition, clones that are induced close to the equator redirect the equator along the clonal border. The gene was named mirror as the opposition of mirror expressing and non-expressing cells was a border of mirror-image symmetry. Clones lacking the whole Iro-C complex are much more efficient at inducing reversals in polarity than clones of mirror alone, indicating that the Iro-C genes act together to establish the equator. The polarity defects associated with mirror and Iro-C clones can be detected in adult eyes and in the imaginal disc (figures 1.9 and 1.10).
Figure 1.9 Mirror expression and mirror phenotypes in the eye

Figure 1.9. All pictures by Helen McNeill. A) Mirror expression pattern (green) in the third instar eye-antennal disc. Mirror protein is found in the dorsal half of the eye disc and expression ends at the midline (arrow-head). B) The Mirror (green) and Fringe (red) expression patterns in the third instar disc are complementary and meet at the equator (arrow head). C) Clones of mirror (marked by the absence of pigment) in the dorsal half of the eye have smooth borders. D) Clones of mirror lead to the generation of ectopic equators and reversal of ommatidial polarity.
Figure 1.10 Iro-C clones show polarity defects in the eye disc

Figure 1.10 Confocal images of third instar eye discs containing Iro-C clones (larvae of the genotype eyFLP; mvh, iro^{DF3D} 80 FRT/ GFP 80 FRT). Dorsal is up, the furrow is marked by an arrow and the midline is marked by an arrow-head. The discs are stained with α-GFP antibodies to visualise the clones (A) and α-BarH1 to visualise the polarity of the ommatidia (B) (GFP in green and BarH1 in red in the merged image, C). BarH1 is a marker for R1 and R6 (Hayashi and Saigo 2001) and reveals the orientation of the photoreceptor clusters. Clones which lack Iro-C function cause the reorientation of clusters around the equatorial border only in the dorsal half of the disc. Also note that the Iro-C clones seem to have smoother borders in the dorsal half compared to the ventral half.
The Iro-C is essential for the establishment of the dorsal compartment and for D/V patterning in the eye disc. Expression of the Iro-C in the dorsal half of the eye restricts the expression of Fringe to the ventral half of the eye allowing the activation of Notch at the midline (Cho and Choi 1998; Dominguez and de Celis 1998). Ectopic expression of any of the Iro-C proteins in the eye disc causes repression of Fringe expression. Combinations of mirror alleles that lead to reduction of mirror expression in the eye, cause dorsal derepression of Fringe (Yang et al. 1999). Loss of Iro-C in clones in the dorsal half of the eye also leads to derepression of fringe expression (Cavodeassi et al. 1999). The establishment of the Fringe expression border is essential for the initiation of photoreceptor development. The localised expression of Hh at the posterior margin marks the position at which differentiation will begin. General over-expression of Ara (and loss of the Fringe border) blocks the activation of Hh expression and the differentiation of the photoreceptors (Cavodeassi et al. 1999).

In addition to the repression of Fringe, the Iro-C also act to establish the D/V boundary by conferring different affinities to cells of the dorsal compartment (Cavodeassi et al. 1999; Yang et al. 1999). Clones lacking mirror or Iro-C expression in the dorsal half of the eye have smooth borders minimising contacts between expressing and non-expressing cells. Clones in the ventral half, where the Iro-C genes are not expressed, have "wiggly" borders. However, clones of cells over-expressing Ara in the ventral compartment also have smooth borders. Establishing compartments with distinct cell adhesion properties is important for maintaining the boundary and in this case would help to ensure a very tight line of Notch activity at the midline. Different cell affinities between Iro-C expressing and non-expressing cells have also been described in the wing disc.

The loss of Iro-C expression in clones in the dorsal half of the eye causes the reversal of planar polarity through the establishment of an ectopic Fringe expression border. If the clones are in contact with the eye disc margin, the clones can cause the formation of ectopic eye fields (Cavodeassi et al. 1999; Cavodeassi et al. 2000). This is
proposed to be due to the intersection of a stripe of Notch activation (due to the border of fringe expression) with the posterior margin. The ectopic eye fields include both mutant and wild type cells and the equator is coincident with the clonal border. The fact that a border of Iro-C expression is sufficient to induce the growth and patterning of secondary eye fields demonstrates the importance of the Iro-C in eye development.

1.4.5 The Iro-C proteins act as dorsal selectors in the Drosophila head

In addition to their roles as dorsal selectors in the eye, the Iro-C genes also have a role in establishing the dorsal head territory (Cavodeassi et al. 2000; Pichaud and Casares 2000). Clones lacking Iro-C function cause the transformation of the dorsal head capsule to ventral structures. As described above, Iro-C clones induced early in development can cause the formation of ectopic eyes in the dorsal head. These ectopic eye structures are often associated with transformation of dorsal structures into ventral ones like ptilinum, suborbital bristles and prefrons. Later induced clones of Iro-C which did not generate ectopic or enlarged eyes, could also cause transformation into ventral head structures. Dorsal structures such as orbital and ocellar bristles as well as the ocelli themselves were missing from these clones. Ectopic eyes generated by Iro-C clones contain both mutant and wild type tissue. However, transformation of head structures into ventral fate was autonomous to the cells lacking Iro-C expression. This indicates that the Iro-C genes are important for specifying dorsal fate as opposed to ventral fate in the head.

Wg is involved in the specification of head structures as opposed to the eye field (Treisman and Heberlein 1998). As expression of the Iro-C is downstream of Wg, these genes could be mediating the effect of Wg in the dorsal half of the eye. In the ventral half of the eye, Homothorax (Hth) has a similar role in specifying ventral head structures as opposed to dorsal ones (Pichaud and Casares 2000). Clones lacking Hth function generate ectopic eyes ventrally and transformation from ventral to dorsal structures. In the ventral
anterior region of the disc Hth and Wg are part of a positive feedback loop regulating each others expression. The Iro-C genes could have an analogous role to Hth in the dorsal anterior region in specifying dorsal fate. Although Iro-C expression is induced by Wg, there is no evidence that the Iro-C genes can induce Wg expression. In fact, it has been reported that loss of Iro-C can lead to upregulation of Wg in some areas (Cavodeassi et al. 2000). The exact mechanisms by which the Iro-C genes specify dorsal fate have therefore yet to be determined.

In addition to the induction of ectopic eyes and transformation of dorsal into ventral structures, loss of Iro-C expression can also induce the formation of ectopic antennae (Cavodeassi et al. 2000). The ectopic antennae were always associated with ectopic eyes and appeared as mirror images of the extant structures with conserved D/V arrangement. The ectopic eye and antennal development could also be seen in the imaginal disc. Dorso-anterior Iro-C clones that touched the disc margin gave rise to large overgrowths in the disc which by looking at Wg expression, could be discerned as ectopic eyes and antennae. It was proposed that the appearance of ectopic antennae was due to upregulation of Distalless in the Iro-C clones. The presence of Wg and Dpp (from the ectopic eye) would act to induce Dll expression and in the presence of Hth this could cause differentiation of antennal structures. It has been shown that ectopic expression of Dll and Hth using the Dpp-Gal4 driver can induce antennal development in the eye-head part of the eye-antennal disc (Dong et al. 2000). However, some combinations of mirror mutations which cause reduction in the size of the eye disc, can also produce duplications of the antennal disc (H. McNeill, unpublished). In this case the antennal duplications are not associated with the generation of ectopic organising centres and Dpp expression. It is therefore possible that the Iro-C genes are important for specification of the dorsal eye-head field as opposed to the antennal field.
1.4.6 Characterisation of the expression of Mirror behind the morphogenetic furrow

As part of this project, the distribution of Mirror protein behind the morphogenetic furrow has been studied in detail. Early reports of the expression patterns of Mirror and Caup indicated that in addition to generalised expression in the dorsal compartment, the proteins were also expressed in a subset of cells behind the MF. By using markers for different photoreceptor subtypes, Mirror expression could be pinpointed to a few developing photoreceptors (figures 1.11 and 1.12). Using the marker Dachshund, which is expressed in the furrow and in R1 and R6 (Mardon et al. 1994), it can be deduced that Mirror is probably expressed in the R2, R5, R3, and R4 cells of the pre-cluster a few columns from the furrow (figure 1.11A). The levels of Mirror expression seem to be higher in the R3 and R4 cells. Spalt protein is expressed in the R3 and R4 cells as they are recruited to the cluster and later in the cone cells (Barrio et al. 1999). Co-staining using Mirror and Spalt antibodies show that Mirror starts to be expressed in the R3 and R4 cells as Spalt expression is fading (figure 1.11B). In more mature clusters towards the posterior margin of the disc, the expression of Mirror seems to resolve to be higher in one of the two cells. Using a reporter construct line for the expression of Enhancer of split mδ in the R4 cell (Cooper and Bray 1999), high levels of Mirror expression were found to coincide with the R4 cell marker (figure 1.12). The mδ enhancer construct is also expressed at low levels in the R7 cell (Cooper and Bray 2000). Mirror expression seemed to coincide with the mδ expression in these cells as well. Co-staining with the R7 marker Pospeso (Kauffmann et al. 1996) indicated that Mirror was expressed in some of the R7 cells (not shown). In summary, Mirror is expressed early in the pre-cluster and expression is later resolved to be high in R4 and low in R3. In addition Mirror may be expressed in the R7 cell.
Figure 1.11 Mirror is expressed in a subset of photoreceptors

A) Co-staining using Mirror (red) and Dachshund (green) antibodies. Dachshund marks the morphogenetic furrow (arrow) and photoreceptors R1 and R6. Mirror expression seems to be restricted to R3, R4, R2 and R5 just behind the furrow, and later becomes restricted to R3 and R4. Note the increased levels of Mirror in one cell of the R3/R4 pair further away from the furrow (arrow head).

B) Co-staining using Mirror (green) and Spalt (red) antibodies. Spalt marks the R3 and R4 cells. Mirror protein is not present in the R3/R4 pair just after the furrow (arrow), but seems to start being expressed in these cells as the Spalt expression is fading.
Figure 1.12 Mirror expression resolves to be high in R4 and lower in R3

Confocal images of third instar eye discs showing expression of the mδ enhancer trap (anti-β-galactosidase staining) in red and Mirror expression in green (anti-Mirror staining). The mδ enhancer trap is expressed at high levels in R4 and at low levels in R7. High levels of Mirror expression coincides with the mδ β-galactosidase expression in the R4 cell. In addition, Mirror expression may also overlap with mδ expression in the R7 cell.
The role of Mirror behind the morphogenetic furrow is not known. The known functions of the Iro-C genes in establishment of the D/V boundary and planar polarity are due to the early expression in the dorsal compartment and the repression of Fringe. Clones of Mirror in the adult eye cause only non-autonomous effects on polarity, whereas development within the clone is wild type indicating that Mirror is not required for normal ommatidial development. It is however intriguing that Mirror is also expressed in developing photoreceptors. The asymmetrical expression in the R3/4 pair could indicate that Mirror also has a late, as yet undiscovered, function in polarity determination. On the other hand, expression of Mirror in photoreceptors could be important for other aspects of their differentiation which can not be detected by looking at discs or sections of adult eyes. It would be very interesting to find out if the Iro-C also have a role in later specification of photoreceptor identity. This could be analogous to their role in specifying lateral identity of bristles on the notum.

1.5 The role of \textit{mirror} in early development

The early investigations of Mirror and Iro-C function were focused on their role in the development of adult structures such as the notum, wing and eye. However, when the expression patterns of \textit{mirror} was investigated in oocytes and embryos, it became clear that these genes also must have important functions during embryonic development. In accordance with this, most alleles of \textit{mirror} and the Iro-C are embryonic lethal. This section outlines \textit{mirror} expression and proposed function in the oocyte as well as the embryonic expression pattern and some embryonic phenotypes associated with \textit{mirror} and \textit{IroC} alleles.
1.5.1 Mirror is expressed in follicle cells and functions in early patterning events

The anterior-posterior and dorsal-ventral axes of the *Drosophila* embryo are determined by patterning events during oogenesis (Dobens and Raftery 2000; van Eeden and St Johnston 1999) (figure 1.13). The *Drosophila* egg chamber assembles within a tube of cells called sheath cells in the germarium. A 16-cell syncytial pro-oocyte forms at the posterior end of the germarium. As the pro-oocyte moves posteriorly, one cell is selected to become the oocyte and the rest of the cells become nurse cells. Follicle cells in the posterior part of the germarium migrate to encase the developing cyst and it buds off from the germarium. The first axis to be determined, the A/P axis, results from the posterior positioning of the oocyte and signalling events to the posterior follicle cells during stages 7-10. The D/V axis is also determined by signalling from the oocyte to the follicle cells at the dorsal anterior region at stage 10. In both cases the signalling involves the EGFR and the ligand Gurken. The A/P and D/V patterning of the follicle cells polarises both the eggshell and the developing embryo. The D/V polarity of the eggshell is characterised by the positions of the dorsal appendages which form at the dorsal anterior side of the egg. The D/V polarity of the embryo is characterised firstly by the repression of the gene *pipe* to the ventral domain of the oocyte. The ventral restriction of *pipe* through a complex signalling cascade ultimately leads to the generation of a gradient of the protein Dorsal which defines the embryonic D/V axis (Peri *et al.* 2002).

The first report of the *mirror* gene described it as being expressed in genital imaginal discs and the oocyte and also reported that some alleles displayed patterning defects in the egg chorion (McNeill *et al.* 1997). A detailed analysis of the expression of *mirror* during oogenesis revealed a dynamic pattern starting in the germarium and lasting until stage 10 of oogenesis (Jordan *et al.* 2000). The *mirror* transcript and protein is present in the follicle cells and inner sheath cells in the most anterior region of the germarium (figure 1.13). In the stage 6 egg chamber *mirror* is expressed in the lateral
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follicle cells. By stage 10 mirror is expressed in anterior dorsal follicle cells and the centripetally migrating cells. The centripetal follicle cells migrate from the nurse cell-oocyte boundary to cover the anterior of the oocyte late in stage 10. It is not known what controls mirror expression during early oogenesis, but at stage 10, mirror expression in the anterior dorsal follicle cells is dependent on Gurken signalling via the EGFR (Jordan et al. 2000; Zhao et al. 2000).

In accordance with the expression patterns, mirror seems to have a role during oogenesis at both early and later stages (Jordan et al. 2000). Analysis of mirror mutants revealed failures in separation of the 16-cell germline cyst from the germarium. In addition expression of Mirror in all follicle cells at stage 6 caused loss of terminal markers indicating that restriction of mirror to the lateral follicle cells is important for patterning. Analysis using combinations of mirror alleles as well as mosaic analysis showed that reduction or loss of mirror leads to ventralisation of the eggshell, characterised by loss of dorsal appendages (Jordan et al. 2000; Zhao et al. 2000). Overexpression of mirror leads to dorsalisation of the eggchamber and embryo (Jordan et al. 2000; Zhao et al. 2000). Dorsalisation of the eggchamber can be seen by an expansion of the dorsal appendages. Dorsalisation of the embryo is characterised by expansion of the dorsal expression of dpp and reduction of the ventral expression of twist.

Interestingly, as described for the eye disc, the mirror expression pattern during oogenesis is complementary to the fringe expression pattern (Jordan et al. 2000). fringe is expressed in the posterior follicle cells in the germarium when mirror is expressed anteriorly. At stage 6 fringe is expressed in the terminal follicle cells when mirror is expressed in the lateral cells. In the stage 10 egg chamber, fringe is expressed in all the follicle cells that are not expressing mirror. This complementary expression pattern together with the known role for Mirror in the repression of fringe in the eye, suggested that Mirror controls fringe expression during oogenesis. Indeed, mutant and overexpression analysis confirm that mirror represses fringe at stage 10 of oogenesis.
(Jordan et al. 2000; Zhao et al. 2000). In addition, fringe loss of function in early oogenesis leads to the same phenotypes as mirror loss of function suggesting that mirror repression of fringe is important in early oocyte development (Jordan et al. 2000).

A model for patterning of the oocyte in which mirror links the EFG pathway to D/V patterning through Notch signalling has been proposed (Jordan et al. 2000; Zhao et al. 2000). As in the eye disc, the generation of a fringe expression border would activate Notch signalling which would then pattern the oocyte. In accordance with this, a temperature sensitive allele of notch causes defects in the dorsal anterior region and loss of dorsal appendages. It was proposed that the downstream effect of Notch activation was repression of pipe to the ventral half of oocyte via an unidentified morphogen (Jordan et al. 2000). The evidence to support this model is that pipe expression is expanded dorsally in loss of function clones of mirror. Overexpression of both Mirror and a constitutively active form of Notch leads to repression of pipe at a distance both in anterior and posterior regions of the oocyte. However, in a recent report, it was found that pipe expression was not affected by the loss of mirror or fringe (Peri et al. 2002). The authors suggest that although pipe can be repressed by ectopic Mirror, Mirror expression and repression of Fringe is not normally required for the restriction of pipe to the ventral oocyte. There is no doubt however, that mirror performs several important functions during oogenesis which all seem to be linked to a restriction of fringe expression and possibly Notch activation.
Figure 1.13 The expression pattern of *mirror* in the oocyte

**Figure 1.13.** From Jordan *et al* 2000. A) Diagram illustrating the stages of oocyte development. B-D) The expression of *mirror* as visualised by in situ hybridisation and antibody staining (inset in B) during oocyte development. *mirror* is expressed in the follicle cells and inner sheath cells in the anterior germarium, in the lateral follicle cells at stage 6, and in the dorsal anterior follicle cells at stage 10.
1.5.2 Mirror has a dynamic expression pattern in the embryo

*mirror* is expressed in a dynamic pattern during embryogenesis (McNeill et al. 1997). Figure 1.14 shows the expression of the *mirror* transcript at various stages of embryonic development. There is no maternal contribution of *mirror* transcript. The first stage at which zygotic expression of *mirror* can be detected is the cellular blastoderm. At stage 5, *mirror* is expressed in an anterior ventral patch close to the site of the presumptive midgut invagination. In addition, *mirror* is also expressed dorsally at sites which will become the dorsal folds. Expression of *mirror* continues anteriorly and in the dorsal folds during gastrulation. During germband extension and until stage 11, the anterior expression seems to mark the stomodeal invagination and from about stage 13, *mirror* is expressed in the region of the proventriculus and the foregut-midgut boundary. The dorsal expression is present in the folds and in the amnioserosa until stage 10. Between stage 10 and 11, the *mirror* transcript becomes expressed in a segmentally repeated pattern in the ectoderm. The segmental expression lasts until the end of stage 11. At this stage, *mirror* is also expressed in the mesoderm. Mirror is expressed in delaminating neuroblasts from about stage 10 and continues to be expressed in the ventral nerve cord and in the brain until at least stage 16. Staining of embryos using anti-Mirror antibodies has confirmed at least the later expression patterns of Mirror (H. McNeill, I. Dahlsveen, unpublished). It is critical that his very dynamic and specific expression pattern be maintained, as ectopic expression of Mirror using a variety of embryonic enhancers is lethal (H. McNeill, I. Dahlsveen, M. Mohns, unpublished).
Figure 1.14 The expression pattern of *mirror* in the embryo

In situ hybridisation pattern of *mirror* in the embryo (H. McNeill *et al*, 1997). A) *mirror* is expressed in dorsal and anterior patches at stage 6. B and C) During gastrulation and germband extension *mirror* is expressed in the dorsal folds and amnioserosa. D) At stage 11, *mirror* is expressed in a segmentally repeated pattern. E) *mirror* is expressed in the brain and the ventral nerve cord as well as the proventriculus at stage 13. F) *mirror* expression continues in the CNS until stage 16.
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The expression patterns of *mirror* and the rest of the Iro-C seem to be more divergent in the embryo than in the imaginal discs. Pictures of the complete expression patterns of *ara* and *caup* transcripts in the embryo have not been published. However, expression of both genes was reported to start at stage 11 in the lateral epidermis and to later occur in the proventriculus and parts of the developing brain (Gomez-Skarmeta *et al.* 1996). As described above, *mirror* expression is present form stage 5 in areas where no *ara* or *caup* expression has been reported. This suggests that only *mirror* in the Iro-C complex has a role in early embryonic development. A segmentally restricted expression has not been reported for *ara* and *caup* suggesting that this pattern is also unique to *mirror*. In addition *ara* and *caup* were only reported to be expressed in the procephalon and not in the ventral nerve cord indicating that the CNS expression pattern of the Iro-C genes also differs. However, all three genes seem to be expressed in the region of the proventriculus.

The neuroblasts in which Mirror is expressed have been defined using the *mirror-lacZ* enhancer trap (Broadus *et al.* 1995; Doe 2002). The development of the CNS in *Drosophila* begins with the delamination of about 30 neuroblasts (NB) per hemi-segment from the embryonic neuroectoderm (Doe 1992). Figure 1.15 shows a schematic representation of *mirror-lacZ* expression in the NBs of one hemi-segment from stage 9 to 11. The NBs can be divided into medial, intermediate and lateral NBs according to their position with respect to the midline. Mirror expressing NBs are found in all three domains. Almost all NBs that express *mirror* delaminate from around the anterior border of each segment which corresponds to the segmental expression of *mirror* at stage 11. In addition, Mirror is also expressed in the median neuroblast. At stage 16 *mirror-lacZ* is found in a subset of neurons and glia in the ventral nerve cord which suggest that the expression of *mirror* could be lineally maintained (Doe 2002).
Figure 1.15 Schematic diagram of *mirror* expression in neuroblasts

Figure 1.15. Taken from the Doe Lab website (Doe 2002). Schematic representation of the development of neuroblasts (NB) in one hemi-segment from stage 9-11. NBs that express the *mirror-lacZ* enhancer trap are marked in black.
1.5.3 The role of mirror and the Iro-C in embryonic development

The mirror expression pattern suggests that the gene may be involved in the development of many different types of structures at different stages of embryonic development. The role of mirror and the Iro-C in the embryo has not been described, but the phenotypes associated with mirror mutations can be used to suggest how they might function during embryogenesis. Phenotypes that may be associated with the early expression pattern of mirror are delays in germ band retraction and dorsal closure (H. McNeill, unpublished). These defects may be overcome as most embryos mutant for mirror survive until later stages. Mirror mutant embryos also show defective head involution.

Mutations in mirror lead to subtle defects in segmentation (McNeill et al. 1997). Cuticle preparations from mirror mutants show loss of anterior denticles in various abdominal segments (figure 1.16). This corresponds to the expression of mirror at the anterior border of each segment. In addition, infrequent fusion of adjacent segments can also be observed. The expression of mirror in neuroblasts, the brain and ventral nerve cord suggests that it has an important role in CNS development. Staining of mirror mutant embryos with the axonal marker BP102 indicates severe CNS defects (figure 1.16). It is not known how Mirror plays a role in the various stages of embryonic development mentioned above. No transcriptional targets for Mirror in the embryo have yet been conclusively identified. However, it is clear that Mirror and probably the rest of the Iro-C have important functions in embryogenesis.
Figure 1.16. Cuticle preparations of wild type (A) and mirror mutant embryos (B and C) (McNeill et al, 1997). Loss of mirror leads to loss of anterior denticles and infrequent fusions of adjacent segments. D and E show staining with the axonal marker BP102 in wild type (D) and mirror mutant (E) embryos (H. McNeill).
1.6 The functions of vertebrate IRO proteins

A summary of the embryonic expression patterns of all six of the mouse \textit{Irx} genes indicates that the vertebrate IRO proteins function throughout development in a number of tissues (Houweling \textit{et al.} 2001). As mentioned in section 1.2, the expression from within the same \textit{Irx} cluster is very similar between \textit{Irx1} and \textit{Irx2} (cluster A) and \textit{Irx3} and \textit{Irx5} (cluster B), whereas \textit{Irx4} and \textit{Irx6} have more divergent expression patterns. Expression of \textit{Irx3} can be detected as early as E6.5 and the onset of expression of the rest of the \textit{Irx} genes occurs between E7 and E10.5. As with the \textit{Drosophila} Iro-C, the mouse \textit{Irx} genes seem to be expressed in larger territories at the onset of expression which are then refined to subdomains later in development. \textit{Irx} expression can be detected early in the ectoderm of the neural plate and in the neural epithelium, whereas later on expression becomes restricted to subdomains within the brain and the spinal cord. In some of these subdomains most of the \textit{Irx} genes show overlapping expression, whereas in other subdomains only one or two of the genes are expressed. Complex semi-overlapping patterns of expression were also observed in the heart and limb bud. In addition \textit{Irx} genes are expressed in the skin, the mammary glands and the developing teeth as well as the lungs, kidneys, pancreas, gonads and intestinal tract. The function of the \textit{Irx} genes in most of these areas of expression remains to be determined. However, a recent report describing the Fused toes mutation in mouse which deletes the entire \textit{Irx} B cluster as well as three other genes, may shed some light on the complex role of the \textit{Irx} genes in development (Peters \textit{et al.} 2002). Mice homozygous for this mutation die between E 10.5 and E14.4 and display defects in many areas in which the \textit{Irx} genes are expressed; ie fusion of digits, malformation of the heart, disorganisation of the ventral spinal cord and deformation of forebrain structures.

The role of IRO proteins during vertebrate development has been studied in more detail using \textit{Xenopus}, \textit{Zebrafish} and chick and it seems that some of the functions have been conserved. \textit{Irx} genes are important in early patterning events in \textit{Xenopus} and
zebrafish that specify dorsal fate in the ectoderm and mesoderm. Studies in chick have revealed a role for the IRO family in the subdivision of neural territories. In addition, the role of Irx genes in heart development has been studied in some detail in chick and mouse. These studies have also revealed more about how the IRO family proteins function as transcription factors.

1.6.1 The role of Irx genes in early patterning events

Chordate development is characterised by the presence of an organiser activity which releases inductive signals important for patterning of the embryo (Harland and Gerhart 1997). In *Xenopus* the Spemann organiser is located in the dorsal marginal zone and specifies the dorso-anterior axis by secretion of factors that promote dorsal fate in the neighbouring mesoderm and formation of the neural plate in the overlying ectoderm. The induction of dorsal and neural fates depends on suppression of the function of Bone morphogenetic protein (BMP), a growth factor in TGFβ superfamily. The secreted factors from the organiser are also part of the TGFβ superfamily (Nodal-related factors) and act antagonistically to inhibit BMP signalling. In addition, the expression of BMP is repressed by Wnt signalling, which is active at the dorsal side of the embryo.

The *Xenopus* IRO proteins were the first vertebrate members of the family to be studied functionally and were found to have a role in neural development. *Xiro*1, 2, and 3 were found to be expressed in neural territories and to be involved in neural specification by regulation of proneural genes (Bellefroid *et al.* 1998; Gomez-Skarmeta *et al.* 1998). Ectopic expression of *Xiro* 3 induced ectopic neural tissue and caused expansion of the neural tube. A more detailed analysis of *Xiro*1 function revealed a role in early specification of the neural territory. *Xiro*1 is essential for neural induction and seems to be downstream of Wnt signalling and upstream of Bmp-4 expression (Gomez-Skarmeta *et al.*
2001). Xirol is expressed in the prospective neuro-ectoderm and this expression is induced by Wnt signalling. Using injection experiments, it was found that Xirol induces neural fate by repressing the expression of Bmp-4. In fact the expression of Xirol and bmp-4 was found to be mutually antagonistic aiding the establishment of distinct territories with different fates. A recent report confirms that zebrafish Zirol and Ziro7 are also essential for neural crest development (Itoh et al. 2002). It was proposed that the Zirol genes establish a distinct compartment in the dorsal ectoderm which gives rise to the neural crest, the midbrain-hindbrain boundary (MHB) and neurons of the trigeminal ganglia. Expression of Zirol and Ziro7 is also affected by Wnt signalling.

IRO proteins were also found to be important for early mesodermal patterning. Xirol was found to be expressed in the Spemann organiser (Glavic et al. 2001). Injection experiments indicated that Xirol could induce secondary axis formation by induction of organiser genes such as chordin and goosecoid. The effect of Xirol on these organiser factors was found to be due to the downregulation of bmp-4 expression. In zebrafish a dorsal region of the mesendoderm called the shield, is the equivalent of the Spemann organiser. The Zirol3 gene was found to be expressed in a unique pattern in the mesendodermal layer which depended on Wnt and BMP signalling (Kudoh and Dawid 2001). Ectopic expression of Zirol3 by mRNA injections caused induction of organiser genes in a non-autonomous manner. In addition, iro3 expression caused a reduction in bmp-4 expression ventrally. Thus it seems that during early Xenopus and zebrafish development, IRO proteins act downstream of Wnt and BMP signalling. It is interesting to note that expression of the Iro-C in flies is also downstream of both Wg (Wnt) and Dpp (BMP) signalling.

In both Xenopus and zebrafish, the role of IRO proteins during early patterning events seems to be that of transcriptional repressors. Injection experiments using hybrid proteins showed that fusions of Xirol with the repressor domain of Engrailed (En) caused
the same induction of neural and mesodermal fates as the wild type protein (Glavic et al. 2001; Gomez-Skarmeta et al. 2001). This could also be seen with a fusion of just the Xiro1 homeodomain to the En repressor domains. The expression of a fusion between the activation domain of VP16 and Xiro1 caused the opposite phenotype, both loss of neural markers and induction of organiser genes. In addition, the Xiro1 homeodomain fused to an activator domain could act as a dominant negative protein rescuing the effects of ectopic expression of the Xrio-1 gene. Similar experiments using the Ziro3 protein fused to activator and repressor domains showed that the VP-16-Iro3 fusion inhibited organiser function whereas the En-Iro3 fusion had the same effect as the wild type protein (Kudoh and Dawid 2001). Thus it seems that in Xenopus and in zebrafish, IRO proteins control ectoderm and mesoderm patterning in a similar way.

1.6.2 The role of Irx genes in subdivision of neural territories

IRO proteins seem to be involved in the subdivision and patterning of the brain in Xenopus, zebrafish and chick. The developing vertebrate brain is divided into three main territories: forebrain, midbrain and hindbrain (Lumsden and Krumlauf 1996). The forebrain is further subdivided into two regions and the hindbrain is divided into rhombomers. The subdivision into different territories is characterised by the expression of overlapping domains of transcription factors. In chick, Irx3 was found to have a role in the regionalisation of the forebrain (Kobayashi et al. 2002). Irx3 is expressed posterior to a boundary of subdivision within the forebrain called the zona limitans intratralamica (ZLI). Anterior to the ZLI, another homeodomain transcription factor is expressed, Six3. The expression of Irx3 and Six3 were found to be mutually repressive. The two regions characterised by Irx3 and Six3 expression were found to respond differently to Fgf8 and Sonic hedgehog (Shh) signalling. Overexpression of Irx3 in the forebrain induced the
formation of abnormal bulges. This may be analogous to the consequence of loss or gain of Iro-C expression in Drosophila which affects cell affinities.

In addition to transcription factor domains, patterning of the brain is also dependent on the establishment of organisers or signalling centres. One such organiser, the isthmic organiser, is established at the midbrain-hindbrain boundary (MHB) (Lumsden and Krumlauf 1996). The MHB is characterised by the border of Otx2 and Gbx2 expression and the localised expression of Fgf8. Experiments in Xenopus and zebrafish have indicated that IRO proteins may be involved in MHB and isthmic organiser formation. Zebrafish Ziro1 and Ziro7 were found to be essential for the establishment of the organiser and it was proposed that Ziro1 and Ziro-7 function upstream of Otx2 and Gbx2 expression (Itoh et al. 2002). In Xenopus Xirol was found to activate the expression of Gbx2 and to be essential for the expression of Fgf8 (Glavic et al. 2002). The specification of distinct territories and the establishment of signalling centres by vertebrate IRO proteins is similar to the role of the Iro-C during Drosophila wing/notum and eye development.

In addition to the role in anterior-posterior patterning of the brain, chick Irx3 also has a role in the dorso-ventral (D/V) patterning of the neural tube (Gomez-Skarmeta and Modolell 2002). Patterning in the D/V axis is also characterised by overlapping domains of transcription factors some of which are repressed by Shh signalling and some of which are induced by Shh. The combinations of transcription factors define the different subtypes of neurons that arise from that subdomain. Irx3 is expressed in a dorsal domain and is repressed by Shh signalling from the floor plate. In addition Olig2, a Shh induced transcription factor, downregulates Irx3 in the ventral nerve cord. The combinatorial effect of Irx3 and other homeodomain transcription factors specifies the identity of a subset of neurons in the ventral nerve cord (Briscoe et al. 2000).
1.6.3 The role of Irx genes in heart development

The chick and mouse Irx genes are also involved in patterning of the developing heart. The vertebrate heart develops from a single tubular structure into ventricular and atrial compartments with different characteristics (Bruneau et al. 2000). The mature atria and ventricles have different contractile and electrophysiologic properties and express different isoforms of myosins. Specification of the different compartments has been proposed to be down to different sets of transcription factors controlling ventricle and atrial specific genes. The role of Irx4 in heart development has been studied in most detail. In mouse Irx4 is expressed early in the linear heart tube in the ventricular segment and is later restricted to the ventricular myocardium (Bruneau et al. 2000). Irx4 is expressed in the chick heart in a similar pattern (Bao et al. 1999). Four other Irx genes are also expressed in the mouse heart in more or less over-lapping patterns (Christoffels et al. 2000). Expression of Irx4 in the mouse heart has been found to be reduced in mice lacking the Nkx2-5 and dHand transcription factors indicating that Irx4 expression is downstream of these genes.

One of the roles of Irx4 in heart development is to ensure expression of the correct isoform of myosin heavy chain in the ventricles. In the chick heart, Irx4 was found to activate the expression of Ventriule myosin heavy chain-1 (VMHC1) and to suppress the expression of Arterial myosin heavy chain-1(AMCH1). Importantly, Irx4 seems to act as an activator in chick heart development. Expression of En-Irx4 fusions led to ectopic expression of AMCH1 and suppression of VMCH1, whereas expression of the wild type protein had the opposite effects. A mouse knockout of the Irx4 gene was generated in order to study the loss of function phenotype in mouse (Bruneau et al. 2001). Irx4 deficient mice were viable but developed cardiomyopathy as adults indicating that other Irx genes may substitute for Irx4 function during early heart development. It was noted that Irx2 expression was upregulated in the ventricles of the Irx4 mutants. The Irx4
knockout did display abnormal expression of various other factors during development which may be responsible for the later deterioration of ventricular function. Thus it seems that Irx genes are involved in specifying ventricle as opposed to atrial fates in the developing heart through regulation of gene expression.

1.7 Project aims: learning more about how Mirror functions as a transcription factor by identifying protein interactors

1.7.1 How does Mirror function as a transcription factor?

The aim of this project was to try to learn more about how Mirror functions as a transcription factor by identifying and characterising protein interactors. Although Mirror has many known functions during Drosophila development, very little is known about how Mirror performs these functions. It is expected that Mirror controls the expression of various target genes, but so far only a few targets have been identified such as fringe in the eye and oocyte. DNA microarray experiments have been performed to try and identify Mirror targets and the results indicated that Mirror can regulate the expression of a large number of genes (M. Mohns and H. McNeill, unpublished). However, only a few of the targets have been verified in vivo.

Transcription factors usually control the expression of genes by binding to upstream enhancer elements and recruiting co-activators and co-repressors. The presence of a homeodomain (HD) within the Mirror protein sequence suggests that it binds DNA directly. Mirror has been shown to bind to specific DNA sequences in vitro (A. Bilioni and H. McNeill, unpublished). The binding of Mirror to DNA is dependent on the HD, but does not require the N-terminus or the IRO box. The preferred DNA sequences for Mirror
binding have been determined using a site selection assay (A. Bilioni and H. McNeill, unpublished). However, so far the binding of Mirror to these sequences has not been confirmed in vivo.

It is not known whether Mirror acts as a transcriptional activator, repressor or both. Mirror can repress the transcription of fringe, but it is not known if this is a direct effect. Results from experiments with other IRO proteins indicate that they can function as activators or repressors. Early reports on Ara and Caup functions suggested that they bind to enhancer elements upstream of the ac-sc complex (Gomez-Skarmeta et al. 1996). Reporter constructs carrying the L3/TSM enhancer seemed to be positively regulated by Ara and Caup in vivo and suggested that the proteins acted as activators. However, in a recent review (Cavodeassi et al. 2001), the authors of the original paper reported that by using En repressor domain fusions with Ara, they had concluded that Ara functions as a repressor. That would indicate that the regulation of the L3/TSM enhancer is not direct. In Xenopus fusions between Xirol and activator or repressor domains also indicated that Xirol acts as a repressor (Glavic et al. 2001; Gomez-Skarmeta et al. 2001). The Xirol protein was found to bind to elements within the Bmp-4 enhancer in vitro suggesting that repression of Bmp-4 by Xirol is direct. In zebrafish however, the results were more confusing. En and VP16 fusions of Ziro7 were both able to incude ectopic expression of one gene, neurogenin1 (ngnl) (Itoh et al. 2002). In the ventral ectoderm, En-Iro7 induced ngn1 in a similar way to wild type protein. The VP16-Iro7 fusion however could only induce ngn1 expression in the neurectoderm. These results suggest that Ziro7 can act as an activator or as a repressor depending on where it is expressed. Studies on Irx4 function in heart development, also using activator and repressor fusions, indicated that Irx4 acts as an activator (Bao et al. 1999). The above results indicate that IRO proteins have the ability to function as both activators and repressors.

Transcription factor function is often regulated to ensure tight control of gene expression. Regulation of transcription factors can be linked to function ie regulation of
DNA binding and co-factor recruitment, nuclear localisation and protein stability. Transcription factors can be regulated by signalling cascades or the cell cycle and the regulation can be implemented via protein-protein interactions and covalent modifications such as phosphorylation. Nothing is known about the regulation of Mirror function. However, experiments on various Mirror constructs expressed in *Drosophila* S2 cells suggested that Mirror is phosphorylated (T. Littlewood and H. McNeill, unpublished). Mirror protein from S2 cells appears to have a higher molecular weight than predicted when analysed by SDS-PAGE. Incubation with phosphatases prior to elecrohoresis, induces a small shift in the apparent size indicating the removal of one or more phosphate groups. The C-terminal part of Mirror contains potential phosphorylation sites for mitogen-activated protein kinase (MAPK).

1.7.2 *What type of proteins is Mirror predicted to interact with?*

As a transcription factor, Mirror would be predicted to interact with transcriptional co-activators and/or co-repressors. Transcription can be controlled at several levels including changes to chromatin structure surrounding the promoter, the rate of initiation ie assembly of the basal machinery, and the rate of elongation by the polymerase. These processes are usually controlled by large protein complexes which are recruited to the target gene by transcription factors. Many co-activators and co-repressors regulate chromatin by the covalent modification of histones or the physical remodelling of nucleosomes. The most common activating modification is acetylation. Many homeodomain proteins have been found to interact with histone acetyl-transferases (Chariot *et al.* 1999). Co-repressors often function as histone deacetylases. In addition, chromatin remodelling complexes can act to reposition nucleosomes and change nucleosome-DNA interactions and in this way affect transcription. Control at the level of
initiation is usually performed by direct interactions with co-factors that contact the basal transcriptional machinery (Berk 1999). TATA-box binding protein (TBP) associated factors (TAFs) are co-activators essential for the assembly of the initiation complex and recruitment of RNA polymerase. Transcription may also be controlled at the level of elongation and elongation factors have also been classified as transcriptional co-activators. Transcription factors may not be limited to interactions with one type of co-factor, but may be involved in several steps of transcriptional control (Berk 1999). It is not known at which step of transcriptional control Mirror is functioning and therefore identification of any co-factors that interact with Mirror would greatly increase our knowledge of how Mirror controls gene expression.

Gene expression in higher eukaryotes is a combinatorial process often requiring the coordinated binding of multiple transcription factors to the promoter-enhancer region. The use of combinations of transcription factors for gene regulation allows complex patterns of regulation to develop. Protein interactions between transcription factors are important for such complex regulatory mechanisms. In addition, cooperative binding to DNA can help to ensure the specificity of transcriptional control. Many HD containing proteins have been found to interact with a variety of other types of transcription factors. Some of these interactions are mediated by the HD. Apart from other IRO proteins the Mirror HD is most closely related to the PBC HD. The HD of the PBC class of proteins is important for their interaction with the Hox proteins. As discussed in section 1.1, this interaction can regulate the Hox specificity of DNA binding as well as the affect on transcription. In the presence of PBC proteins, Hox proteins act as activators, whereas in the absence of PBC proteins, they acts as repressors. Apart from the HD, Mirror does not contain other characterised domains making it difficult to predict other types of transcription factor interactions. It is however possible that Mirror binds cooperatively to DNA with other proteins through as yet unidentified interaction domains. Apart form the HD Mirror contains a motif with slight homology to the EGF repeat in the N-terminus,
and a short motif of unknown function, the IRO box, in the C-terminus (figure 1.17). In addition, there is an acidic patch of residues following the HD and some glutamine and proline rich motifs which could be interaction domains.

In addition to co-factors and other transcription factors, Mirror may interact with a number of proteins that somehow regulate its function. Mirror could be regulated by or act synergistically with signalling cascades. As mentioned above, this may include phosphorylation and/or dephosphorylation by kinases and/or phosphatases. Other post-translational modifications may also be important for Mirror function. Localisation of Mirror in the nucleus may be a regulated process and this will certainly involve protein interactions. In addition, the stability of the Mirror protein could also be regulated. The identification of Mirror binding-proteins could shed light on many aspects of Mirror function as well as the function of other IRO proteins.

1.7.3 Identification of Mirror binding proteins

There are many in vitro and in vivo techniques commonly used for identification and characterisation of protein interactions. When searching for novel interactions the protein of interest must be somehow “screened” against possible partners. In vitro methods for such screening include expression library screening and phage display. These techniques involve the production of labelled protein which is then tested for interactions with an \textit{E. coli} expression library or a phage library. Using in vitro methods ensures that the interactions identified are direct between the protein of interest and the library clone. Techniques for identifying interactions in vivo include two-hybrid systems which can be performed in yeast or mammalian cells as well as immunoprecipitation or affinity purification methods. In two-hybrid systems, the protein of interest is usually fused to a DNA binding domain and screened against a cDNA library fused to an activation domain.
Interactions are detected by the expression of reporter genes. Immunoprecipitation and affinity purification methods can be used to pull out interacting proteins from cells and/or tissues in which the protein of interest is normally expressed. This allows the identification of endogenous protein interactors as well as protein complexes.

When this project was initiated, the materials needed for affinity purification of Mirror were not available. Therefore, an in vitro approach to identifying Mirror interactors was initially considered. This would involve production of reasonable amounts of Mirror protein in *E. coli* for labelling. A number of different Mirror constructs were made by amplification of various parts of the cDNA by PCR. Primers used for the amplification introduced restriction sites for cloning (see materials and methods). Figure 1.18 shows a schematic representation of all the Mirror constructs produced during this project. This includes full length, N-terminal and C-terminal constructs as well as a C-terminal construct which does not include the HD. Constructs generated by site-directed mutagenesis which delete the IRO box were made later during this project, but are included in the summary. Most of the Mirror constructs were cloned into expression vectors for use in *E. coli* in frame with tags for affinity purification. Three different types of tag were used: the calmodulin binding protein, glutathione-S-transferase, and a 6 x His tag. Unfortunately it seemed that the Mirror HD is incompatible with high levels of expression in *E. coli*. A variety of different *E. coli* strains and conditions were used, but only the fusion proteins which did not contain the homeodomain could be expressed at reasonable levels (I. Dahlsveen and A. Bilioni, data not shown). Production of recombinant Ara protein was also found to be difficult in *E. coli* (personal communication, J. Modolell) As the production of full length Mirror protein in *E. coli* was not possible, it was decided to use the yeast-two-hybrid system for identifying Mirror interactors. The yeast-two-hybrid system will be introduced in chapter 2.
Figure 1.17. Mirror cDNA and protein sequence. The EGF-like domain is marked in blue, the homeodomain in red, the acidic domain in green and the IRO box in purple.
Figure 1.18 Schematic representation of Mirror constructs

- Mirror Full length, aa 1-641
- Mirror N-terminal, aa 1-333
- Mirror C-terminal, aa 195-641
- Mirror C-terminal HD', aa 341-641
- Mirror Full length ΔIRO, Aaa 451-459
- Mirror C-terminal ΔIRO, Aaa 451-459

**Figure 1.18.** Mirror constructs generated by PCR amplification or site directed mutagenesis. N, Notch or EGF-like motif; HD, homeodomain; A, acidic region; I, IRO domain.
CHAPTER 2

Identifying Mirror interactors by yeast-two-hybrid screening

2.1 Introduction.

The yeast-two-hybrid system is a well established in vivo system for identifying proteins interactors. Initial experiments suggested that this system could be used to identify Mirror-binding proteins and several screens were subsequently performed using two different Mirror constructs. This chapter describes how the yeast-two-hybrid system was established for identification of Mirror interacting proteins, the screens that were performed and the clones that were selected from these screens. It also includes some further characterisation some of the interactions identified.

2.1.1 Introduction to the yeast-two-hybrid system

The yeast-two-hybrid system is an in vivo assay for protein-protein interactions (Fields and Sternglanz 1994; Luban and Goff 1995). It takes advantage of the fact that the DNA-binding domain (BD) and the transcription activation domain (AD) of some transcription factors are separable and can function independently. The two domains do not need to be part of the same polypeptide, but need only be brought into close proximity to each other to activate transcription. In the yeast-two-hybrid system, putative binding
partners are fused to the BD and AD respectively and their interaction assayed by the transcription of reporter genes. The reporter genes contain binding sites for the BD domain upstream of the promoter and only if the two hybrid proteins interact bring the AD domain close to the promoter, will transcription occur. The original two-hybrid system developed by Fields and Song used the BD and AD domains of the yeast Gal4 protein which binds to specific upstream activating sequences (UAS) of galactose responsive genes (Fields and Song 1989). Another commonly used system employs the LexA BD and the Herpes virus VP16 activation domain (Luban and Goff 1995).

The yeast-two-hybrid system is a highly sensitive assay which can reveal interactions that cannot be detected by other methods (Fields and Stemglanz 1994). Due to the amplification afforded by repeated translation of mRNAs, weak or transient interactions can be detected using this system. In addition, the participation in a transcription activation complex may also help to stabilise weak interactions between hybrid proteins. The two-hybrid system has been used to investigate known protein interactions in order to identify specific residues critical to the interaction, but has also proved an important tool in screening for novel interactions with a known target (Fields and Stemglanz 1994; Luban and Goff 1995).

2.1.2. The MATCHMAKER two-hybrid system 2 from Clontech

The yeast-two hybrid system used in screening for Mirror-binding proteins was the MATCHMAKER two-hybrid system from Clontech. The main features of this system were developed by S. Elledge and coworkers who used it to screen for novel interactors with the retinoblastoma protein (Durfee et al. 1993). The main advantage of this system was the introduction of a second reporter gene, HIS3, which allowed for nutritional selection on medium lacking histidine. Direct selection for reporter gene activation made
screening large numbers of clones, such as a cDNA library, easier. Vectors were
developed that fused the Gal4 BD (amino acids 1-147) to the protein of interest and the
Gal4 activation domain II (amino acids 768-881) to a cDNA library. The vectors used in
the Clontech system are derived from these original vectors and are illustrated in figure
2.1 (Clontech 1999). Each vector contains a different nutritional marker for transformation
in yeast. Protein expression is controlled by the full length ADH1 promoter which leads to
high levels of expression during logarithmic growth. The BD and AD fusion proteins also
contain nuclear localisation signals.

The cDNA library that was used in the screens below is a Drosophila embryonic
cDNA library from Clontech (Clontech 1999). The mRNA source for the library was
0-21 hour Canton S. embryos. The library was constructed using oligo(dT) priming and
was cloned directionally (by using an adaptor sequence) into a λACT2 vector from which
the pACT2 vector was released. Directional cloning ensures that all the cDNA inserts are
cloned 5’ to 3’ behind the GAL4 AD. The cDNA inserts were size fractionated and the
insert size varies from 0.5-3 kb. The number of independent clones was determined to be
3.0 x 10^6 which should ensure that the library is representative of mRNA complexity.
(Information on insert size and number of independent clones was obtained as quality
control data with the library).
Figure 2.1. MATCHMAKER two-hybrid system 2 vectors.

Figure 2.1 Schematic representation of the vectors used in the MATCHMAKER two-hybrid system pAS2-1 (A) and pACT2 (B).
2.1.3. Yeast strains and reporter constructs

A variety of different yeast strains with different reporter constructs exist. Although they all contain a \textit{HIS3} and a \textit{lacZ} reporter gene, the promoters that control their expression can be slightly different. The promoters have two elements, the Gal4 responsive UAS and the TATA minimal promoter region (Clontech 1999). The strains used in the screens for proteins that interact with Mirror were HF7c and Y190. HF7c uses the native GAL1 UAS with the native GAL1 TATA sequence for high but tightly controlled expression of the \textit{HIS3} gene. However, the \textit{lacZ} reporter is under the control of a synthetic UAS consensus sequence (UAS$_{G17\text{mer}}$) and the minimal promoter from the \textit{CYCl} gene. This combination gives very low expression levels of the reporter. The Y190 strain uses the native GAL1 UAS and TATA sequences to control the expression of the \textit{lacZ} gene. The \textit{HIS3} gene is also controlled by the GAL1 UAS, but retains the endogenous \textit{HIS3} minimal promoter which causes leaky expression of this reporter. The yeast can still be used for nutritional selection by including an inhibitor of the \textit{HIS3} gene product (imidazole glycerol phosphate dehydratase), 3-aminotriazole (3AT), in the medium. This feature was introduced in the original system so that the levels of \textit{HIS3} activation could be varied to enable selection of even weak interactors (Durfee \textit{et al.} 1993). The complete genotype for the yeast strains is listed in chapter 6.
2.2 Establishing the yeast-two hybrid system

2.2.1 Cloning and expression of Mirror constructs in yeast

In order to identify as many putative binding partners for Mirror as possible, the yeast-two-hybrid screens should ideally be performed using the full length Mirror protein. Many proteins, especially transcription factors, do however activate the reporter genes autonomously in the presence or absence of the Gal4 AD. In addition, some constructs may be toxic to the yeast and their expression levels thereby affected. Therefore three different Mirror constructs were initially produced. The full length, N-terminal and C-terminal Mirror constructs described in the introduction were cloned into the pAS2-1 vector using the NcoI site to create GAL4 BD-Mirror fusion proteins. To check that the constructs were in frame, the resulting plasmids were sequenced using the GAL-DB primer (Clontech 1999). Schemes of the resulting fusion proteins can be seen in figure 2.2A.

The pAS2-1-Mirror constructs were transformed into the HF7c and Y190 yeast strains in order to check the expression of the fusion protein. Protein extracts were made as described in materials and methods and analysed by SDS-PAGE followed by Western blotting. Blots of extracts from both strains expressing the full length and C-terminal constructs can be seen in figure 2.2B. The N-terminal construct can not be detected with the Mirror antibody used for these blots.
Figure 2.2A. GAL4-DNA binding domain-Mirror fusion proteins.

GAL4-DB-Mirror FL: The GAL4 DNA binding domain includes amino acids 1-147 of the GAL4 protein. Mirror FL: Full length construct (amino acids 1-621), Mirror N: N-terminal construct (amino acids 1-333), Mirror C: C-terminal construct (amino acids 195-641).

Figure 2.2B. Expression of GAL4-DB-Mirror fusion proteins in yeast.

Figure 2.2B. Western blots of protein extracts from untransformed and transformed yeast strains HF7c and Y190, probed with a Mirror rabbit antibody. Lane 1: HF7c, lane 2: HF7c + pAS-Mirror FL, lane 3: HF7c + pAS-Mirror C, lane 4: HF7c + pAS2-1, lane 5: Y190, lane 6: Y190 + pAS-Mirror FL, lane 7: Y190 + pAS-Mirror C.
2.2.2 Checking Mirror constructs for autoactivation of reporter genes

In order to check if any of the Mirror constructs caused activation of the reporter genes, they were transformed into yeast cells alone and in combination with the empty pACT2 library vector which produces the Gal4 AD. The transformations were plated on medium lacking histidine and in the case of the Y190 strain, the medium included 25mM 3AT. Growth on selective medium was scored as slight, moderate or strong according to the number of colonies and their size after 5-7 days. Activation of the lacZ reporter was assayed by the β-galactosidase filter assay as described in chapter 6. Activation was scored as moderate or strong according to the intensity of the colour produced and the time it took for colour to appear. The results for both yeast strains used can be seen in table 2.1 below.

<table>
<thead>
<tr>
<th>Construct</th>
<th>HF7c</th>
<th>Y190</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HIS3</td>
<td>lacZ</td>
</tr>
<tr>
<td>pAS2-1-Mirror full length</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>pAS2-1-Mirror N-terminal</td>
<td>−</td>
<td>(+)</td>
</tr>
<tr>
<td>pAS2-1-Mirror C-terminal</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

Table 2.1 Growth on his\(^{-}\) medium and lacZ activation scored as slight (+); moderate +; or strong ++.

The N-terminal and the full length Mirror constructs cause activation of the HIS3 and lacZ reporter genes in the Y190 strain to varying degrees. Due to the stringency of the HIS3 reporter and the low levels of the lacZ reporter in the HF7c strain, no activation was detectable by any of the Mirror constructs.
Chapter 2

2.3 Yeast-two-hybrid screens using full length Mirror as bait

2.3.1 Yeast-two-hybrid screens I-III

The first three library screens (I-III) were performed using the full length Mirror protein as bait. Due to the activation of reporter genes by this construct in Y190 cells, the HF7c strain was used in these experiments. Library scale transformations were carried out using the pAS2-1-Mirror FL construct and amplified library DNA (see chapter 6). A small aliquot of the transformations were diluted and plated on selective medium lacking tryptophan and leucine, but not histidine. The colonies from these plates were counted and the transformation efficiency per µg of library DNA calculated. The total number of library clones screened was calculated by multiplying the transformation efficiency with the amount of library DNA used in µg (formula from the MATCHMAKER Yeast-Two-Hybrid manual). In the first two screens the transformation efficiency was not high enough to ensure that all the clones in the library had been screened, so three screens were performed in total. The rest of the transformation was plated on selective medium lacking histidine and containing 1.5mM 3AT. The plates were incubated at 30°C for up to 14 days and growing colonies picked and re-streaked on selective medium. A summary of the numbers of clones screened and the numbers of HIS3 positive colonies picked form screens I-III can be found in table 2.2.

2.3.2 Titration of HIS+ clones on 3AT

All the HIS3 positive colonies picked in screens I-III were tested for activation of the lacZ reporter using the β-galactosidase filter assay, but they were all negative. This is probably due to the very weak promoter used for the lacZ gene in this strain. In order to try to select for true positives, a more stringent test for activation of the HIS3 reporter was
employed. The colonies were streaked onto selective medium containing increasing amounts of 3AT to titer the activity of the *HIS3* gene product, so that only colonies that expressed high levels of the reporter could be picked. Titering growth on 3AT in this way has been used to identify true yeast-two-hybrid interactors even in the absence of *lacZ* activation (Alberts *et al.* 1998). Table 2.2 below contains the results of screens I-III.

Table 2.2 Summary of results form screen I-III.

<table>
<thead>
<tr>
<th>Screen</th>
<th>Library clones screened</th>
<th><em>HIS3</em>+ colonies</th>
<th>Total colonies tested on 3AT</th>
<th>Colonies growing on 3AT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2mM</td>
</tr>
<tr>
<td>I</td>
<td>250 000</td>
<td>65</td>
<td>45</td>
<td>29</td>
</tr>
<tr>
<td>II</td>
<td>500 000</td>
<td>70</td>
<td>48</td>
<td>35</td>
</tr>
<tr>
<td>III</td>
<td>4 000 000</td>
<td>83</td>
<td>50</td>
<td>37</td>
</tr>
</tbody>
</table>

Table 2.2. The number of library clones screened is calculated by multiplying the transformation efficiency with the amount of library DNA (in μg) used in the transformations. *HIS3*+ colonies were streaked onto plates with increasing concentrations of 3AT and scored as growing if large colonies had appeared within 7 days.

2.3.3 Sequencing of putative yeast-two hybrid interactors form screens I-III.

In order to investigate what types of proteins had been selected during screens I-III, plasmid DNA was isolated from most of the colonies that grew on 10mM 3AT. The plasmids were then used as templates for PCR reactions to amplify the cDNA inserts. The PCR products were sequenced at the 5' end and the sequence used to search the *Drosophila* genome database for matches. Due to the failure of some inserts to amplify or sequence well, the final number of sequenced clones were 8 for screen I, 7 for screen II and 10 for screen III. The results of the sequencing are presented in table 2.3.
Table 2.3 Sequencing results from screen I-III

<table>
<thead>
<tr>
<th>Clone</th>
<th>CG number</th>
<th>Protein name, domains or homologies to other proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>I 5a</td>
<td>CG10642</td>
<td>Klp64D (Kinesin-like protein at 64D)</td>
</tr>
<tr>
<td>I 8a/b</td>
<td>CG8286</td>
<td>Chaperone J-domain, Tetratricopeptide repeat (TPR)</td>
</tr>
<tr>
<td>I 10a</td>
<td>none</td>
<td>P element sequence</td>
</tr>
<tr>
<td>I 16b</td>
<td>CG11604</td>
<td>Zn-finger C2HC, NLS</td>
</tr>
<tr>
<td>I 23a</td>
<td>CG4027</td>
<td>Act5C (Actin at 5C)</td>
</tr>
<tr>
<td>I 36a</td>
<td>CG18642</td>
<td>Bem46, Esterase, Hydrolase</td>
</tr>
<tr>
<td>I 42a</td>
<td>CG1135</td>
<td>FHA domain (homologous to nucleolar protein Ms/Hu)</td>
</tr>
<tr>
<td>I 50b</td>
<td>CG12262</td>
<td>Acyl-CoA dehydrogenase domains</td>
</tr>
<tr>
<td>II 3a</td>
<td>CG2103</td>
<td>Polypeptide N-acetylgalactosaminyltransferase</td>
</tr>
<tr>
<td>II 9b</td>
<td>CG9184</td>
<td>rhodopsin C-terminal tail</td>
</tr>
<tr>
<td>II 12a</td>
<td>CG1135</td>
<td>FHA domain (homologous to nucleolar protein Ms/Hu)</td>
</tr>
<tr>
<td>II 13a</td>
<td>none</td>
<td>Mitochondrial Cytochrome C oxidase I</td>
</tr>
<tr>
<td>II 31b</td>
<td>CG1467</td>
<td>Syx16 (Syntaxin 16)</td>
</tr>
<tr>
<td>II 48a</td>
<td>CG11584</td>
<td>C-terminal homology to mucins/phosphoproteoglycans</td>
</tr>
<tr>
<td>II 48c</td>
<td>CG12919</td>
<td>TNF (Tumor Necrosis Factor) family motif</td>
</tr>
<tr>
<td>III 1d</td>
<td>CG7558</td>
<td>Arp66B (Actin-related protein 66B)</td>
</tr>
<tr>
<td>III 6a</td>
<td>CG2102</td>
<td>Castor (Zn-finger transcription factor)</td>
</tr>
<tr>
<td>III 11a</td>
<td>CG13389</td>
<td>RpS13 (Ribosomal protein)</td>
</tr>
<tr>
<td>III 12b</td>
<td>CG8448</td>
<td>Several polypeptides predicted, all contain DNAJ domain.</td>
</tr>
<tr>
<td>III 13c</td>
<td>CG7558</td>
<td>Arp66B (Actin-related protein 66B)</td>
</tr>
<tr>
<td>III 14c</td>
<td>CG1846</td>
<td>inositol-3,4,-bisphosphate 4-phosphatase</td>
</tr>
<tr>
<td>III 17d</td>
<td>CG6163</td>
<td>No homologues or motifs</td>
</tr>
<tr>
<td>III 27a</td>
<td>CG1135</td>
<td>FHA domain (homologous to nucleolar protein Ms/Hu)</td>
</tr>
<tr>
<td>III 39a</td>
<td>CG5740</td>
<td>C-terminal coiled-coil region (homo. non-muscle myosins)</td>
</tr>
<tr>
<td>III 41a</td>
<td>CG13067</td>
<td>No homologues or motifs</td>
</tr>
<tr>
<td>III 50a</td>
<td>CG4954</td>
<td>eIF3-S8 (translation initiation factor)</td>
</tr>
</tbody>
</table>

Table 2.3. Each partial cDNA sequence was used to search the entire Drosophila genome sequence using the BDGP Fly-BLAST server to identify the predicted gene product (CG numbers). Information about the predicted protein sequences was taken from GadFly where identified domains and homologues can be found for each gene.

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As the sequencing of selected clones form screens I-III shows, most of the different yeast colonies selected contained different cDNA clones. Two genes were represented more than once, CG1135 which was found three times, once in each screen, and Arp66B which was found twice in screen III.

2.4 Yeast-two-hybrid screen using C-terminal Mirror as bait

2.4.1. Yeast-two-hybrid screen IV

Due to the lack of lacZ reporter gene activation in the HF7c yeast strain during screens I-III, another library screen was undertaken using the Y190 strain and the C-terminal Mirror construct as bait. It was necessary to use the C-terminal part of Mirror as both the N-terminal and full length constructs caused activation of the reporter genes in this strain. The library transformation was carried out as for screens I-III and plated on selective medium lacking histidine and containing 30mM 3AT to combat the leaky HIS3 reporter. HIS3 positive clones were picked and re-streaked before being tested for lacZ activation using the β-galactosidase filter assay. Some of the HIS3 positive clones could not be assayed as they were contaminated with bacteria. The activation of the lacZ reporter was graded according to the length of time before the appearance of colour and the intensity of the colour in the filter assay. A summary of screen IV is shown in table 2.4 below.
Table 2.4 Summary of yeast-two-hybrid screen IV.

<table>
<thead>
<tr>
<th>Screen</th>
<th>Library clones screened</th>
<th>HIS3+ colonies</th>
<th>Colonies tested for β-Gal</th>
<th>lacZ+ colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td>IV</td>
<td>4 000 000</td>
<td>59</td>
<td>55</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>22</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>8</td>
</tr>
</tbody>
</table>

Table 2.4. lacZ positive colonies scored as follows: +, weak activation; ++, moderate activation; ++++, strong activation.

2.4.2 Analysis and sequencing of putative yeast-two hybrid interactors from screen IV

In order to sequence the library clones selected in screen IV, plasmid DNA was isolated from the 44 lacZ positive colonies and the library inserts amplified by PCR. Over half of the inserts amplified were of nearly exactly the same size and when a selection of these were sequenced, it was found that they all were from the same cDNA. All of the amplified inserts were therefore analysed by RsaI digest, and grouped according to their fragment sizes. Most of the inserts (32) had the same basic pattern of fragments with one fragment varying slightly in size. A representative number of these inserts were sequenced and all found to match the same cDNA, CGI 135. Two other groups of inserts were also found to be the same due to their RsaI fragment sizes. One or two from each group were sequenced as well as the rest of the unique inserts. A summary of the sequencing results can be found in table 2.5, which also indicates the number times each insert was selected in this screen.
Table 2.5. Sequencing results from screen IV

<table>
<thead>
<tr>
<th># of clones</th>
<th>Clones sequenced</th>
<th>CG number</th>
<th>Protein name, domains or homologies to other proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>32</td>
<td>1a, 4a, 8a, 9a, 9c, 10a, 16a, 23d, 41a, 42a</td>
<td>CG1135</td>
<td>Forkhead associated (FHA) domain (homologous to nucleolar protein/cell cycle regulated protein in mouse and human.)</td>
</tr>
<tr>
<td>3</td>
<td>22b, 38a</td>
<td>CG5740</td>
<td>C-terminal coiled-coil region (homology to non-muscle myosins)</td>
</tr>
<tr>
<td>2</td>
<td>3a</td>
<td>CG3987</td>
<td>No homologies or motifs</td>
</tr>
<tr>
<td>1</td>
<td>7a</td>
<td>CG10395</td>
<td>Weak homology to Arabidopsis hypothetical protein</td>
</tr>
<tr>
<td>1</td>
<td>23c</td>
<td>CG3733</td>
<td><strong>CHD1</strong> (Chromo-Helicase-DNA binding domain protein)</td>
</tr>
<tr>
<td>1</td>
<td>27a</td>
<td>CG5893</td>
<td><strong>Dichaete</strong> (HMG-box transcription factor)</td>
</tr>
<tr>
<td>1</td>
<td>29a</td>
<td>CG30337</td>
<td>N-terminal coiled-coil motif</td>
</tr>
<tr>
<td>1</td>
<td>31b</td>
<td>CG1962</td>
<td>Homology to paraneoplastic cerebellar degeneration antigen, cdr2</td>
</tr>
<tr>
<td>1</td>
<td>41d</td>
<td>CG2957</td>
<td><strong>mRpS9</strong> (mitochondrial ribosomal protein S9)</td>
</tr>
<tr>
<td>1</td>
<td>46a</td>
<td>CG13367</td>
<td>Homologous to human EST, no motifs</td>
</tr>
<tr>
<td>1</td>
<td>48a</td>
<td>CG4903</td>
<td><strong>MESR4</strong> (Misexpression suppressor of ras 4)</td>
</tr>
</tbody>
</table>

**Table 2.5.** Each partial cDNA sequence was used to search the entire *Drosophila* genome sequence using the BDGP Fly-BLAST server to identify the predicted gene product (CG numbers). Information about the predicted protein sequences was taken from GadFly where identified domains and homologues can be found for each gene.
2.5 Re-testing of selected yeast-two-hybrid positives

2.5.1 Testing positive clones for activation of reporter genes and for interaction with the C-terminal Mirror construct.

In order to confirm the putative Mirror interactors selected during the library screens, the clones need to be re-tested to rule out independent activation of the reporter genes. This requires the pACT2 library plasmid to be isolated from the selected yeast clone. As both the pAS2-1 and pACT2 plasmids carry the same antibiotic resistance gene, this was done using auxotrophic KC8 bacterial cells. The pACT2 vector can be selected by transforming KC8 cells with yeast plasmid DNA and plating the bacteria on medium lacking leucine. Some of the selected library clones could not be isolated in bacteria, and could therefore not be re-tested. Based on sequence information a number of the library clones, especially from screens I-III, seemed to be false positives or not be relevant as interactors with a transcription factor and these were therefore not re-tested.

Due to the weak activity of the \textit{lacZ} reporter in the HF7c yeast strain it was decided to re-test clones in the Y190 strain. Individual library clones were transformed into Y190 cells in combination with the pAS2-1 vector on its own and with the pAS2-1-C-terminal Mirror construct. Activation of the \textit{HIS3} reporter was tested by growth on medium lacking histidine (+ 30mM 3AT). Transformations were monitored for 7 days and growth scored as minimal, moderate or solid according to the number and size of the colonies and how long they took to appear. Activation of the \textit{lacZ} reporter was tested using the \( \beta \)-galactosidase filter assay and scored as weak, moderate or strong according to the intensity of the colour and the time it took for colour to appear. Assays were done in triplicate and the results averaged. Results from the re-testing in Y190 cells can be seen in table 2.6 below.
Table 2.6. Summary of yeast-two-hybrid re-tests.

<table>
<thead>
<tr>
<th>Library clone</th>
<th>+ pAS2-1</th>
<th>+ pAS-C-terminal Mirror</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ref.</td>
<td>GC number</td>
<td>HIS3</td>
</tr>
<tr>
<td>I 8a</td>
<td>CG8286</td>
<td>+</td>
</tr>
<tr>
<td>I 16b</td>
<td>CG11604</td>
<td>-</td>
</tr>
<tr>
<td>I 36a</td>
<td>CG18642</td>
<td>-</td>
</tr>
<tr>
<td>II 48a</td>
<td>CG11584</td>
<td>+</td>
</tr>
<tr>
<td>II 48c</td>
<td>CG12919</td>
<td>+</td>
</tr>
<tr>
<td>III 6a</td>
<td>CG2102</td>
<td>-</td>
</tr>
<tr>
<td>III 12b</td>
<td>CG8448</td>
<td>+</td>
</tr>
<tr>
<td>III 14c</td>
<td>CG1846</td>
<td>+</td>
</tr>
<tr>
<td>III 17d</td>
<td>CG6163</td>
<td>++</td>
</tr>
<tr>
<td>III 41a</td>
<td>CG13067</td>
<td>+</td>
</tr>
<tr>
<td>IV 3a</td>
<td>CG3987</td>
<td>-</td>
</tr>
<tr>
<td>IV 7a</td>
<td>CG10395</td>
<td>-</td>
</tr>
<tr>
<td>IV 10a</td>
<td>CG1135</td>
<td>-</td>
</tr>
<tr>
<td>IV 23c</td>
<td>CG3733</td>
<td>-</td>
</tr>
<tr>
<td>IV 27a</td>
<td>CG5893</td>
<td>-</td>
</tr>
<tr>
<td>IV 29a</td>
<td>CG1931</td>
<td>-</td>
</tr>
<tr>
<td>IV 31b</td>
<td>CG1962</td>
<td>-</td>
</tr>
<tr>
<td>IV 38a</td>
<td>CG5740</td>
<td>-</td>
</tr>
<tr>
<td>IV 41d</td>
<td>CG2957</td>
<td>-</td>
</tr>
<tr>
<td>IV 46a</td>
<td>CG13367</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 2.6. Summary of re-testing library clones with pAS2-1 and pAS-C-terminal Mirror. +, minimal growth or weak activation; ++ moderate growth or moderate activation; +++ solid growth or strong activation.
2.5.3 Investigating the relative strengths of reporter gene activation

To investigate the relative strength of reporter gene acitvation by the yeast-two-hybrid positive clones identified, a liquid β-galactosidase assay was performed using a chemiluminescent substrate. Cultures of yeast transformed with selected library clones and pAS2-1 or pAS-C-terminal Mirror were used in the assay as described in material and methods. The read-out of the assay is Relative Light Units (RLU) from the chemiluminescent substrate divided by the cell density in OD$_{600}$ units. The results can be seen represented as bar graphs in figure 2.3. Although maximal amounts of yeast culture was used for the liquid β-galactosidase assays, most of the clones had very low β-galactosidase activities and were outside the linear range of the assay. This means that only clones that give quite high lacZ reporter activity can be compared using this assay. Most of the clones that gave low RLU readings have been shown to activate the lacZ reporter in the filter assay, which may be more sensitive to low β-galactosidase levels.
**Figure 2.3.** Results of the liquid β-Galactosidase assays for yeast-two-hybrid positives from screen I-III (A) and screen IV (B). Each library clone was transformed into Y190 yeast cells in combination with the pAS2-1 vector (light blue bars) or the pAS-Mirror C construct (purple bars). Relative light units (RLU) were read using a plate luminometer and divided by the OD<sub>600</sub> units of the sample.
Chapter 2

2.6 Further investigation of the Mirror interaction domains

2.6.1 New Mirror constructs for testing interactions

Many of the library clones selected in the yeast-two hybrid screen that were re-tested appear to be genuine yeast-two-hybrid interactors. Most of the clones do not seem to activate the reporter genes on their own, and interaction with the Mirror C-terminal construct can be confirmed. To determine if any of the clones identified interact with certain domains in the C-terminal part of the Mirror protein, two new constructs were cloned into the pAS2-1 vector. To test for interactions with the homeodomain (HD), a C-terminal construct of Mirror starting just after the HD was used. Interactions between library clones and the Iro domain were tested using a C-terminal construct made by site directed mutagenesis, which lacks nine amino acids of the IRO domain. Schematic diagrams of the new Mirror fusion proteins can be seen in figure 2.4.

2.6.2 Results of testing new constructs with yeast-two-hybrid positives.

To test the interaction of selected library clones with the HD or Iro domain, they were transformed into Y190 yeast cells in combination with the pAS2-1 vector, the pAS-Mirror C construct, the pAS-Mirror C HD construct, and the pAS-Mirror C ΔIRO construct. HIS3 reporter activity was assayed on selective medium and lacZ reporter activity using the filter assay. Both HIS3 and lacZ activation was scored as described for the re-tests above. The results of the interaction of the selected library clones with the new Mirror constructs can be seen below in table 2.7.
Figure 2.4. New GAL4-DB-Mirror fusion proteins for testing putative interactors.

Mirror C HD

Mirror C ΔIRO

Figure 2.4. The GAL4 DNA binding domain includes amino acids 1-147 of the GAL4 protein. Mirror C HD: Mirror C-terminal construct lacking the homeodomain (amino acids 341-641); Mirror C ΔIRO: Mirror C-terminal construct (amino acids 195-641) lacking the Iro domain (amino acids 451-459).
<table>
<thead>
<tr>
<th>Clone</th>
<th>pAS2-1</th>
<th>pAS-Mirror C</th>
<th>pAS-Mirror C HD-</th>
<th>pAS-Mirror ΔIRO</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HIS</td>
<td>lacZ</td>
<td>HIS</td>
<td>lacZ</td>
</tr>
<tr>
<td>I 8a</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>II 48c</td>
<td>(+)</td>
<td>+</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>III 6a</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>III 12b</td>
<td>+</td>
<td>(+)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>III 14c</td>
<td>(+)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IV 3a</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>IV 7a</td>
<td>(+)</td>
<td>-</td>
<td>++</td>
<td>-</td>
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<tr>
<td>IV 10a</td>
<td>-</td>
<td>(+)</td>
<td>+++</td>
<td>+++</td>
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<tr>
<td>IV 23c</td>
<td>+</td>
<td>-</td>
<td>++</td>
<td>(+)</td>
</tr>
<tr>
<td>IV 27a</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>IV 29a</td>
<td>(+)</td>
<td>+</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>IV 31b</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>IV 38a</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>(+)</td>
</tr>
<tr>
<td>IV 41d</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>IV 46a</td>
<td>(+)</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>

Table 2.7. Summary of testing library clones with pAS2-Mirror C HD- and pAS-Mirror C ΔIRO. (+), minimal growth or very weak activation; +, slight growth or weak activation; ++ moderate growth or moderate activation; +++ solid growth or strong activation.
2.7 Discussion

2.7.1. Establishing the yeast-two-hybrid system.

The yeast-two-hybrid system is an established method for investigating protein-protein interactions and has been used in these experiments to try and identify interaction partners for the transcription factor Mirror. There are several factors which are important for the success of two-hybrid screens. Firstly, the "bait" protein used in the screen has to be expressed in the yeast as a stable fusion protein with the GAL4 BD. It needs to be properly folded and able to enter the nucleus. The first part of these criteria was addressed by extracting protein from yeast expressing different Mirror constructs and analysing them by western blot. Both the HF7c and Y190 yeast strains were shown to contain the Mirror full length and Mirror C-terminal fusion proteins. A common way of confirming the correct folding and nuclear translocation of fusion proteins is to check for interaction with a known partner (Luban and Goff 1995). As there were no known binding partners for Mirror, this type of control could not be performed.

A very important criterion for the success a library screen is that the bait protein does not activate the reporter genes either alone or in combination with the GAL4 AD. As described in the introduction, two yeast strains were used in the screens, Y190 which has high reporter gene activity and HF7c which has low reporter gene activity. In the HF7c strain none of the Mirror constructs caused significant activation of the reporter genes. The HIS3 gene in HF7c is under very tight control and may have a high threshold for activation whereas the lacZ gene is only expressed at very low levels after activation. However, in the Y190 strain the full length and N-terminal Mirror constructs were found to activate both the HIS3 and lacZ reporter genes. This might indicate that there is a domain or motif in the N-terminal part of Mirror that can directly or indirectly cause the activation of transcription from these yeast promoters. Activation of reporter genes does in
some cases reflect a true function of the protein in transcription, but in many cases reporter gene activation can be caused by random proteins or domains (Fields and Sternglanz 1994). It is not unexpected that the transcription factor Mirror contains an activation domain, but the fact that it seems to lie within the N-terminal part of the protein is somewhat surprising. The predicted activation/repression domain is a stretch of acidic amino acids following the homeodomain in the C-terminal part of the protein. The activation caused by these constructs is not as high as that of the GAL4 activation domain for example, as it is not detectable in the HF7c strain.

Another important consideration when setting up a yeast-two-hybrid screen is the library of GAL4-AD fusion proteins to use. In this case, a commercially available *Drosophila* embryonic library was used. Some of the concerns associated with the use of such libraries, such as many clones not containing inserts and certain highly transcribed cDNAs being over-represented, does not seem to have been a major problem in this case. However, the library was made by polyT priming and consequently most of the clones will contain only the C-terminal parts of proteins. It is therefore possible that many interactions that are mediated by domains in the N-terminal parts of proteins (especially large proteins) were not picked up in the screens performed.

2.7.2 Problems associated with the MATCHMAKER two-hybrid system

The main problems encountered when using the MATCHMAKER two-hybrid system from Clontech were with reporter gene expression. In order to try and identify proteins which might bind to any part of the Mirror protein, it was desirable to use the full length Mirror construct in the yeast-two-hybrid screens. Due to activation of reporter genes by this construct in the Y190 strain, the HF7c strain was used in three screens with the full length construct. Unfortunately, it was found that in this strain only very high affinity interactions could activate the lacZ reporter gene (data not shown) and none of the
selected clones from screens I-III had β-galactosidase activity. This made it difficult to identify true yeast-two-hybrid interactors from these screens using only the activity of the HIS3 reporter. In order to try and titrate the activity from the HIS3 reporter, the colonies were grown on increasing amounts of 3AT, an inhibitor of the HIS3 gene product. Although this allowed for selection of fewer colonies, once sequenced, it was clear that there must have been quite a few false positives identified.

The fourth screen was performed using the Y190 strain of yeast which has a much stronger lacZ promoter so positive clones that activate both reporters could be selected. The Y190 strain does however have leaky expression of the HIS3 gene and selection has to be performed in the presence of 3AT. Although most of the HIS3 positive clones selected in this screen did show activation of the lacZ reporter, the activation was somewhat variable. The filter assays were generally done in triplicate and some clones would show different levels of lacZ activation on different occasions. In addition, there were significant differences observed in reporter gene activation between the first round of re-testing and the second round of testing new Mirror constructs (see tables 2.6 and 2.7). The levels of activation were generally higher in the second re-tests and this was most likely due to a fresh aliquot of Y190 cells being used in these tests. The variability of the reporter gene activation questioned the reliability of re-confirming the interactions as well as eliminating auto-activation by library proteins using this system.

2.7.3 Identification of putative Mirror interaction proteins

Identification of proteins that interact with Mirror depended on many factors that could not be controlled for. In addition to the correct folding and cellular localisation of the fusion protein, putative interactions may depend on post-translational modifications like phosphorylation. There is some evidence that Mirror is phosphorylated, at least in Drosophila tissue culture cells (T. Littlewood and H. McNeill, unpublished), and this may
be important for interactions with other proteins. Due to the problems with reporter gene activation, the clones which have been finally selected are only those that interact with the C-terminal part of Mirror. This means of course that many interactions could have been missed. In addition, fusion of the Gal4 BD upstream of the Mirror homeodomain may have affected the ability of proteins to interact in close proximity to this domain due to steric constraints or problems with folding.

Out of about 200 HIS3 positive clones from library screens I-III, 37 were considered to show strong activation of the HIS3 reporter gene and most of these were sequenced to get an idea of what types of proteins had been identified. Many of these clones were not considered to be likely interaction partners for the transcription factor Mirror, such as Cytochrome C oxidase and Acetyl-CoA dehydrogenase. These proteins were possible picked out as they are relatively highly expressed and may be highly represented in the library. Due to the difficulties with the HF7c \textit{lacZ} reporter, though, it is likely that many of the other clones selected were also not relevant as Mirror interactors.

As the interaction with the full length Mirror protein could not easily be re-tested, selected clones were re-tested with the C-terminal construct. Many of the clones re-tested were found to have background activation of the \textit{HIS3} reporter gene, but some were also found to interact with C-terminal Mirror. The remaining clones could be re-tested in the future if an N-terminal construct could be made that did not activate the reporter genes or using another system, such as co-immunoprecipitation. The most interesting clones that were confirmed as positives from screens I-III were the Zinc-finger transcription factor Castor and a novel protein containing a DNAJ domain.

In screen IV, using the C-terminal Mirror construct, 44 HIS3+, lacZ+ clones were selected, and surprisingly 32 of these were from the same cDNA. This cDNA represented the novel gene CG1135 which was also pulled out in each of the first three screens. Two other cDNAs were represented more than once, CG5740 and CG3987. Although this screen afforded a more reliable selection of positives using both reporter genes, it is clear
that some of the clones pulled out are probably not Mirror interactors, such as the mitochondrial ribosomal protein, S9. During the re-testing of clones from screen IV it was also became clear that fewer of these turned out to activate the reporter genes in the absence of Mirror compared to clones re-tested from screens I-III. Most of the positive clones from screen IV are previously uncharacterised proteins which are only represented by CG numbers in the \textit{Drosophila} genome. At the time when these screens were performed, only two of the proteins had been characterised, Dichaete, a HMG domain containing protein and CHD1, a putative chromatin remodeling protein. Some of the novel proteins have interesting homologues in other organisms whereas others are completely novel in that they do not contain any recognised domains.

It is apparent that the positive clones selected in the library screens cause activation of reporter gene transcription to different levels. Differences in activation may depend on many parameters such as the expression levels of the AD fusion protein and extent of nuclear import, but may also reflect the affinity of the interacting proteins (Fields and Sternglanz 1994). In order to investigate the relative strength of reporter gene activation, liquid β-galactosidase assays were performed where the activity of the β-galactosidase enzyme is measured using a chemiluminescent substrate. Unfortunately, most of the interactors did not cause strong enough activation of the \textit{lacZ} gene to be detectable in this system. Of the clones, CG1135 caused much higher activation of \textit{lacZ} than any of the other clones. This clone was selected 35 times in total in four screens. Other clones with high β-galactosidase activity were CG30337 and CG12919 although the latter showed significant activation in the absence of Mirror.
2.7.4 Investigation of the Mirror interaction domains.

All the yeast-two-hybrid clones that were re-tested and found to be positive interact with the C-terminal part of Mirror including the homeodomain, the acidic domain, and the IRO domain. In order to investigate if any of these domains may be important for the binding of the identified clones to Mirror, two new Mirror constructs were used to test the yeast-two-hybrid interactions. These constructs consisted of the C-terminal part of Mirror either lacking the entire homeodomain or the IRO domain. An interesting result from these experiments is that most of the positive clones seem cause a stronger activation of reporter gene activity in the presence of the C-terminal construct lacking the homeodomain. Increased activation of reporter genes has been associated with deletion constructs in other labs (Fields and Sternglanz 1994). The reason for this may be that the shorter construct is expressed at higher levels than the C-terminal construct (although this has not been confirmed) or that the absence of the homeodomain leaves the rest of the protein more exposed and available for interaction with other proteins. The absence of the homeodomain may also simulate certain conformations of the Mirror protein. One of the positive clones however, CG5740, does seem to depend completely on the homeodomain for its interaction with Mirror. This putative interactor was pulled out twice in screen IV and once in screen III.

None of the putative interactors seems to require the presence of the IRO domain for its interaction with Mirror. Although the activation of the lacZ reporter gene was in some cases reduced in the presence of the ΔIRO construct, the HIS3 reporter gene activation was comparable to that of the original C-terminal construct for most positive clones. As the absence of the homeodomain from the C-terminal construct seemed to increase the activation of the reporter genes in combination with most of the putative interactors it would be interesting to see if removing the Iro domain from this construct affects any of the interactions. The C-terminal part of the Mirror protein contains in
addition to the HD and IRO domain, glutamine and proline rich motifs, which may serve as protein-protein interaction motifs. The contribution of these motifs to any of the identified interactions could be tested with further deletion constructs of the C-terminal part of Mirror.

2.7.5 Concluding remarks

The yeast-two-hybrid system has been used to identify proteins from a cDNA library that may interact with the transcription factor Mirror. The putative interactors have been confirmed as yeast-two-hybrid positives in that they only activate the reporter genes in the presence of the Mirror construct. A more rigorous way confirming the interactions would be to test the selected library clones for interaction with other Gal4 BD fusions. This would confirm that the interaction with Mirror was specific. However, other yeast-two-hybrid screens have been performed using various portions of the Fat intracellular domain and the same amplified library. None of the clones that have been re-tested with Mirror were selected in those screens. This indicates that at least the positive clones from this screen are not generally selected in yeast-two-hybrid screens due to being over-represented in the library.

Although four screens were performed, it is very unlikely that most of the proteins that interact with even the C-terminal part of Mirror have been identified in these experiments. Many of the selected clones encode completely novel proteins and a lot of the anticipated types of interactors have not been found. One of the putative interactors was pulled out 35 times in four screens, whereas most other interactors were only selected once. An interaction in yeast is however only an indication that these proteins actually associate with Mirror in vivo. The interactions need to be confirmed as functionally important in Drosophila.
CHAPTER 3

Further characterisation of selected yeast-two-hybrid interactors

3.1 Introduction

As the main aim for identifying proteins that interact with Mirror was to discover more about how Mirror acts as a transcription factor in *Drosophila*, the primary concern when analysing the interactors from the yeast-two-hybrid screen was to establish if an in vivo interaction would be possible. There were three main criteria that were thought to be important for investigating the biological relevance of the putative interactions. Firstly, it was reasoned that if the protein interacts with Mirror in vivo, it must be co-expressed with Mirror in the same cells and at the same time. Therefore, a comparison of the expression patterns between the putative interactors and *mirror* was undertaken. In addition to confirming the likelihood of a physical interaction, studying the expression patterns of putative interactors could also provide hints as to the nature of the interaction. The transcription factor Mirror might be expected to have both general interaction partners such as co-factors and regulators, and specific interaction partners such as other transcription factors or signalling molecules. These different types of interactors would be predicted to have different expression patterns, the former to be ubiquitously expressed and the latter to overlap with Mirror at specific sites. It was also thought that a comparison of the phenotypes of mutant alleles with the *mirror* phenotype might aid the establishment
of a functional interaction. Unfortunately, this type of analysis would only be possible for two of the putative interactors, as most of the proteins were previously unknown and lacked any characterised alleles. Finally, an important way of ascertaining if there was an in vivo association between the proteins would be to demonstrate a genetic interaction. This could either be an interaction between alleles of mirror and the gene in question or it could be the effect of loss of the putative interactor on an over-expression phenotype of Mirror. The types of genetic interaction observed might also imply the relationship between the two proteins, ie if the putative interactor is a regulator of Mirror function or a necessary cofactor. The results of the above investigations would then be used to determine which of the putative interactors would be studied in detail.

3.1.1 Selected clones for further analysis

The main parameter for selecting putative interactors for further analysis was that the interaction seemed to be a true yeast-two-hybrid interaction. Therefore, some of the proteins selected that gave strong or variable background activation of reporter genes in the absence of Mirror were not selected. In addition, some of the yeast-two-hybrid interactors that had known or predicted functions that did not seem compatible with a role as a transcription factor binding partner were also not analysed further. Table 3.2 below lists the yeast-two-hybrid clones that were selected for further analysis in Drosophila, including information on any known domains or homologies to proteins from other species. The table also lists the exact portion of the protein that was selected in the yeast-two-hybrid screens and which is the putative Mirror interaction domain. Of the proteins of known function, Castor and Dichaete will be introduced in separate sections below and CHD1 is discussed in detail in chapter 5.

Some of the previously uncharacterised proteins contain recognised domains or motifs or share limited homology with proteins from other species. The novel protein
CG1135 which contains a forkhead associated (FHA) domain is discussed in detail in chapter 4. CG30337 and CG5740 both have regions that are predicted to form coiled-coils, which are protein interaction domains. CG30337 consists of mainly coiled-coil regions and is moderately homologous to a novel mouse protein termed Rab6 interaction protein 2 (unpublished submission to GenBank). CG5740 contains an N-terminal coiled-coil region which shows weak homology to myosins. CG1962 also contains an N-terminal coiled-coil region, which is specifically homologous to an auto-antigen associated with paraneoplastic cerebellar degeneration in humans (Fathallah-Shaykh et al. 1991). CG13367 shows limited homology to mouse and human cDNAs which are highly expressed during eye development (unpublished submission to GenBank). The novel gene CG12919 contains a tumour necrosis factor-like (TNF) motif. The TNF motif is a trimeric motif found in all TNF ligands and is involved in the interaction with the TNF receptor via cystein-rich motifs (Bodmer et al. 2002). The presence of a TNF-like motif indicates that this protein may be involved in extracellular signalling. Finally, CG8448 contains a DNAJ domain. DNAJ domains were first studied in E. coli molecular chaperones and are believed to function as co-chaperones with Hsp70-type proteins (Hartl 1996). Mammalian relatives of DNAJ (Mrj) have been found to have a role in placental development (Hunter et al. 1999) and as chaperones for neuronal proteins (Chuang et al. 2002).
### Table 3.1 Putative interactors chosen for further analysis.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Location</th>
<th>Function/known domains/homologies</th>
<th>Total aa</th>
<th>Interaction domain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dichaete</td>
<td>70D3</td>
<td>HMG domain transcription factor</td>
<td>384</td>
<td>324-384</td>
</tr>
<tr>
<td>Castor</td>
<td>83C1</td>
<td>Zn-finger transcription factor</td>
<td>799</td>
<td>428-799</td>
</tr>
<tr>
<td>CG13367</td>
<td>1B13</td>
<td>No motifs, Limited homology mouse and human cDNA</td>
<td>488</td>
<td>75-488</td>
</tr>
<tr>
<td>CHD1</td>
<td>23C4</td>
<td>Chromo-Helicase-DNAbinding domain protein</td>
<td>1883</td>
<td>1481-1883</td>
</tr>
<tr>
<td>CG1962</td>
<td>38E1-E2</td>
<td>N-terminal Homology to paraneoplastic cerebellar degeneration antigen, cdr2</td>
<td>790</td>
<td>525-790</td>
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<tr>
<td>CG10395</td>
<td>41D1</td>
<td>No motifs or homologies</td>
<td>173</td>
<td>1-173</td>
</tr>
<tr>
<td>CG30337</td>
<td>45F1-F3</td>
<td>N-terminal coiled-coil motif</td>
<td>1456</td>
<td>917-1456</td>
</tr>
<tr>
<td>CG12919</td>
<td>46F1</td>
<td>Tumour necrosis factor (TNF)- like motif</td>
<td>325</td>
<td>117-325</td>
</tr>
<tr>
<td>CG8448</td>
<td>53B4-C1</td>
<td>Several polypeptides predicted, all contain DNAJ domain.</td>
<td>CG8448-RC 259</td>
<td>69-256</td>
</tr>
<tr>
<td>CG1135</td>
<td>64A4</td>
<td>Forkhead associated (FHA) domain</td>
<td>578</td>
<td>169-578</td>
</tr>
<tr>
<td>CG3987</td>
<td>88E2</td>
<td>No motifs or homologies</td>
<td>404</td>
<td>213-404</td>
</tr>
<tr>
<td>CG5740</td>
<td>94A2</td>
<td>N-terminal coiled-coil region, weak homology to myosins</td>
<td>1483</td>
<td>1026-1483</td>
</tr>
</tbody>
</table>

Table 3.1 Information on location of genes, known functions, domains, homologies and total length in amino acids from Gad-Fly and Flybase. Interaction domain indicates amino acids present in the cDNA clone isolated from the yeast-two-hybrid screens.

### 3.2 The putative interactors Dichaete and Castor

#### 3.2.1 Introduction to Dichaete.

Dichaete or Fish-hook as it is also known, is a High Mobility Group (HMG) domain containing protein of the SOX family. The founding member of the SOX family is the testis-determining gene Sry, and SOX family proteins are divided into six sub-groups
based on their homology within the HMG DNA-binding domain or Sry box (Penvy and Lowell-Badge 1997). Due to the fact that HMG domains interact with the minor groove and thus induce a 70-85° bend in the DNA, it has been suggested that these types of protein function in stabilising chromatin architecture and facilitating assembly of protein/DNA complexes (Wegner 1999). Some SOX proteins can activate transcription directly, but detailed studies of in vivo functions have revealed that most act in concert with other transcription factors.

Dichaete was originally named after a dominant phenotype causing out-held wings and was discovered independently by two groups to be a SOX domain protein required for embryonic segmentation and CNS development (Nambu and Nambu 1996; Russell et al. 1996). The expression pattern during embryogenesis is dynamic starting at stage 4 in a central domain which by late stage 5 resolves into seven transient stripes. At this stage expression is also seen in the procephalic region. Dichaete is expressed in the neuroectoderm and the brain from stage 7 and later in some developing neuroblasts and neurons as well as in various cells of the midline. From about stage 10 Dichaete is also expressed in the hindgut (Ma et al. 1998; Soriano and Russell 1998). Dichaete is expressed post-embryonically in restricted patterns in the eye-antennal and leg discs as well as in the CNS and the salivary glands (Mukherjee et al. 2000).

Early functional studies showed that the segmental defects in dichaete mutants were due to defects in the expression of the pair-rule genes even-skipped (eve), hairy and runt and the axonal CNS phenotype was found to be due to defective development of midline glia (Russell et al. 1996; Soriano and Russell 1998). The Dichaete protein has been demonstrated to bind to the SOX consensus DNA binding motif as well as fragments of the eve regulatory region containing such motifs in vitro (Ma et al. 1998). Dichaete was also found to be important for the development of the brain and hindgut through regulation of genes such as wingless, engrailed and decapentaplegic (Sanchez-Soriano and Russell 2000). Post-embryonic functions of Dichaete also seem to be mediated
through regulation of these genes as *dichaete* clones in the leg disc show reduced *wg* expression and clones in the antennal disc show reduced *en* expression (Mukherjee et al. 2000). The dominant Dichaete phenotype in the wing is due to ectopic expression in the wing disc resulting from chromosome aberrations.

The closest vertebrate homologue to Dichaete is Sox2 and the HMG domains of these proteins are 88% identical. In fact, mouse Sox2 can rescue the midline defects seen in dichaete mutants to a similar degree as a Dichaete transgene (Soriano and Russell 1998). SOX2 has been show to interact with the POU-domain protein OCT-3 during early mouse embryogenesis to control FGF4 expression (Wegner 1999). Interactions between Dichaete and *Drosophila* POU-domain proteins have been detected at several stages of development. The early function of Dichaete in segmentation is probably due to interactions with one or both of the POU domain proteins Pdm-1 and Pdm-2. Early studies showed a genetic interaction between *dichaete* and *pdm* mutants (Nambu and Nambu 1996). In the midline, Dichaete is co-expressed with another POU-domain protein, Drifter (also called *ventral veins lacking*), and genetic interactions have been demonstrated between *dichaete* and *vvl* mutants (Soriano and Russell 1998). Recent studies confirmed that Dichaete interacts physically with both Pdm1 and 2 as well as Drifter and the basic-helix-loop-helix-PAS transcription factor Single-minded in a yeast-two-hybrid assay (Ma et al. 2000).

3.2.2 Comparison of the Dichaete and Mirror expression patterns

The very dynamic embryonic expression pattern of dichaete as determined by both in situ hybridisation and antibody staining is described above. There are three possible locations for overlapping expression between Dichaete and Mirror, the neuroectoderm, neuroblasts and the brain. Mirror is expressed in the neuroectoderm in a tight stripe at the anterior border of each segment and in NBs that delaminate from this region. Dichaete is
expressed in the medial and intermediate neuroectoderm and weakly in the NBs as they are delaminating, but then fades (Zhao and Skeath 2002). Therefore Mirror and Dichaete overlap in the Mirror expression domain in the neuroectoderm. Two medial NBs have been identified that continue to express Dichaete at high levels, 1-1 and 7-1 (Overton et al. 2001), whereas several lateral NBs activate expression at stage 10-11, namely 7-4, 5-6, 2-5 and 3-5. This means that Mirror and Dichaete are only known to be coexpressed in NBs 1-1 and 2-5. In addition, Dichaete and Mirror may also be coexpressed in the brain. There is no overlap in the Dichaete and Mirror expression domains in the imaginal discs.

3.2.3 Introduction to Castor

Castor or Ming as it is also known, was identified independently by two groups studying enhancer trap lines expressed in a subset of neuroblasts. Castor is a zinc finger transcription factor involved in CNS development (Cui and Doe 1992; Mellerick et al. 1992). Both groups found that the gene was required for the correct expression of engrailed in the CNS and ming alleles show subtle defects in the axonal scaffold of the ventral nerve cord. Later studies showed that Castor is required for the full expression of drifter and I-POU and for repression of pdm-1 and pdm-2 in the CNS (Kambadur et al. 1998). The Castor protein contains four consecutive C\textsubscript{2}-H\textsubscript{2}-C\textsubscript{2}-H\textsubscript{2} repeats which make up the zinc finger domain and has been shown to bind to DNA using electrophoretic mobility shift assays (EMSA). The consensus DNA binding sites for Castor is very similar to that of Hunchback, another zinc finger protein expressed in neuroblast (Kambadur et al. 1998). The expression of Castor in the embryo is restricted to specific cells at the midline of the CNS at stage 9 and is later found in a subset of neuroblasts (NB) and ganglion mother cells (GMC) from stage 11 to 16 (Cui and Doe 1992). Castor is also expressed in brain cell clusters at larval and pupal stages and is required for correct axon pathfinding (Hitier...
et al. 2001). A detailed analysis of the temporal expression patterns of Hunchback, Kruppel, Pdm and Castor found that they were expressed sequentially in all NBs examined and that this sequential expression was important for specification of cell fates (Isshiki et al. 2001). To establish this temporal expression Castor is positively regulated by Pdm and negatively regulated by Kruppel (Brody and Odenwald 2000) (Isshiki et al. 2001).

3.2.4 Comparison of the Castor and Mirror expression pattern

The embryonic expression pattern of Castor has been determined in detail by both in situ hybridisation and staining with specific antibodies (Cui and Doe 1992; Kambadur et al. 1998) and is strictly limited to the developing nervous system. During their efforts to identify markers for all the neuroblasts in the Drosophila CNS, Chris Doe and colleagues have described the expression pattern of the mirror and ming-lacZ enhancer traps (Broadus et al. 1995; Doe 1992; Doe 2002). It is therefore known that Mirror and Castor are co-expressed in the medial NBs 1-1, 1-2, 2-2, 2-1 and 6-1, the intermediate NB 3-2, the lateral NBs 2-4, 2-5 and 3-4 and the MNB. NBs 2-1 and 3-4 co-express Castor and Mirror as they delaminate, whilst most of the other NBs (except 6-1) express Mirror first and castor slightly later in their development. In addition to specific NBs Castor and Mirror are also both expressed in the brain. In order to visualise the co-expression of Castor and Mirror proteins in the embryo, double antibody staining was performed using fluorescently labelled secondary antibodies. Confocal images showing the localisation of Mirror and Castor can be seen in figure 3.1.
Figure 3.1. Co-localisation of Mirror and Castor in the CNS

Figure 3.1 Double antibody staining using anti-Castor and anti-Mirror antibodies. All panels are confocal sections showing the ventral nerve cord of a stage 11 embryo. Castor staining is shown in red (A) and Mirror staining is shown in green (B). The merge (C) shows that Castor and Mirror are co-expressed in several developing neural cells as indicated by yellow staining.

The close up of the double stainings in D clearly shows that both proteins are present in a number of neural cells, specifically in the medial (arrow) and intermediate domains (arrowhead).
3.3 Comparison of the expression patterns of novel genes with that of mirror

In order to study the expression patterns of previously uncharacterised genes, in situ hybridisation in *Drosophila* embryos to detect the predicted transcripts was performed. In most cases, the full length pACT2 library clone isolated from yeast was first subcloned into pBluescript using the appropriate restriction enzymes. The pBluescript clones which contain both T7 and T3 RNA polymerase promoters, could then be used as templates for the DIG-labeled RNA probe. Due to the fact that the clones often include the 3'UTR and C-terminal portions of the protein, it was hoped that the detected expression patterns would be specific for the selected gene. In some cases, when the library clone was not suitable for use as a template, usually due to problems with restriction sites for linearization, a *Drosophila* EST clone (ResGen) from the gene was used. All the ESTs used as templates were in the pOT2 vector which uses the SP6 polymerase promoter for anti-sense RNA production.

The in situ hybridisation patterns of most of the genes studied seemed to be ubiquitous. An example of what was termed ubiquitous staining can be seen in figure 3.2. One of the anti-sense probes did not give significant staining over background (in situ hybridisation protocol followed using no RNA or sense probe) and was therefore termed inconclusive. Two of the patterns, although mainly ubiquitous, seemed to show an enrichment for the transcript in some embryonic structures. CG1931 seems to be expressed at a consistent level in the whole embryo up until stage 11-12 before the expression seems to become stronger in the CNS (figure 3.2). CG8448 is also expressed throughout the embryo at earlier stages, but at later stages it also seems to be enriched in narrow segmental bands close to the epidermis (figure 3.2).
Figure 3.2. Examples of ubiquitous expression patterns

Figure 3.2. In situ hybridisations on *Drosophila* embryos using RNA probes made from various pACT2 library clones in pBluescript. In all panels anterior is left and dorsal up. A and B: in situ hybridisations using a probe for IV 10a (CG1135) showing ubiquitous expression at stage 10 (A) and stage 14 (B). C: in situ hybridisation using a probe for IV 29a (CG30337) showing ubiquitous staining and enhanced expression in the CNS at stage 13-14. D: in situ hybridisation using a probe for III 12b (CG8448) showing ubiquitous staining with enhanced expression in a segmental pattern at stage 14-15.
Three of the previously uncharacterised genes have distinct expression patterns in the embryo. As seen in figure 3.3, CG1962 is expressed exclusively in the pole cells from stage 5 to about stage 10. From stage 10 or 11 the transcript can be seen in a few cells which may be neuroblasts and expression continues and becomes more widespread in cells of the ventral nerve cord and brain until stage 16. Mirror is also expressed in the developing CNS and it is therefore possible that the two proteins are coexpressed. A comparison of the in situ hybridisation patterns of CG 1962 and mirror can be seen in figure 3.4.

Figure 3.5 shows the expression pattern of CG12919. Expression of GC12919 starts at about stage 4-5 in dorsal regions of the embryo. Expression continues in the dorsal folds and amnioserosa at stages 6-7, but is not present at germband retraction. Later, at stage 12-14, expression reappears in distinct structures in the head region of the embryo and in two dots posteriorly, which could be part of the PNS. The expression pattern of CG12919 overlaps with the mirror expression patterns in early development as can be seen from the comparison in figure 3.6.

The in situ hybridisation pattern for CG5740 was quite weak, but the transcript seemed to be present in the embryonic gut at the invagination between the proventriculus and the midgut as well as in the hindgut at stages 13-15, figure 3.7. Mirror is also expressed in the area of the proventriculus and could therefore overlap with CG5740 (figure 3.7). Table 4.2 summarises the expression patterns determined for the novel interactors and the possible overlaps with mirror expression.
Figure 3.3. Expression pattern of CG1962

In situ hybridisation on *Drosophila* embryos using an RNA probe made from the pACT2 library clone IV31b cloned into pBluescript. A-E are lateral views with anterior left and dorsal up. F is a ventral view with anterior left. Embryos of approximately these stages are shown: A) stage 5, B) stage 9, C) stage 10-11, D) late stage 11, E) stage 13-14, F) stage 14-15. The CG1962 transcript can be detected in the pole cells from stage 5 to about stage 10 when expression fades. CG 1962 is expressed in the CNS from about stage 10-11 until stage 16. Initially expression seems limited to a few cells, probably delaminating neuroblasts, but the expression becomes more widespread and by stage 13 the transcript seems to be present in numerous cells in the ventral nerve cord and brain.
Figure 3.4. Comparison of the expression patterns of CG1962 and *mirror*

A and C are in situ hybridisations using the pBluescript IV31b probe and B and D are in situ hybridisations using the *mirror* 9B probe. A and C are about stage 11 and B and D are about stage 13-14. Both CG1962 and *mirror* are expressed in cells of the CNS from stage 10 – 16, so their expression could overlap.
Figure 3.5. In situ hybridisation on Drosophila embryos using a pBluescript clone of library clone pACT2 II-48c (CG12919) as template. In all panels anterior is right, A-D lateral view with dorsal up, E and F dorsal view. Embryos of these stages are shown: A) stage 5, B) stage 6, C) stage 7-8, D) stage 9-10, E) stage 10-11, F) stage 14-15.

Expression of the mRNA can be seen dorsally at stage 5 and in the dorsal folds and amnioserosa at stages 6-10. At about stage 14-15, expression can be seen in two posterior structures as well as in the head.
Figure 3.6. Comparison of the in situ hybridisation patterns of CG 12919 and *mirror*. In all panels anterior is left and dorsal is up. A-C: in situ hybridisation patterns for CG 12191, D-F: in situ hybridisation patterns for *mirror*. A and D are stage 5, B and E are stage 7, C and F are stage 9-10. Both CG12919 and *mirror* are expressed dorsally early in development and both are expressed in the dorsal folds during germband retraction as well as in the amnioserosa.
Figure 3.7. Expression pattern of CG5740 and comparison with the mirror expression pattern

Figure 3.7. In situ hybridisations of Drosophila embryos using EST GH11144 from CG5740 and pBluescript clone mirror 9B as templates. All panels are dorsal views of embryos at stage 12-14, anterior is up. A-C: in situ hybridisation patterns of CG5740, D and E: in situ hybridisation pattern of mirror. Expression of CG5740 can be seen at the junction between the proventriculus and the midgut at stages 13-15 and in the hindgut at the same stages (A-C). Mirror is expressed in the proventriculus and may overlap with CG5740 (D and E).
Table 3.2 Summary of expression patterns of putative interactors

<table>
<thead>
<tr>
<th>Putative interactor</th>
<th>Expression pattern</th>
<th>Overlap with mirror expression pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dichacte</td>
<td>Published</td>
<td>Neuroectoderm, neuroblasts, brain</td>
</tr>
<tr>
<td>Castor</td>
<td>Published</td>
<td>Neuroblasts (figure 3.1), brain</td>
</tr>
<tr>
<td>CG13367</td>
<td>Ubiquitous</td>
<td>Yes</td>
</tr>
<tr>
<td>CHD1</td>
<td>Ubiquitous</td>
<td>Yes</td>
</tr>
<tr>
<td>CG1962</td>
<td>Expressed in pole cells stage 5-11, CNS from stage 10-11.</td>
<td>Possibly ventral nerve cord and brain (figure 3.4)</td>
</tr>
<tr>
<td>CG10395</td>
<td>Ubiquitous</td>
<td>Yes</td>
</tr>
<tr>
<td>CG30337</td>
<td>Ubiquitous, enriched in CNS late.</td>
<td>Yes</td>
</tr>
<tr>
<td>CG12919</td>
<td>Expressed dorsally stage 4-5, dorsal folds and amnioserosa stage 6-10, head and posteriorly stage 12-14.</td>
<td>Early dorsal, dorsal folds and amnioserosa (figure 3.6)</td>
</tr>
<tr>
<td>CG8448</td>
<td>Ubiquitous, possibly enriched in segmental pattern late.</td>
<td>Yes</td>
</tr>
<tr>
<td>CG1135</td>
<td>Ubiquitous</td>
<td>Yes</td>
</tr>
<tr>
<td>CG3987</td>
<td>Inconclusive</td>
<td>N/D</td>
</tr>
<tr>
<td>CG5740</td>
<td>Expressed foregut-midgut boundary and hindgut stage 12-15.</td>
<td>Possibly anterior gut (figure 3.7)</td>
</tr>
</tbody>
</table>

Table 3.2. Summary of expression patterns as determined by in situ hybridisation and possible overlaps with mirror expression. N/D, not determined.

3.4 Genetic interactions

3.4.1 Genetic interactions between putative protein interactors and mirror alleles

In order to investigate possible genetic interactions between mirror and the genes coding for the putative protein interactors, different mirror alleles were used in simple heteroallelic interaction tests. The mirror alleles used were \( mrr^{P1} \), \( mrr^{P2} \) and \( mrr^{48} \) (McNeill et al. 1997). \( mrr^{P1} \) is a homozygous viable P element insertion within 300 bp of...
the *mirror* transcriptional unit. \textit{mrr}^{P2} is a lethal P-element insertion situated 234 bp upstream of *mirror*. Both \textit{mrr}^{P1} and \textit{mrr}^{P2} are enhancer-traps for *mirror* expression. The \textit{mrr}^{48} allele is an excision of the \textit{mrr}^{P1} P-element and is the strongest *mirror* allele available. The excision does not however remove the *mirror* gene from the genome, but is probably a regulatory mutation as the upstream enhancer and promoter regions for *mirror* are affected by the excision (H. McNeill, unpublished). Characterised alleles exist for only two of the identified yeast-two-hybrid interactors, \textit{dichaete} and \textit{castor}. There are no alleles known for CHD1 and although a lethal P-element allele might exist for CG1135, this has not been characterised. Interactions between *mirror* and this allele will be discussed in chapter 4. \textit{dichaete} alleles D^{72} and D^{513} were obtained from Steve Russell (Soriano and Russell 1998), and \textit{castor} allele \textit{ming}^{24} from Chris Doe (Cui and Doe 1992). For the other interactors, deficiencies covering the genes were obtained from the Bloomington stock center. The breakpoints of the deficiencies used are listed in table 3.3. There were no deficiencies available that covered CG10395, CG30337 and CG3987 so these could not be tested for genetic interactions.

Genetic interactions were first assayed by crossing the *mirror* alleles to alleles for \textit{dichaete} and \textit{castor} as well as to deficiencies covering most of the novel interactors and looking at survival of progeny that carried both alleles. Table 3.3 below summarises the results of the crosses performed. As can be seen from the results, there seems to be no effect of reducing the amount of any of the putative interactors on the survival of heterozygous *mirror* alleles. In addition to survival, the progeny from the crosses were also checked for any phenotypes that might indicate an interaction with the *mirror* allele, but no such phenotypes were observed.
Table 3.3. Heteroallelic interactions between *mirror* and putative protein interactors.

<table>
<thead>
<tr>
<th>Putative interactor</th>
<th>Allele/ Deficiency</th>
<th>Comments/ Breakpoints</th>
<th>Percent viable progeny over <em>mirror</em> alleles</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>mrr&lt;sup&gt;P2&lt;/sup&gt;</em></td>
</tr>
<tr>
<td>Dichae te</td>
<td><em>dichaete&lt;sup&gt;72&lt;/sup&gt;</em></td>
<td>EMS alleles from Steve Russell</td>
<td>33% (33%)</td>
</tr>
<tr>
<td>Dichae te</td>
<td><em>dichaete&lt;sup&gt;531&lt;/sup&gt;</em></td>
<td></td>
<td>28% (33%)</td>
</tr>
<tr>
<td>Castor</td>
<td><em>ming&lt;sup&gt;24&lt;/sup&gt;</em></td>
<td>P element excision allele from Chris Doe</td>
<td>38% (33%)</td>
</tr>
<tr>
<td>CG13367</td>
<td>Df(1)su(s)83</td>
<td>1B10;1D6-E1</td>
<td>N/D</td>
</tr>
<tr>
<td>CHD1</td>
<td>Df(2L)JS17</td>
<td>23C1-2;23E1-2</td>
<td>40% (25%)</td>
</tr>
<tr>
<td>CG1962</td>
<td>Df(2L)TW2</td>
<td>37D6-E1;38E6-9</td>
<td>17.5% (25%)</td>
</tr>
<tr>
<td>CG8448</td>
<td>Df(2R)Jp4</td>
<td>52D3-53A1</td>
<td>17% (25%)</td>
</tr>
<tr>
<td>CG8448</td>
<td>Df(2R)Jp6</td>
<td>51F13;52F8-9</td>
<td>25% (25%)</td>
</tr>
<tr>
<td>CG12919</td>
<td>Df(2R)X1</td>
<td>46C2;47A1</td>
<td>25% (25%)</td>
</tr>
<tr>
<td>CG12919</td>
<td>Df(2R)stan1</td>
<td>46D7-9;47F15-16</td>
<td>39% (25%)</td>
</tr>
<tr>
<td>CG1135</td>
<td>Df(3L)GN24</td>
<td>63F6-7;64C13-15</td>
<td>31% (25%)</td>
</tr>
<tr>
<td>CG5740</td>
<td>Df(3R)e-GC3</td>
<td>93C6;94A1-4</td>
<td>32% (33%)</td>
</tr>
</tbody>
</table>

Table 3.3. Crosses were allele or deficiency/balancer (chromosomes I, II or III) x *mrr<sup>P2</sup>/TM6 or *mrr<sup>48</sup>/TM3*. Percentages shown are the percent of progeny which carry one copy of the allele or deficiency and one copy of the *mrr<sup>P2</sup>* or *mrr<sup>48</sup>* (expected percentage of progeny according to mendelian ratios in brackets).

In order to investigate the possibility of a genetic interaction between *castor* and *mirror* further, the *mrr<sup>P1</sup>* and *mrr<sup>P2</sup>* *mirror* alleles were recombined onto the *ming<sup>24</sup>* chromosome. Double mutant chromosomes were confirmed to contain both the *ming* and *mirror* alleles by backcrossing to the original stocks. The effect of loss of one copy of *castor* on *mrr<sup>P1</sup>* homozygous viability and the survival of *mrr<sup>P1</sup>/mrr<sup>P2</sup>* flies was then tested. The results summarised in table 3.4 below show that there is no affect on survival.
Table 3.4. Genetic interactions between castor and mirror alleles

<table>
<thead>
<tr>
<th>Alleles</th>
<th>Percent viable progeny (expected percentage)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mrr&lt;sup&gt;pl&lt;/sup&gt;</td>
</tr>
<tr>
<td>mrr&lt;sup&gt;pl&lt;/sup&gt;</td>
<td>viable</td>
</tr>
<tr>
<td>mrr&lt;sup&gt;p2&lt;/sup&gt;</td>
<td>33% (50%) lethal</td>
</tr>
</tbody>
</table>

Table 3.4. The mrr<sup>pl</sup>/ mrr<sup>pl</sup> viable stock and the mrr<sup>p2</sup>/TM6 stock were crossed to mrr<sup>pl</sup>/ mrr<sup>pl</sup>; mrr<sup>p2</sup>/TM6; ming<sup>24, mrr<sup>pl</sup></sup>/TM3 or ming<sup>24, mrr<sup>p2</sup></sup>/TM3. Percentage of viable progeny (with expected percentages according to mendalian ratios in brackets) are indicated.

Further investigations of genetic interactions between *dichaete* and *mirror* by the creation of double mutant chromosomes was not attempted as the two genes are located less than 1.5 Mb apart on 3L and isolating recombinants would have been difficult.

3.3.2 Affects of alleles of putative interactors on mirror over-expression phenotypes

As detecting heteroallelic interactions may be difficult, a second approach using Mirror over-expression as a sensitised background was attempted. Two over-expression systems were used. Firstly, a mirror transgene controlled by the hsp70 promoter was used. Raising these flies at 29°C causes low levels of ubiquitous Mirror expression which leads to a reduction in viability (see table 3.5). The reduction in viability can be rescued by a deficiency for the Iro-C which has been shown to lack mirror transcript (iro<sup>DPM</sup>) (Diez del Corral *et al.* 1999). The effect of the reduced levels of the putative interactors on viability was determined by crossing homozygous hsp70-*mirror* flies with the mutant alleles or deficiencies and raising the progeny at 29°C. The results of the crosses can be seen in table 3.4. The *dichaete* allele D<sup>513</sup> caused a slight increase in viability for flies over-expressing Mirror, although not as much as the Iro deficiency. Both the ming<sup>24</sup> allele and the deficiency covering CHD1 showed slight affects on survival, but these may not be significant. Deficiencies covering CG13367, CG8448, CG5740 and CG 1135 did cause a
significant reduction in viability, whereas the deficiencies covering CG12919 lead to a dramatic drop in survival.

The second over-expression system for detecting interactions with mirror used a UAS-*mirror* transgene and the glass multiple repeat (GMR) Gal4 driver. The GMR driver causes expression of Gal4 in all differentiating cells in and behind the morphogenetic furrow during eye development (Freeman, M. 1997). When Mirror is expressed using GMR-Gal4, the eyes become very rough with disorganised ommatidia. At higher temperatures (which increases the activity of the Gal4 transcription factor) pigment cells and possibly photoreceptor cells are lost and the number of bristles seem increased (this might be due to the bristles being closer together due to loss of photoreceptors), figure 3.8. The effect of reducing the amount of any of the interactors on this phenotype was investigated by crossing flies containing both the UAS-*mirror* and GMR-Gal4 transgenes to the deficiencies. The eye phenotype was scored as suppressed or enhanced over the deficiency compared to progeny which did not contain the deficiency from the same cross. A summary of the results can be seen in table 3.5 below. The observed effects were however very mild and difficult to document. Removing one copy of the Iro-C only caused a slight suppression of the UAS-*mirror*, GMR-Gal4 phenotype as seen in figure 3.8. The deficiency covering the CHD1 gene also caused a slight suppression of the phenotype whilst deficiencies covering CG1962, CG8448 and CG1135 caused a slight enhancement (figure 3.8).
<table>
<thead>
<tr>
<th>Putative interactor</th>
<th>Chromosomes for testing</th>
<th>Viable progeny with ( 1x \text{hsp}70 \text{mirror} )</th>
<th>Effect on phenotype caused by GMR-Gal4, UAS-Mirror</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Wild type</td>
<td></td>
<td>37%</td>
<td>No effect</td>
</tr>
<tr>
<td>Control TM3/+</td>
<td></td>
<td>39%</td>
<td>N/D</td>
</tr>
<tr>
<td>Control TM6/+</td>
<td></td>
<td>45%</td>
<td>N/D</td>
</tr>
<tr>
<td>Control Cyo/+</td>
<td></td>
<td>56%</td>
<td>N/D</td>
</tr>
<tr>
<td>Control ( iro^{DFM3} )/TM6</td>
<td></td>
<td>79%</td>
<td>Slight suppression (figure 3.8)</td>
</tr>
<tr>
<td>Dichacte ( dichaete^{551} )/TM3</td>
<td></td>
<td>70%</td>
<td>Not done</td>
</tr>
<tr>
<td>Castor ( ming^{23} )/TM3</td>
<td></td>
<td>65%</td>
<td>Not done</td>
</tr>
<tr>
<td>CG13367 Df(1)su(s)83/ Dp(1;Y)y^{sc}</td>
<td></td>
<td>17%</td>
<td>No obvious effect</td>
</tr>
<tr>
<td>CHD1 Df(2L)JS17/CyO</td>
<td></td>
<td>41%</td>
<td>Slight suppression (figure 3.8)</td>
</tr>
<tr>
<td>CG1962 Df(2L)TW2/CyO</td>
<td></td>
<td>N/D</td>
<td>Possible enhancement</td>
</tr>
<tr>
<td>CG8448 Df(2R)Jp4/CyO</td>
<td></td>
<td>26%</td>
<td>Slight enhancement</td>
</tr>
<tr>
<td>Df(2R)Jp6/CyO</td>
<td></td>
<td>38%</td>
<td>Slight enhancement</td>
</tr>
<tr>
<td>CG12919 Df(2R)X1/CyO</td>
<td></td>
<td>8%</td>
<td>No obvious effect</td>
</tr>
<tr>
<td>Df(2R)stan1/ CyO</td>
<td></td>
<td>0%</td>
<td>No survivors</td>
</tr>
<tr>
<td>CG1135 Df(3L)GN24/TM3</td>
<td></td>
<td>16%</td>
<td>Slight enhancement (figure 3.8)</td>
</tr>
<tr>
<td>CG5740 Df(3R)e-GC3/TM6</td>
<td></td>
<td>20%</td>
<td>No obvious effect</td>
</tr>
</tbody>
</table>

Table 3.5. The homozygous stock hsp70 \textit{mirror}/hsp70 \textit{mirror} was crossed to flies of the genotype indicated in column 2. In each case the chromosome for testing is listed first. The crosses were raised at 29°C and eclosed progeny scored. Percentages indicate surviving progeny which contain the chromosome tested and the \textit{hsp70 mirror} chromosome. In each case, the expected survival is 50%. For interactions with the GMR \textit{mirror} phenotype, crosses were GMR-Gal4/Cyo;UAS-mirror/UAS-mirror x lines listed in column 2. Flies carrying both the GMR-Gal4, UAS-\textit{mirror} and deficiency were compared to the flies carrying the GMR-Gal4, UAS-\textit{mirror} and balancer chromosome.
Figure 3.8. Effects of deficiencies on the GMR-Mirror ectopic expression phenotype

Figure 3.8. Pictures of adult heads (dorsal is up) of flies expressing Mirror under the control of the GMR promoter. A) GMR-GAL4/+; UAS-mirror/TM3 Sb, Tb. D) GMR-GAL4/+; UAS-mirror/iro Df(2L)JS17; UAS-mirror/+. C) GMR-GAL4/+; UAS-mirror/TM3 Sb. E) GMR-GAL4/ Df(2L)JS17; UAS-mirror/+. G) GMR-GAL4/+; UAS-mirror/TM3 Sb. F) GMR-GAL4/+; UAS-mirror/ Df(3L)GN24. Removing one copy of the Iro complex gives rise to a slight suppression of the GMR-mirror phenotype (A and D). The deficiency covering CHD1 also causes suppression of the rough eye phenotype, although there are other notable effects on the phenotype possibly caused by the difference in genetic backgrounds (C and E). A deficiency covering the novel gene CG1135 gives rise to a slight enhancement of the GMR-Mirror phenotype (C and F).
3.5 Discussion

3.5.1. Comparison of expression patterns between mirror and novel interactors

One of the most important criteria for establishing the possibility of an in vivo interaction between Mirror and the selected yeast-two-hybrid proteins is that they are expressed in the same cells at the same time. Studying the expression patterns of putative interactors could also provide hints as to the nature of the interaction. To investigate if the putative interactors co-localize with Mirror, the embryonic expression patterns of novel genes was characterised by in situ hybridisation using either the partial cDNA clones isolated in the yeast-two-hybrid screen or ESTs from the appropriate gene. Only one probe for each gene was tested and it is therefore possible that some of the patterns obtained do not truly reflect the presence of the transcript, in particular those which show ubiquitous expression patterns. One of the probes tested did not produce any signal above the background control developed for the same length of time. In order to confirm these results, in situ hybridisations could be performed using probes from a different part of the cDNA. Another caveat to this type of analysis is that it has to assume that the presence of a transcript indicates the presence of a functional protein. This not always the case as mRNAs may be regulated post-transcriptionally so that translation of the protein only occurs under certain circumstances. In addition proteins may be post-translationally modified and may be inactive although present in the cell.

Out of ten novel genes analysed, four seemed to have completely ubiquitous expression patterns and two seemed to be ubiquitous, but might be more enhanced in certain structures. The proportion of novel genes giving a non-specific expression pattern (70%) is similar to that of other studies where expression patterns of previously uncharacterised genes have been investigated. An in situ hybridisation screen for genes with a dorso-ventrally restricted expression pattern showed that approximately 30% of the
Chapter 3

embryonic cDNAs tested were expressed in specific patterns (Scuderi and Letsou 2001). Although the selection of genes in this study is not random, it would not be expected that all putative Mirror interactors are expressed exclusively in all or some of the mirror expression domains. In fact, it would be reasonable to assume that most of the interaction partners of Mirror would be general co-factors or regulators which also have other roles and interact with other proteins and therefore have a much wider expression profiles.

The proteins that are ubiquitously expressed include CHD1 and CG1135, which are discussed in detail in separate chapters. The only other ubiquitous gene that contains a specific domain which might hint at its function is CG8448. As described in the introduction, CG8448 contains a DNAJ domain, which is associated with a function as a co-chaperone. In addition to protein folding, molecular chaperones may have other roles in the cell such as protein complex assembly. Two mammalian DNAJ containing proteins have been described. They are expressed ubiquitously, but are enriched in certain areas and have been found to have specific roles in these tissues (Chuang et al. 2002; Hunter et al. 1999). Indeed, the expression pattern of CG8448 did seem to be more enriched in specific structures late in development.

Proteins that are required to co-operate with Mirror or affect Mirror function in specific cell types would be expected to have partially overlapping expression patterns. Three out of ten novel genes studied by in situ hybridisation gave specific patterns of expression. CG1962 shows expression in two specific cell types, the pole cells from stage 5-10, and in cells of the developing ventral nerve cord and brain from stage 11-16. Mirror is also expressed in neuroblasts and the CNS and it is possible that they overlap in various cells. A detailed analysis of both expression patterns would resolve if there is a specific overlap. CG1962 contains an N-terminal coiled-coil domain that is specifically homologous to a leucine zipper protein called PCD17 or cdr2, which is associated with neoplastic cerebellar degeneration in humans (Fathallah-Shaykh et al. 1991). This protein is found predominantly in the cytoplasm of Purkinje neurons and has been show to
interact with a putative transcription factor, MRG X (Sakai et al. 2002), and with c-Myc. PCD17 acts to down regulate the activity of c-Myc by sequestering it to the cytoplasm (Okano et al. 1999). It is intriguing to note that this protein also seems to have a role in the CNS of *Drosophila*, even though the C-terminal parts of the protein are not homologous. In fact the similarity between CG1962 and PCD17 is limited to about 100 amino acids in the N-terminus of both proteins and it is difficult to speculate if they would have similar functions.

The in situ hybridisation pattern detected for CG12919 in early development is very similar to the early *mirror* expression pattern. CG12919 expressed at stage 5 in dorsal parts of the embryo where expression continues in the dorsal folds and amnioserosa until stage 7. This exactly overlaps with the early expression patterns of *mirror*. The presence of a TNF-like domain however could indicate that CG12919 is a ligand of the TNF family and would therefore be expected to be extracellular or to be associated with the cell membrane. In fact, during the preparation of this manuscript, CG12191 was identified as Eiger, a TNF ligand that triggers the *Drosophila* INK pathway (Igaki et al. 2002). The authors reported the expression pattern of Eiger to be restricted to the CNS. In light of this the in situ hybridisation for the CG12919 probe was repeated, but found to be same as in figure 3.5. The differences in the observed patterns may be due to different probes having been used in each experiment.

CG5740 showed a very restricted expression and although this needs to be confirmed, it indicates that this gene has a very specific role during development. Although CG5740 does not contain any domains that indicate its role in the cell, the presence of an N-terminal coiled-coil motif could indicate that the protein acts as a dimer or higher multimer, or that it interacts with coiled coils from other proteins. The expression of *mirror* and CG5740 might overlap around the border between the midgut and the proventriculus and careful examination of double in situ hybridisations would be required to verify this. The interaction between CG5740 and Mirror is especially
interesting as this interactor was identified three times during the yeast-two-hybrid screens and seems to interact specifically with the homeodomain.

3.5.2. Genetic interactions between mirror and novel protein interactors

One of the main ways of demonstrating that an in vitro physical interaction is reflecting a functional in vivo interaction would be to prove that mutations in the genes in question interact genetically. The effects observed can be used to speculate on the function of the interaction. Positive regulators and necessary co-factors would be expected to effect over-expression phenotypes most dramatically. Negative regulators and cooperating co-factors would be expected to affect the mutant alleles more than over-expression phenotypes. Genetic interactions were investigated by testing the effect of removing one copy of a putative interactor on mutant alleles of mirror and on ectopic expression of Mirror. Specific genetic interactions can only be investigated using characterised mutant alleles which were only available for two of the putative interactors, castor and dichaete. However, it was thought that deficiencies covering genes of other putative interactors could be used to get an indication of possible interactions. The deficiencies covered from about 50-200 genes and it was therefore possible that the interactions observed were due to any of the genes covered. On the other hand, if no interaction could be detected, it would indicate that even a specific allele would not show any effect in the systems used.

Removal of one copy of any of the putative interactors did not seem to affect the survival or produce any obvious phenotypes in flies carrying various mirror mutations (none of which are null mutations). As these interactions were only carried out using single alleles of mirror which are perfectly viable, it would only be possible to detect interactions which caused a decrease in survival or created an adult phenotype. Such heteroallelic interactions are difficult to detect, even between proteins that have been
demonstrated to interact by other means. A more rigorous way of testing interactions between genes would be to create double mutant flies in which the effect of loss of one copy of the interactor can be tested on various semi-viable combinations of *mirror* alleles.

As heteroallelic interactions are difficult to detect, the effect of the deficiencies on the ectopic expression of Mirror was also investigated. First the effects on low levels of ubiquitous Mirror expression were detected by examining the survival of flies carrying an *hsp70 mirror* transgene at 29°C. Additionally, the effect on ectopic expression of Mirror in the eye was investigated using the GMR-Gal4 driver to express a UAS-*mirror* transgene. In addition to the deficiencies covering the yeast-two-hybrid interactors, a deficiency covering the Iro complex was also tested for interactions. It would be expected that reducing the endogenous levels of *mirror* would partly suppress the over-expression phenotype, and this is indeed the case for low level ubiquitous expression from the *hsp70* promoter. The rough eye phenotype caused by ectopic expression of Mirror using GMR was not affected to a great extent. This is probably due to the fact that GMR drives expression at very high levels in many cells which do not normally express *mirror*.

Most of the deficiencies had an effect on the Mirror over-expression phenotypes. Only one deficiency, covering CHD1, caused a slight suppression of the rough eye phenotype. The suppression effect was similar to that seen with the Iro deficiency. The putative interaction between CHD1 and Mirror will be discussed in chapter 5. Deficiencies covering CG13367, CG8448, CG5740 and CG 1135 caused a reduction in survival of the *hsp70 mirror* flies at 29°C and a slight enhancement of the GMR Mirror phenotype. Both of the deficiencies covering CG12919 had a dramatic effect on survival of flies with low level ubiquitous Mirror expression and also on the survival of flies expressing Mirror using the GMR driver, although escaping flies did not show an effect on the GMR Mirror phenotype.
The above results can only be taken to suggest that there might be a genetic interaction between mirror and the yeast-two-hybrid interactors covered by the deficiencies. The main caveat with this type of analysis is of course that the interactions observed could be due to any of the genes covered by the deficiencies. In addition the interactions could be affected by the balancer chromosomes present in each cross and the genetic background in general. In the flies expressing the hsp-70 mirror transgene, the balancers tested did not seem to have a significant effect on survival. However, some variation in the rough eye phenotype caused by the expression of Mirror with the GMR driver was seen in different backgrounds (compare A, C and E in figure 4.8). The effects seen on the GMR driven expression of mirror also assume that the gene in question is expressed in the eye disc after the furrow in order to affect the ectopic Mirror protein. The fact that the effects seen on the ectopic expression of Mirror using GMR were so slight could be due to the extremely high expression levels of this system. Lowering the temperature or using a different driver could have been useful for investigating the effects further.

3.5.3. Investigating the possibility of an interaction between Mirror and Dichaete

In respect to function, the HMG domain containing protein Dichaete appears to be a good candidate for a Mirror interacting protein. The minor groove binding activity of HMG domains causes a bend in the DNA and also makes the binding of other transcription factors in close proximity possible. Many proteins of the SOX family of which Dichaete is a member, cooperate with other factors in transcriptional regulation, some of which contain homeodomains (Wegner 1999). Dichaete itself has been shown to interact genetically and physically with Pdm1 and 2, Drifter and Single-minded. The specific portion of the Dichaete protein involved in these interactions has not been mapped, but it is thought that the HMG domain is important (Ma et al. 2000). The yeast-
two-hybrid clone selected as interacting with the C-terminal part if Mirror contains only the last 60 amino acids of the Dichaete protein. The C-terminus is the only region of similarity, apart from the HMG domain, between Dichaete and its closest vertebrate homologue, SOX2. A truncated Dichaete construct lacking the last 75 amino acids can rescue the midline defects seen in *dichaete* mutants, but the same construct does not produce the dominant wing phenotype associated with ectopic expression in wing discs (see below) (Soriano and Russell 1998). This indicates that the C-terminus of Dichaete may have a context dependent function.

Another aspect which in theory makes Dichaete a good interaction candidate to study, is the availability of characterised mutant alleles. Demonstrating a genetic interaction is a very good way of confirming a functional association in vivo, and mutant alleles also allow for comparison of phenotypes. Comparing specific phenotypes is however only relevant if both proteins are expressed and are thought to have a role in the process in question. Most of the embryonic phenotypes of *dichaete* have been characterised and fall into two categories, segmentation defects and CNS defects. Some *mirror* mutants show mild segmentation and CNS defects (see introduction). The segmentation phenotypes of *mirror* may seem quite similar to those of *dichaete* (data not shown), but Dichaete has a role very early in segmentation in regulation of segment polarity genes, whereas Mirror is only expressed at later stages and may be involved in controlling segmental boundaries.

Mutations in *dichaete* cause various CNS defects. The overlap between Mirror and Dichaete expression is limited to very restricted parts of the neuroectoderm, a few neuroblasts and the brain, and therefore any overlap in phenotypes should be restricted to these areas. In the neuroectoderm, Dichaete acts as a regulator of drosophila patterning and cell fate (Zhao and Skeath 2002). The role of Dichaete in specific neuroblasts is not known. A study of regulatory mutants showed that even if expression of *dichaete* was lost in neuroblasts, no obvious phenotype could be detected. Dichaete expression is
widespread in the CNS, but so far a specific role for *dichaete* in the brain is limited to the
development of the tritocerebrum (Sanchez-Soriano and Russell 2000). The CNS
phenotypes of *mirror* mutants have not been characterised in detail and the downstream
targets of Mirror in the embryo are not known. A detailed investigation of the mirror and
dichaete phenotypes concentrating on the areas of overlapping expression would be
necessary in order to uncover any overlapping functions.

The investigation of genetic interactions between *mirror* and *dichaete* are
complicated by the fact that the two genes are found within 1.5Mb of each other on 3L.
No significant interactions could be detected using simple hetero-allelic interactions or
Mirror over-expression systems. Most of the original dominant *dichaete* alleles are due to
chromosomal aberrations and a detailed study of these, revealed that two of the alleles, D^1
and D^3, also affect *mirror* expression (Russell 2000). D^3 is a partial revertant of D^1 in
which there is an additional lesion close to the 3' end of the dichaete gene which makes it
a protein null (Russell et al. 1996). Several studies of dichaete phenotypes have been
performed using the D^3 allele in combinations with other dichaete alleles, and this would
in fact include a heterozygous reduction of *mirror*. Although the phenotypes observed
were sometimes more severe compared to other combinations this is most likely due to the
fact that the D^3 allele was the only protein null available (Russell et al. 1996; Soriano and
Russell 1998).

The dominant wing phenotype associated with expression of Dichaete in the wing
disc may be the strongest argument in favour of an interaction between Dichaete and
Mirror. The phenotype, which is characterised by out-held wings and loss of proximal
structures such the alula is extremely similar to the phenotype observed in some
combinations of *mirror* alleles (Kehl et al. 1998). It was also found that that the phenotype
of the D^3 allele (which is a dichaete null) is actually due to a significant reduction in
*mirror* levels in the wing disc (Russell 2000). The dichaete phenotype can also be
replicated by the ectopic expression of a Dichaete in the wing hinge region using the Gal4
system. However, as mentioned above, a truncated Dichaete construct lacking the last 75 amino acids does not produce any phenotype. It is therefore very interesting that this is the part of Dichaete which interacts with Mirror in the yeast-two-hybrid system. It could be hypothesised that the Dichaete phenotype is therefore due to an ectopic interaction with Mirror which mimics a loss of function for \textit{mirror}. A detailed investigation of the effects of ectopic Dichaete and reduction of Mirror in the wing disc may uncover if this is indeed the case.

3.5.4. Investigating the possibility of an interaction between Mirror and Castor

Castor is a zinc finger transcription factor expressed in the embryonic and larval CNS and is required for the development of a subset of neuroblasts and neurons. Castor contains four zinc-fingers of the C2H2 or TFIIA type. The putative Mirror interaction domain lies within the last 371 amino acids, which includes the last three zinc-fingers. No Castor-binding proteins have been identified, but there are examples of C2H2 zinc-finger proteins interacting with other transcription factors. The zinc finger GATA-4 protein and the Nkx 2-5 homeodomain protein interact physically and act synergistically to activate transcription of the atrial natriuretic peptide promoter during vertebrate cardiac development (Durocher and Nemer 1998). In yeast, a physical interaction between the C2H2 zinc finger transcription factor Swi5 and the homeodomain containing Pho2 protein is necessary for cooperative binding to the HO promoter (Bhoite and Stillman 1998). Although Castor binds to DNA independently in vitro and regulates transcription in vivo, it is possible that Castor interacts synergistically with other proteins, such as Mirror, to control gene expression in the CNS. Such an interaction would require the binding sites for both proteins to be found in upstream enhancer regions of the same genes and that these sites make a physical interaction between the two proteins possible.
Chapter 3

The availability of castor mutants would make it possible to investigate the likelihood of an interaction by comparing phenotypes and testing genetic interactions. The possibility of a functional interaction between Castor and Mirror is limited to the specific neuroblasts in the ventral nerve cord and brain that express both proteins. Loss of Castor leads to a reduction in engrailed expressing cells and to subtle defects in CNS axons (Cui and Doe 1992). Castor is also required for the correct expression of drifter, 1-POU and Pdm 1 and 2 (Kambadur et al. 1998). The detailed CNS phenotype of mirror has not been described, and it is not known which genes Mirror regulates in the CNS. In order to investigate if Mirror and Castor act synergistically to control gene expression, the effect of loss of mirror on Castor targets could be investigated as well as the effect of loss of both mirror and castor. In an attempt to investigate if Mirror controls the expression of Pdm 2 or nubbin, as it is also known, mirror embryos were stained with Nubbin antibodies. No effect on the expression pattern could be seen (data not shown). In addition, Mirror was ectopically expressed using the Paired-Gal4 driver. Castor expression using this driver caused a reduction in Nubbin levels, but the expression of Mirror did not (data not shown). Further investigation to uncover any synergistic effects could be performed using castor, mirror double mutants (see below) as well as flies expressing both proteins under UAS control.

Genetic interactions were investigated between mirror and the castor allele ming

No interactions were detected between single alleles and there was no effect of loss of one copy of castor on the Mirror over-expression phenotypes. Double mutant castor, mirror chromosomes were generated by recombination, but there was no detectable effect on survival of reducing the levels of Castor on different combinations of mirror alleles. Although there does not seem to be any synergistic interactions which can be detected by reduced survival between the genes, it is still possible that early effects are being masked by a later lethality of either of the two mutations. An investigation of the embryonic CNS of the double mutants may still reveal an interaction.
3.5.5 Summary

Table 3.6 summarises the results obtained from comparing expression patterns and testing for genetic interactions between Mirror and selected yeast-two-hybrid interactors. In addition, it lists the “tools” that were available for studying the putative interaction. As the main aim of these investigations were to determine which of the putative interactors to study in more detail, it was important to consider if mutant alleles and antibodies existed that could be used for further studies. The detailed investigations of two of the putative interactors CHD1 and CG1135 will be described in the next two chapters.

<table>
<thead>
<tr>
<th>Putative interactor</th>
<th>Overlap in expression pattern</th>
<th>Genetic interaction</th>
<th>Available tools</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dichaete</td>
<td>Yes, specific but limited.</td>
<td>None detected (double mutants not obtained)</td>
<td>Mutant alleles, Antibodies</td>
</tr>
<tr>
<td>Castor</td>
<td>Yes, specific</td>
<td>None detected</td>
<td>Mutant alleles, Antibodies</td>
</tr>
<tr>
<td>CG13367</td>
<td>Yes, ubiquitous</td>
<td>Slight enhancement of ubiquitous overexpression phenotype</td>
<td>N/A</td>
</tr>
<tr>
<td>CHD1</td>
<td>Yes, ubiquitous</td>
<td>Slight suppression of GMR overexpression phenotype</td>
<td>Antibodies</td>
</tr>
<tr>
<td>CG1962</td>
<td>Yes, specific</td>
<td>Not obvious</td>
<td>N/A</td>
</tr>
<tr>
<td>CG10395</td>
<td>Yes, ubiquitous</td>
<td>No deficiency</td>
<td>N/A</td>
</tr>
<tr>
<td>CG30337</td>
<td>Yes, ubiquitous</td>
<td>No deficiency</td>
<td>N/A</td>
</tr>
<tr>
<td>CG12919</td>
<td>Yes, specific</td>
<td>Enhancement of ubiquitous overexpression phenotype</td>
<td>N/A</td>
</tr>
<tr>
<td>CG8448</td>
<td>Yes, ubiquitous</td>
<td>Slight enhancement of overexpression phenotypes</td>
<td>N/A</td>
</tr>
<tr>
<td>CG1135</td>
<td>Yes, ubiquitous</td>
<td>Slight enhancement of overexpression phenotypes</td>
<td>Uncharacterised P element allele</td>
</tr>
<tr>
<td>CG3987</td>
<td>Inconclusive</td>
<td>No deficiency</td>
<td>N/A</td>
</tr>
<tr>
<td>CG5740</td>
<td>Possibly specific</td>
<td>Enhancement of ubiquitous overexpression phenotype</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Table 3.6. N/A, not available.
CHAPTER 4

Further characterisation of CG1135, a putative interactor for Mirror

4.1 Introduction

The novel protein CG1135 was represented by 35 clones pulled out of the yeast-two-hybrid screen for interactors with Mirror. All the CG1135 cDNA clones identified in the screen contained the C-terminal part of the predicted open reading frame, varying only slightly in size. The interaction between CG1135 and Mirror was found to lead to very high levels of reporter gene activation several times stronger than any of the other putative interactors, especially in the liquid β-galactosidase assay (figure 2.3). It was decided to characterise CG1135 further and to try to establish if the interaction between Mirror and CG1135 occurs in vivo. During the characterisation of expression patterns of selected yeast-two-hybrid interactors (chapter 3), CG1135 was found to be expressed ubiquitously in the embryo. In addition, CG1135 is represented in all EST databases available, indicating that it is expressed in a widespread fashion in larvae and adults as well as embryos. A deficiency covering the CG1135 locus could modify phenotypes caused by the over-expression of Mirror protein indicating a possible genetic interaction. As opposed to many of the other novel proteins identified in the yeast-two-hybrid screens, a lethal P element insertion mutation is available for the CG1135 locus. Although this mutation has not been characterised, it is predicted to be an allele of CG1135 (see below). This
mutation allows possible genetic interaction between mirror and CG1135 to be investigated properly and allows detailed studies of the phenotypes associated with this mutation to be performed. This chapter presents the further analysis of CG1135 and its putative interaction with Mirror.

4.1.1 CG1135 is a novel FHA containing protein.

CG1135 is a novel protein which contains a forkhead associated (FHA) domain in the C-terminus. Figure 4.1 shows the entire CG1135 predicted ORF indicating the FHA homology domain and the putative Mirror interaction domain. The FHA domain was first identified as a domain of unknown function in a group of forkhead transcription factors (Hofmann and Bucher 1995). FHA domains can now be found in over 200 different proteins from both prokaryotes and eukaryotes and are known to be phosphopeptide-binding motifs (Durocher and Jackson 2002). The functions of FHA domain containing proteins varies from roles in intracellular signalling cascades, cell proliferation, protein transport and degradation as well as transcription. All these different processes can however be regulated by protein phosphorylation which is where the FHA domain and its interactions with phosphorylated peptides is important.

One of the first FHA domain interactions to be studied in detail was the interaction between the Arabidopsis kinase-associated protein phosphatase (KAPP) and a plant receptor-like protein kinase (RLK) (Li et al. 2000). This interaction is important for the CLAVATA1 signal transduction pathway. Some of the best studied FHA domains are found in proteins involved in the cellular response to DNA damage or mitotic stress. The checkpoint kinase Rad53 in yeast contains two FHA domains. The C-terminal FHA2 domain interacts with phosphorylated Rad 9 and this interaction is important for induction of G2/M arrest following DNA damage (Li et al. 2000). The human homologue of Rad53,
the checkpoint kinase Chk2, contains one FHA domain which has been shown to be important for targeting Chk2 to binding partners such as BRAC1 (Li et al. 2002). Some FHA domains may be involved in targeting proteins to specific cellular compartments (Durocher and Jackson 2002). The nuclear inhibitor of protein phosphatase 1 (NIPP1) is associated with the spliceosome and the FHA domain of NIPP1 is known to be important for its subnuclear localisation. Many proteins contain FHA domains for which the specific targets are not known. These include certain kinesins, the forkhead transcription factors and some nucleolar proteins (Durocher and Jackson 2002).

The FHA domain consists of 120-140 amino acids of which 55-75 residues make up the FHA homology region (Li et al. 2000). Primary sequence homology between different FHA domains is limited to a few highly conserved residues, but all FHA domains are predicted to have very similar tertiary structures. Figure 4.2 includes a comparison of the sequence of the CG135 FHA domain to some other well studied FHA domains. The tertiary structure of at least four different FHA domains has been solved by X-ray crystallography and nuclear magnetic resonance (NMR) (Durocher and Jackson 2002; Li et al. 2000; Stavridi et al. 2002). It consists of a sandwich of two twisted antiparallel β-sheets made up of 11 β-stands (figure 4.2 B). The overall fold of the domain is similar to that of the SMAD MH2 domain. The most conserved residues within the FHA domain are found in the loops and turns between the β-strands. The importance of the loops was revealed by the co-crystallisation of the Rad-p53 FHA1 domain with a phosphopeptide (Durocher et al. 2000). The peptide is bound in an extended conformation by the loops connecting β-strands 3/4, 4/5, and 6/7 (figure 4.2). This type of binding is reminiscent of the binding of antigen epitopes to antibodies.
Figure 4.1 DNA and protein sequence of the CGI 135 ORF indicating in blue the FHA homology domain and in **bold** the Mirror interaction domain, * marks the insertion site of the l(3)rG166 P element.
Figure 4.2 A. The FHA domain homology region

CG1135  EITFGRDAKDCVVDVDLGLEGPAAKISRRQGTIKLRSNGDPFIANEGKRA-IFIDGTPL
Cons... TVTIGRSSEDCDIQLPG-------PSISRRHAVIVYDGGGRFYLIDLGSTNGTFVNGKRV
Radp53...WTPGRNPACDYHLGNIS-------RLSNKHFPQILLGEGDLNLL-NDISTNGTWLNGQ
KAPP.....ILTGRVPPSDLVLKDSE-------VSGKHAQINWNGKTLKWEVLDGLNGTFLNSQ

Figure 4.2 Comparison of the FHA homology region of CG1135 with a consensus FHA sequence (Cons, PROSTIE) as well as the FHA domains from yeast Radp53 and Arabidopsis KAPP.

Figure 4.2B Structure of the FHA domain of Chk2

Figure 4.2B From Li et al, 2002. Structure of the Chk2 FHA domain bound to a phosphothreonine peptide. The loops between the β-strands make contacts with the peptide which is bound in an extended conformation.
FHA domains have been found to interact mainly with phosphothreonine (pT) containing epitopes, but have also been shown to interact with phosphotyrosines and phosphoserines (Durocher and Jackson 2002; Li et al. 2000). The binding specificity of the Rad53 FHA domains has been most intensely studied. The specificity of individual FHA domains seems to depend mainly on the residue at position pT +3 in the phosphopeptide. Within the FHA domain, the conserved residues within the connecting loops make contacts with the peptide backbone and the pT residue. As mentioned in the introduction, Mirror might be phosphorylated in vivo. The phosphorylation site or the kinase responsible is not known. However, the identification of a phosphopeptide-binding domain containing protein as a putative Mirror interactor is very intriguing.

4.1.2 The C-terminus of CG1135 is homologous to human, mouse and quail proteins

CG1135 is homologous to a small group of FHA containing proteins which may be part of a novel family of proteins. So far homologues have been identified in human, mouse and quail. There are two human proteins of 78KDa and 58KDa which may be different isoforms, and they have been called nucleolar and cell cycle regulated protein p78 or microspherule protein 1 (MCRS1) and microspherule protein p58 (MSP58) (Bruni and Roizman 1998; Ren et al. 1998). The p78 and p58 versions of the protein are almost identical in the last 458 amino acids. The mouse MCRS1 is the same size and highly homologous to MSP58. The quail homologue of these proteins has been recently identified and called target of Jun 3 (TOJ3) (Bader et al. 2001). TOJ3 is highly homologous to the C-terminal 448 amino acids of all three proteins, and contains an unconserved N-terminal extension. Figure 4.3 shows that CG1135 is homologous to the human, mouse and quail proteins within the last 350 amino acids.
Cell cycle regulated factor p78 or MCRS1 was first identified as an interaction partner of the regulatory protein ICP22 from herpes simplex virus 1 (Bruni and Roizman 1998). ICP22 is a nuclear protein required for the expression of a subset of viral genes. The p78 protein was found to be expressed in a cell cycle dependent fashion accumulating in early S phase. Staining using p78 antibodies showed that the protein was mostly nuclear in HeLa cells and localised to nucleoli in Hep-2 cells. As ICP22 also accumulates in early S phase it was suggested that p78 might somehow regulate the ICP22 protein. The MSP58 protein was isolated in a screen for interactors with the proliferation related nucleolar protein p120 (Ren et al. 1998). The interaction between MSP58 and p120 was found to be dependent on the FHA domain. MSP58 was found to accumulate in dense bodies or microspherules within the nucleoli. The microspherules are regions of the nucleolus that are involved in pre-ribosome formation and maturation. Apart from interacting with the proteins mentioned above, the specific function of both p78 and MSP58 is unknown.

The quail homologue of MCRS1 and MSP58, TOJ3, was recently described as a target of the v-jun oncogene (Bader et al. 2001). TOJ3 was proposed to be a direct target of v-jun and to mediate part of the transforming activities of this gene. Over-expression of TOJ3 in fibroblasts allowed anchorage-independent growth, a characteristic of a transformed state. It is interesting to note that nucleolar hyper-reactivity and elevated levels of nucleolar proteins such as p120 is a characteristic of many tumour cells (Bader et al. 2001). Although very little is known about the functions of the human, mouse and quail homologues of CGI 135, they all seem to be related to the regulation of cell proliferation and/or the cell cycle. Further analysis of the function of Drosophila CG1135 should reveal if it too has a similar role.
Figure 4.3 Alignment of the CG1135 amino acid sequence with human cell cycle regulated factor p78 (Hsapiens_CCRF), mouse MSP58 (Mmus_msp58), and quail Target of jun 3 (Ccot_toj3). Conserved residues are indicated in red. The alignment was done using ClustalW (1.82) multiple sequence alignment.
4.2 Dissecting genetic interactions between mirror and CG1135

4.2.1 The lethal P element l(3)G166 is an insertion within the CG1135 ORF

The CG1135 locus was found to contain an uncharacterised lethal P element insertion, l(3)G166. Sequencing of the insertion site had been performed (AQ026355, (Flybase 1998)) and was found to match sequences within the CG1135 gene. Further analysis of this sequence found it to be in the last exon of the transcript within the ORF. Figure 4.12 shows a scheme of the genomic region of CG1135 and the position of the P element. The insertion site is also marked in the CG1135 sequence in figure 4.1. The P element is the ry\(^+\) lacZ enhancer trap P\{PZ\} (Rubin and Spradling 1983). The lacZ transcription unit is oriented in the opposite direction to the orientation of the CG1135 gene. Staining using a β-galactosidase antibody found that the enhancer trap is expressed in a restricted pattern in the eye and leg disc (figure 4.4), but not in the embryo (not shown). Due to the orientation of the P element and the fact that CG1135 has been shown to be expressed ubiquitously, at least in the embryo, it is probable that the P element is not a CG1135 enhancer trap. It may however represent the expression of the CG11359 gene which is transcribed in the same direction as the enhancer trap and starts just upstream of the beginning of the CG1135 transcription unit (see figure 4.12). However, it is still formally possible that the enhancer trap does represent a subset of the CG1135 expression domain which is controlled by elements 3’ of the transcription unit.

As the l(3)G166 P element is inserted within the ORF of CG1135, it assumed to be an allele of this gene. Insertion of the P element would in the least disruptive case cause the production of a truncated CG1135 protein missing most of the FHA domain. This protein would be expected to be non-functional and could possibly represent a dominant
negative version. However, the P element insertion would also lead to the production of a truncated mRNA lacking the 3'UTR and poly A signal. Therefore the allele may represent a protein null for CGI 135.

4.2.2 Investigation of genetic interactions between l(3)rG166 and mirror

Genetic interactions between l(3)rG166 and mirror were first assayed by crossing the P element line to various mirror alleles and looking at viability of progeny carrying both mutations. The mirror alleles mrr<sup>pl</sup>, mrr<sup>p2</sup> and mrr<sup>48</sup> have been described previously (chapter 3). As can be seen from table 4.1, the presence of the l(3)rG166 allele did not affect the survival of any of the mirror alleles. The percentage of surviving progeny carrying both mutations was increased compared to the expected mendelian ratios, but this is probably due to the deleterious effects of the balancer chromosomes which also cause reduced viability. In order to investigate a genetic interaction further, the l(3)rG166 allele was recombined with the three mirror alleles. Double mutant chromosomes were confirmed to contain both the l(3)rG166 and mirror mutations by backcrossing to the original stocks. The effect of the l(3)rG166 mutation on mrr<sup>pl</sup> homozygous viability and the survival of mrr<sup>pl</sup>/ mrr<sup>p2</sup> flies was then tested. The results in table 4.1 indicate that the presence of the CGI135 allele does not drastically affect the survival of flies with reduced amounts of Mirror, although a slight decrease in viability was observed.
Figure 4.4 Expression of β-galactosidase from the l(3)rG166 enhancer trap

Figure 4.4 Confocal images of an eye-antennal disc (A, anterior left, dorsal up) and a leg disc (B) from larvae of the l(3)rG166 enhancer trap line. The discs were stained with anti-β-galactosidase antibody (green). The enhancer trap shows expression posterior to the morphogenetic furrow (arrow) and in patches in the dorsal head region and the antennal disc. The enhancer trap is also expressed in distal parts of the leg disc.
Table 4.1 Genetic interactions between CG1135 and mirror alleles

<table>
<thead>
<tr>
<th>Alleles</th>
<th>mrr\textsuperscript{P1}</th>
<th>mrr\textsuperscript{P2}</th>
<th>mrr\textsuperscript{48}</th>
</tr>
</thead>
<tbody>
<tr>
<td>l(3)rG166</td>
<td>46% (50%)</td>
<td>44% (33%)</td>
<td>36% (25%)</td>
</tr>
<tr>
<td>l(3)rG166, mrr\textsuperscript{P1}</td>
<td>37% (50%)</td>
<td>16% (33%)</td>
<td>ND</td>
</tr>
<tr>
<td>mrr\textsuperscript{P1}</td>
<td>viable</td>
<td>33% (50%)</td>
<td>ND</td>
</tr>
<tr>
<td>l(3)rG166, mrr\textsuperscript{P2}</td>
<td>25% (50%)</td>
<td>lethal</td>
<td>lethal</td>
</tr>
<tr>
<td>mrr\textsuperscript{P2}</td>
<td>33% (50%)</td>
<td>lethal</td>
<td>lethal</td>
</tr>
</tbody>
</table>

Table 4.1. Crosses were l(3)rG166/TM3 x mrr\textsuperscript{P1}/mrr\textsuperscript{P1}; l(3)rG166/TM3 x mrr\textsuperscript{P2}/TM6; l(3)rG166/TM3 x mrr\textsuperscript{48}/TM3; l(3)rG166, mrr\textsuperscript{P1}/TM3 x mrr\textsuperscript{P1}/mrr\textsuperscript{P1}; l(3)rG166, mrr\textsuperscript{P1} x mrr\textsuperscript{P2}/TM6; l(3)rG166, mrr\textsuperscript{P2} x mrr\textsuperscript{P1}/mrr\textsuperscript{P1}. Viable progeny of the indicated combination of alleles were counted (expected percentage of progeny in brackets). ND: not determined.

As described in the introduction, various combinations of mirror alleles which lead to reduced expression levels, have been shown to exhibit a mild Iroquois phenotype characterised by the loss of some lateral bristles and duplication in scutellar bristles. (Kehl et al. 1998). These phenotypes are also associated with the mrr\textsuperscript{P1}/mrr\textsuperscript{P1} and mrr\textsuperscript{P1}/mrr\textsuperscript{P2} allelic combinations (I. Dahlsveen and H. McNeill, figure 4.5). It was found that the presence of the l(3)rG166 allele affects these phenotypes. Figure 4.5 shows the absence of bristles in mrr\textsuperscript{P1}/mrr\textsuperscript{P1} and mrr\textsuperscript{P1}/mrr\textsuperscript{P2} flies carrying the l(3)rG166 allele. Adults heterozygous for the CG1135 mutation did not show any bristle defects (not shown). In order to quantify the effect, the presence of bristles in the notum of double mutant flies was recorded. The results are presented in table 4.2 and show that the CG1135 mutation increases the loss of the posterior superalalar and the anterior postalar bristles and increases the frequency of duplication of the anterior scutellar bristle.
Figure 4.5 Loss of lateral bristles in CG1135, mirror double mutants

Figure 4.5 Adult thorax from flies carrying mirror and CG1135 mutations. A) mirr^{P2}/+; B) mirr^{P1}/mirr^{P1}; C) l(3)rG166, mirr^{P1}/mirr^{P1}; D) l(3)rG166, mirr^{P1}/mirr^{P2}. The presence of the l(3)rG166 allele causes increased frequency of loss of the posterior super-alar (pSA, white arrow) and anterior post-alar (aPA, black arrow) as well as increased frequency of doubling of the anterior scutellar (aSC, red arrow) bristles. Please see table 4.2 for details.
Table 4.2 The effect of the CG1135 allele on the mirror bristle phenotype

<table>
<thead>
<tr>
<th>Allelic combination</th>
<th>Frequency of presence</th>
<th>Frequency of duplication of aSC</th>
<th>N=</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>aSA</td>
<td>pSA</td>
<td>aPA</td>
</tr>
<tr>
<td>mrr^{P1}/mrr^{P1}</td>
<td>1</td>
<td>0.75</td>
<td>0.53</td>
</tr>
<tr>
<td>mrr^{P1}/l(3)rG166, mrr^{P1}</td>
<td>1</td>
<td>0.86</td>
<td>0.27</td>
</tr>
<tr>
<td>mrr^{P1}/mrr^{P2}</td>
<td>0.99</td>
<td>0.59</td>
<td>0.12</td>
</tr>
<tr>
<td>mrr^{P2}/l(3)rG166, mrr^{P2}</td>
<td>0.99</td>
<td>0.15</td>
<td>0.08</td>
</tr>
<tr>
<td>mrr^{P2}/l(3)rG166, mrr^{P1}</td>
<td>1</td>
<td>0.22</td>
<td>0.06</td>
</tr>
</tbody>
</table>

Table 4.2. Frequency of the presence of bristles (aSA: anterior superalar; pSA: posterior superalar; aPA: anterior postalar; pPA: posterior postalar) per hemi-notum (N= number of hemi-nota scored) for various combinations of mirror and CG1135 alleles. Frequency of duplication of the aSC (anterior scutellar) bristle also included.

The above results indicate that mirror interacts genetically with the l(3)rG166 allele. It seems that reducing the amounts of CG1135 somehow impairs Mirror function.

In chapter 3, genetic interactions were also investigated using two over-expression systems for Mirror. A deficiency covering CGI135 was found to enhance the phenotypes associated with ectopic Mirror expression. However, these effects could not be reproduced using the l(3)rG166 allele (not shown).

4.3 Investigating the CG1135 phenotype

In order to find out if the loss of CG1135 generated similar defects to the loss of Mirror, the phenotypes associated with the l(3)rG166 allele were investigated in more detail. Individuals homozygous for the P element insertion were found to die as late embryos or early larvae with no obvious cuticle defects (not shown). In order to examine any phenotypes later in development, the l(3)rG166 allele was recombined onto the 80
FRT chromosome. The FLP-FRT system takes advantage of the site-specific FLP recombinase (FLPase) from yeast which catalyses recombination between FRT recognition sequences (Golic 1991). The combination of an FRT chromosome carrying a mutation with an FRT chromosome which is marked (and the addition of the FLP recombinase) allows mitotic clones of the mutation to be generated and identified by the absence of the marker.

4.3.1 Attempting to generate mitotic clones of the CG1135 allele in adults.

To determine if the CG1135 allele has a phenotype in the adult eye, mitotic clones were generated using an FRT chromosomes marked by the mini-white gene (P[w+,pMyc]80FRT). Initially, induction of clones was attempted using a FLPase under the control of a heatshock promoter. Subjecting larvae carrying the mutant and marked FRT chromosomes to heatshock should induce a limited number of recombination events and lead to the generation of a few clones in each eye. The clones should be marked by the absence of pigment. In addition, the clone of cells homozygous for the marked chromosome (called the twin spot) should also be distinguishable by the presence of two copies of the mini-white gene. As seen in figure 4.6, when clones of l(3)rG166 where induced during larval stages, only the twin spot could be distinguished in adult eyes. No mutant tissue marked by the lack of pigment could be detected. This suggests that mutation of CG1135 may affect cell viability, at least in the eye.
Figure 4.6 Clones of \( l(3)rG166 \) in adult eyes

A and B) Clones of the \( l(3)rG166 \) allele generated using hsFLP (hs FLP; \( l(3)rG166 \) 80FRT/P\([w^+,pMyc] \) 80FRT). No mutant tissue is visible, only the white+ homozygous twin spot. C-E) Clones of the \( l(3)rG166 \) allele generated using eyFLP (ey FLP; \( l(3)rG166 \) 80FRT/P\([w^+,pMyc] \) 80FRT). Ey FLP induced clones lead to a reduction in the size of the eye, slight roughness and occasional transformation of eye into head cuticle.
Clones of cells homozygous for the l(3)rG166 allele were also induced using FLPase under the control of Eyeless enhancer elements (Bohni et al. 1999). As the recombinase will be expressed throughout eye development, many recombination events will take place and a large number of clones can potentially be generated within the eye. As with the heatshock induced FLPase, no clonal tissue lacking the marker was visible in the adult eye. However, eyes in which clones of the CG1135 mutation have been generated using eyeless FLP, were often reduced in size and had a slightly rough appearance (figure 4.6). In a small number of cases, part of the eye field had been replaced by head cuticle. These transformations occurred most often around the ventral margin (figure 4.6).

To investigate the affect of the l(3)rG166 clones further, adult eyes containing eyeless induced clones were embedded and sectioned (chapter 6). As seen in figure 4.7, clones of the CG1135 mutation generate small defects in a number of ommatidia such as missing and/or mis-specified photoreceptors. These defects could be due to the presence of very small clones within the eye or could be indicative of a clone which was present earlier in development, but had later disappeared.

The above results suggest that the mutation in CG1135 may be cell lethal or may lead to reduced viability. If the lack of clones in the adult is due to reduced viability, then it might be possible to get clones to survive by giving them a growth advantage over the surrounding tissue. This can be done by using marked FRT chromosomes which contain mutations or elements which leads to slow growth or death. The GMR-hid FRT chromosome carries a P element insertion which leads to expression of the pro-apoptotic gene head involution defective (hid) in all cells posterior to the furrow (Stowers and Schwarz 1999). This generates a very small bar-shaped eye which contains only a few pigment cells (figure 4.8). When this chromosome is combined with wild type (with respect to cell viability) FRT chromosomes and clones are induced with the eyeless FLP recombinase, the eye is rescued due to the fact that it is made up of only clonal cells and
not GMR-hid cells ((Stowers and Schwarz 1999) and figure 4.8). The $l(3)rG166$ FRT chromosome was crossed to the GMR-hid FRT stock which also contains the eyeless FLP in order to see if any CG1135 mutant cells would survive to be part of the adult eye. However, most of the adults had eye phenotypes indistinguishable from the GMR-hid FRT flies. On few occasions, small patches of ommatidia that presumably contained CG1135 mutant cells could be seen (figure 4.8), but these were not very common. This suggests that even with a growth advantage, cells homozygous for the $l(3)rG166$ allele in the eye have severely reduced viability.
Figure 4.7 Section of adult eye from fly with the genotype eyFLP; l(3)G166 80FRT/P[w+,pMyc]80 FRT. Several ommatidia are missing photoreceptors (red arrows) and some have mis-specified photoreceptors (white arrows).
Figure 4.8 Generation of l(3)rG166 clones using the GMR-hid FRT chromosome

Figure 4.8 Pictures of adult heads, dorsal is up, anterior is left. A) eyFLP; GMR-hid 80FRT; B) eyFLP; GMR-hid 80FRT/ P[w+,pMyc] 80FRT; C) eyFLP; GMR-hid 80FRT/l(3)rG166 80FRT; D) eyFLP; GMR-hid 80FRT/l(3)rG166 80FRT. The GMR-hid FRT chromosome leads to the death of nearly all cells in the eye (A). Induction of wild type clones using eyFLP rescues the eye (B). However, induction of l(3)rG166 clones only occasionally gives rise to small patches of eye tissue (C,D).
4.3.2 Investigating phenotypes of clones of the CG1135 allele in eye discs

To find out if clones of the \( l(3)rG166 \) allele are visible earlier in development, clones were generated using eyeless FLP and an FRT chromosome marked with either the Myc epitope tag or GFP. Eye discs from third instar larvae were stained with anti-Myc or anti-GFP antibodies to visualise the clones. In contrast to the adult eye, clones of the CG1135 allele were visible in third instar discs. As seen in figure 4.9, clones in the eye disc can be detected both anterior and posterior to the morphogenetic furrow. Clones of \( l(3)rG166 \) posterior to the furrow are generally smaller, especially towards the posterior margin. In addition, the clones seem to have a dispersed appearance without distinct borders. The borders are not smooth like in \textit{mirror} clones indicating that CG1135 does not function in setting up differences in cell affinities. As these clones are not visible in the adult, the cells homozygous for the CG1135 mutation must be lost during pupal stages, probably due to apoptosis.

Clones of \textit{mirror} in the eye induces planar polarity defects. These defects can be detected in the eye disc by staining with a marker for the photoreceptors R1 and R6. Figure 1.10 in the introduction shows staining of R1 and R6 with the Bar antibody in discs containing clones of the Iro-C. The reorientation of photoreceptor clusters around the equatorial borders of the Iro C clones is visible. In order to determine if clones of the CG1135 allele affect planar polarity of photoreceptor clusters, discs with eyeless induced clones of the \( l(3)rG166 \) allele were stained with the Bar antibody. Although the effects are much less dramatic compared with the effects of the Iro-C clones, defects in the orientation of photoreceptor clusters are associated with clones of the CG1135 allele. As seen in figure 4.10, photoreceptor clusters can be seen to be rotated abnormally where there are clones of \( l(3)rG166 \). However, due to the difficulties in distinguishing the borders of the clones and the number of clones present, it was not possible to discern if the defects in polarity were exclusively inside or outside the clones. In addition, clones of
CG1135 might sometimes affect differentiation, as in some clones Bar staining seems to be absent in occasional cells. However, these results suggest that clones of the CG1135 allele may have a similar phenotype to clones of *mirror* in the eye disc.

In order to investigate how CG1135 might be affecting photoreceptor polarity further and to gain insight into how CG1135 might be affecting Mirror function, discs carrying clones of the *l(3)rG166* allele were stained with the Mirror antibody. As described in the introduction, Mirror is expressed in all dorsal cells anterior to the furrow (although staining for Mirror is strongest in the dorsal anterior region). Posterior to the furrow, Mirror expression is concentrated in a few photoreceptor cells identified as R3, R4 and possibly R7. In addition, Mirror expression after the furrow seems to be more concentrated to the nuclei of the developing photoreceptors. Preliminary results shown in figure 4.11 indicate that Mirror staining in *l(3)rG166* clones after the furrow differs to that of surrounding, wild type tissue. Mirror staining within the clones seems to be less concentrated within the photoreceptors or possibly less nuclear. However, Mirror staining in clones anterior to the furrow seemed normal. The distribution of Mirror protein within the CG1135 clones needs to be studied in more detail, but these results may suggest that CG1135 might be involved in inducing or maintaining the nuclear localisation or the stability of the Mirror protein.
Figure 4.9 Third instar discs with clones of the l(3)rG166 allele

Clones of l(3)rG166 generated by eyFLP and marked by GFP (A and B: eyFLP; l(3)rG166 80FRT/69 GFP 80FRT) or Myc (C: eyFLP; l(3)rG166 80FRT/ P[w+,pMyc]80 FRT).

A and B) discs stained using rabbit anti-GFP and fluorescent anti-rabbit. C) discs stained using mouse anti-Myc and fluorescent anti-mouse antibodies. Clones of the l(3)rG166 allele are visible anterior and posterior to the morphogenetic furrow (white arrows).
Figure 4.10 Discs with clones of the l(3)rG166 allele stained with Bar antibodies.

Figure 4.10 Collapsed confocal Z sections of third instar discs, dorsal is up and anterior left. Two examples (A-C and D-F) of discs from larvae of the genotype eyFLP; l(3)rG166 80FRT/69 GFP 80FRT. A and D) anti-GFP staining; B and E) anti-BarH1 staining; C and E) merged image with GFP in green and BarH1 in red. BarH1 marks the R1 and R6 cells and can be used to infer the direction of rotation of the photoreceptor clusters. Several clusters can be seen to rotate abnormally in and/or around clones of the l(3)rG166 allele (red arrows).
Figure 4.11 Mirror antibody staining in clones of the CG1135 allele

Figure 4.11 Confocal image (single section) of third instar discs (dorsal half posterior to the furrow) from larvae of the genotype eyFLP; l(3)G166 80 FRT/GFP 80 FRT. A) GFP antibody staining; B) Mirror antibody staining; C) Merged image of GFP staining (green) and Mirror staining (red). The lack of GFP staining indicates the clones of the CG1135 allele. Mirror is present in a subset of photoreceptor nuclei. Within the CG1135 clones, Mirror staining seems to be less distinct (possibly less nuclear).
4.4 Excision of the CG1135 P element

In order to confirm that the lethality of the \textit{l(3)rG166} allele is due to the P element insertion and to generate new CG1135 alleles, it was decided to excise the P element (O'Hare and Rubin 1983). Perfect excision of the \textit{l(3)rG166} insertion should generate viable flies if this is the only lethal mutation on the chromosome. In addition, some excision events will be imprecise possibly deleting part of the surrounding locus. Details of how the excision was done can be found in chapter 6 (materials and methods). Males in which an excision event had taken place were identified by the loss of the \textit{rosy} reporter gene from the P element. In total 35 excision lines were established, 32 of which were homozygous lethal and three of which were viable. The excision lines were also backcrossed to the original P element stock and nine were found to complement the \textit{l(3)rG166} mutation (table 4.3).

To characterise the excision events further, primers for PCR were made that bind 5' and 3' of the insertion site (figure 4.12). Single-fly PCR reactions were performed on the 35 excision lines using these primers and a primer for the P element inverted repeat sequence. The position of the primers and combinations used are indicated in figure 4.12. The results of the PCR reactions were used to infer if the excision lines contained an intact 5' and/or 3' P element inverted repeat sequence. Surprisingly, even though the flies had rosy eyes, most of the lethal excision lines still seemed to contain both sides of the P element. Alternatively, primers could be binding to partial inverted repeat sequences which can remain in the genome post-excision (Takasu-Ishikawa et al. 1992). Six of the excision lines had lost the 5' end of the P element which is the side at which the \textit{rosy} gene is located (figure 4.12). The nine lines which complemented the original P element stock (three of which are homozygous viable) had lost both sides of the P element indicating a precise excision. Two lines which did not complement the \textit{l(3)rG166} allele had also lost both sides of the P element indicating that a deletion within the locus might have taken...
place. PCR reactions across the insertion site (using the 5’ and 3’ primer) of heterozygous individuals did not reveal any large internal deletions. However, even very small deletions or remaining P element sequences at the insertion site would presumably affect the integrity of the GC1135 ORF. Further investigation of these excision lines would be necessary to establish the exact nature of the putative mutations. However, the fact that viable lines can be generated by the excision of the P element indicate that the lethality and clonal phenotypes seen are due to the l(3)rG166 insert.

4.5 Investigating in vivo interactions between Mirror and CG1135

To confirm that the physical interaction detected using the yeast-two-hybrid system reflects a true association between Mirror and CG1135, the interaction has to be proved to take place in vivo. Co-immunoprecipitation (IP) from Drosophila embryos or tissue culture cells is an attractive way to confirm interactions. Mirror is not expressed endogenously in Drosophila S2 cells, but in order to study Mirror in a tissue culture system, a construct has been generated to express a full length FLAG-tagged Mirror protein from an inducible promoter. The pMT FLAG-Mirror construct was made by A. Bilioni using the full-length Mirror construct described in the introduction. It was decided to use this construct to investigate if an interaction between CG1135 and Mirror could be detected by co-transfection and IP from S2 cells.
Figure 4.12 Scheme representing the \( l(3)rG166 \) P element insertion

![Diagram of genomic region around the CG1135 gene (green) showing the position of the CG11593 gene (blue) and the rG166 P element (red). The position of the rosy and lacZ reporter genes within the P element are indicated. The primers used for the amplification of genomic DNA are represented by black arrows (P INV = primer for the inverted repeat). PCR reactions on flies from each line were performed using the P (INV) primer in combination with the P 5' or P 3' primer to determine if the 5' or 3' side of the P element was still intact.]

Table 4.3 Characterisation of excision lines

<table>
<thead>
<tr>
<th>Number of lines</th>
<th>Homozygous viability</th>
<th>Complementation of ( l(3)rG166 )</th>
<th>Intact P element sides</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>5'</td>
</tr>
<tr>
<td>3</td>
<td>viable</td>
<td>yes</td>
<td>3</td>
</tr>
<tr>
<td>6</td>
<td>lethal</td>
<td>yes</td>
<td>6</td>
</tr>
<tr>
<td>24</td>
<td>lethal</td>
<td>no</td>
<td>16</td>
</tr>
</tbody>
</table>

Table 4.3 The excision lines were characterised by checking homozygous viability and complementation of the original P element allele \( l(3)rG166 \). The results of PCR reactions on each line were used to determine if the 5' and/or 3' side of the P element was still intact.
4.5.1 Expression of a full length and truncated CG1135 construct in S2 cells

In order to investigate the interaction with Mirror and to study the function of CG1135 further, the full length ORF was cloned by PCR from an EST clone containing the entire CG1135 cDNA (LD26139). The 5' primer was designed to generate a 5' EcoRI site just upstream of the ATG allowing cloning of the CG1135 ORF in frame with the HA-tag in the pMT HA vector. The 3' primer included a XbaI site just 3' to the stop codon. The PCR product was first cloned using the TOPO TA Kit into the pCR2.1-TOPO vector and fully sequenced. The full length construct was then cloned into the pMT HA vector using the EcoRI and XbaI sites. A truncated version of CG1135 excluding the last 164 amino acids was generated by using an internal XhoI site (CG1135 I-414). A scheme for the cloning as well as maps of the final vectors is shown in figure 4.13.

To test the expression of the CG1135 proteins, the pMT-HA-CGI135 constructs were transfected into S2 cells by A. Bilioni. Expression was assayed 24 hours post-transfection by Western blotting of samples and detection of the HA tag using an anti-HA antibody. As seen in figure 4.14, both the truncated and full-length proteins are present in transfected and induced cells.

4.5.2 Immunoprecipitation of Mirror and CG1135 from S2 cells

To try to determine if Mirror and CG1135 interact in S2 cells, the pMT-FLAG-Mirror and the full length pMT-HA-CGI135 constructs were co-transfected into S2 cells by A. Bilioni. Expression of both proteins was confirmed by western blotting of cell lysates and detection using the HA11 and Mirror antibodies (figure 4.14 and not shown). IPs were performed from cells expressing FLAG-Mirror alone, HA-CG1135 alone and FLAG-Mirror + HA-CG1135 using affinity beads with covalently linked FLAG and HA antibodies. The IP of FLAG-Mirror and full length HA-CG1135 with the appropriate
affinity beads could be confirmed (not shown). FLAG-Mirror also seemed to be enriched in the IP using HA beads from cells expressing both FLAG-Mirror and HA-CG1135. However Mirror protein was also present in the control IP from FLAG-Mirror only cells (not shown). The HA-CG1135 protein seemed to be present at similar levels in FLAGIPs from co-expressing cells and HA-CG1135 only cells (not shown). The putative interaction between Mirror and CG1135 will be further investigated using this expression system and more stringent IP conditions.
Figure 4.13. Cloning of CG1135 by PCR

Full length CG1135 was cloned by PCR using primers with EcoRI and Xbal sites (blue arrows) into the pCR2.1-TOPO vector. Following sequencing, full length CG1135 was subcloned into the pMT HA (pMT-HA-CG1135a) vector using EcoRI and Xbal. Truncated CG1135 was cloned using EcoRI and an internal XhoI site (pMT-HA-CG1135c).
Figure 4.14 Expression of full length and truncated HA-CG1135 in S2 cells

Western blot of extracts from S2 cells. Proteins are detected using the HA11 antibody and anti-mouse horseradish peroxidase conjugated secondary antibody. The lanes are loaded as follows: 1: molecular weight marker; 2 and 3: S2 cells expressing FLAG-Mirror; 4 and 5: S2 cells expressing full length HA-CG1135; 6 and 7: S2 cells expressing FLAG-Mirror and full length HA-CG1135; 8 and 9: S2 cells expressing truncated HA-CG1135. The full length and truncated CG1135 proteins have predicted molecular weights of ~64 KDa and ~52 KDa respectively.
4.6 Discussion

4.6.1 The lethal P element insertion l(3)rG166 is probably an allele of CG1135

The lethal mutation l(3)rG166 is due to a P element insertion in the ORF of CG1135. The P element therefore interrupts the CG1135 transcript and would be expected to at least lead to the production of a truncated protein. However, the insertion might also cause the production of an unstable mRNA which lacks the 3’ UTR and poly A signals. This would lead to reduced or abolished translation making the allele a protein null for CG1135. So far there is no antibody to CG1135 available so the absence of full-length protein or presence of a truncated version can not be investigated. Attempts to produce CG1135 specific antibodies are on-going. Two different peptides from CG1135 were used to make four polyclonal antibodies (H. McNeill). So far, these antibodies have been shown to recognise bacterially produced proteins, but do not recognise a specific band in S2 cell extracts. One of these antibodies is being affinity purified. In addition, the partial CG1135 cDNA clone from the yeast-two-hybrid library was cloned into an E. coli His-tag expression vector to produce recombinant protein for antibody production. The fusion protein was insoluble, but inclusion bodies containing CG1135 were purified and used to produce two additional antibodies (not shown). However, these antibodies have not yet been shown to specifically recognise CG1135 on Western blots of S2 cell extracts or in discs.

The P element insertion in the CG1135 ORF contains a lacZ reporter gene transcribed in the opposite orientation to the CG1135 gene. Antibody staining for β-galactosidase shows a restricted expression pattern. Due to the direction of transcription however, it is unlikely that this is an enhancer trap for the CG1135 gene. However, it may
be an enhancer trap for the novel gene CG11593 which is transcribed in the same direction as the lacZ reporter and is found about 1.5 kb upstream of the insertion site. CG1135 and CG11593 are transcribed from opposite strands with start sites that are very close together. This genomic organisation means that some regulatory elements for the CG11593 gene may be part of the CG1135 transcription unit. It also means that the I(3)rG166 P element may be a regulatory mutant for the CG11593 gene as well as a mutant for CG1135. To investigate if the P element affects CG11593 expression, in situ hybridisation using a probe for CG11593 would have to be performed on discs carrying clones of the I(3)rG166 allele.

Another way to investigate if the allele is due purely to disruption of the CG1135 gene is to rescue the mutation by expressing CG1135 protein from a transgene. The full length CG1135 ORF is therefore being cloned into a P element vector for production of transgenic flies. A UAS-CG1135 construct would also allow the targeted expression of the protein which could be used to further investigate the phenotype and genetic interactions with mirror.

In order to rule out additional mutations on the I(3)rG166 chromosome and to generate new CG1135 alleles, the P element insert was excised. Three out of the 35 excision lines generated were homozygous viable and PCR analysis of the insertion site indicated that the P element had been precisely excised. Six other lines also seemed to contain precise excision events, and these lines could complement the original P element mutation. Homozygous lethality in these lines could be due to insertion of the P element at another locus. The fact that most of the excision lines remained lethal is not surprising as the insertion site lies within the ORF of the CG1135 gene. Any partial or imprecise excision events would be expected to still cause disruption in the gene. Indeed, PCR analysis of most of the lines revealed that some of the P element was still present at the insertion site. Two lines were generated which did not complement the I(3)rG166 allele, but did not seem to contain any part of the P element as judged by PCR analysis. These
lines may represent small deletions within the CG1135 gene and will be investigated further.

4.6.2 The phenotype of the CG1135 allele suggests a role in cell proliferation or survival

Mitotic clones of the \(l(3)\)rG166 allele are generated in the eye imaginal disc, but fail to appear in the adult eye. Induction of small numbers of clones using heatshock, leads to patches of cells homozygous for the marked chromosome, but no homozygous mutant cells can be detected. When many clones are induces using eyeless FLP, the size of the eye is reduced and sometimes loss of eye tissue can be seen suggesting that the cells homozygous for the CG1135 mutation have failed to contribute to the adult eye. This could be due to a defect in cell proliferation or survival. Clones of the \(l(3)\)rG166 allele were visible in the third instar disc. However, the clones were often quite small and had irregular borders. Clones were especially reduced in size towards the posterior margin. The above observations suggest that CG1135 may be involved in proliferation or survival of cells, especially during late larval and/or pupal development. In addition to the affect on cell survival, clones of cells mutant for the CG1135 mutation also seemed to affect the rotation of photoreceptor clusters in the eye disc. This phenotype will be discussed in the next section.

Cell proliferation and survival is linked to the differentiation process during Drosophila eye development (Baker 2001). As mentioned in the introduction, cells entering the morphogenetic furrow are arrested in G1. The cells that do not form part of the pre-cluster (which is made up of R8, R2, R5, R3 and R4) enter S phase synchronously posterior to the furrow to reach G2. Mitosis in these cells is regulated by activation of the EGFR pathway via the ligand Spitz which is secreted from the pre-cluster cells. Without EGFR activation, the cells do not enter mitosis and arrest in G2. In addition to being required for mitosis, the EGFR and Spitz are also required for survival of cells posterior to the furrow and later in pupal development. The death of cells lacking \textit{egfr} can be rescued
by the expression of baculovirus p35 indicating that EGFR activation is important for suppressing apoptosis.

The phenotype associated with the CG1135 mutation suggest that it may be important for the proliferation or survival of cells after the furrow. CG1135 could for example be required for EGRF induced mitosis. This might explain why some clones lacked cells staining with the R1/6 marker Bar. However, this can not be the only requirement for CG1135, as no mutant cells are present in the adult. In addition, there are many cells within the \( l(3)rG166 \) clones that do stain with Bar indicating that although they may die later, cells within the clone are still able to differentiate as photoreceptors. Both neuronal differentiation and survival are dependent on EGFR signalling. However, if apoptosis is suppressed (by expression of p35), cells survive, but do not differentiate. It is possible therefore that CG1135 is important for survival of cells after the furrow. To find out if clones of the \( l(3)rG166 \) allele are subject to apoptosis, discs containing clones could be stained with markers such as acridine orange or TUNEL. In addition, expression of p35 could be used to try to rescue the CG1135 cells. It should also be investigated if CG1135 is working downstream of the EGFR pathway. For example, binding of CG1135 to its targets is expected to require phosphorylation which could be regulated by downstream effectors of EGFR activation.

CG1135 has been reported to interact physically with the cyclin-dependent kinase inhibitor Roughex (Chen and Thomas 2002). Roughex (Rux) plays an important role in the G1 arrest associated with the morphogenetic furrow by negatively regulating cyclin A levels through a physical interaction (Avedisov et al. 2000). Clones of cells mutant for Rux in the eye enter S phase early (Baker 2001). Rux has also been shown to have a more general role in regulation of the cell cycle by contributing to exit from mitosis (Foley and Sprenger 2001). This function is probably also related to the regulation of cyclin A. The function of the interaction between CG135 and Rux is not known. However, as the
phenotype of the CG1135 mutation seems to be related to cell proliferation and survival, this interaction may provide a link between CG1135 and cell cycle regulation.

The phenotype of the CG1135 allele has so far only been investigated in the eye. It is possible that CG1135 also has a more general role in cell proliferation than suggested by the above results. Generation of marked clones in other imaginal discs should be performed to study CG1135 function further. It is very interesting that the human and quail homologues of CG1135 have been linked to the cell cycle and to proliferation. The expression of the p78 or MCSR1 protein was found to peak at early S phase. It would be interesting to find out if expression of CG1135 is somehow linked to the cell cycle. The quail CG 1135 homologue, TOJ3, was found to have transforming activities when over-expressed. This suggests that it is normally required to control proliferation and/or survival.

4.6.3 Does CG1135 interact with Mirror in vivo?

One of the main aims of characterising the CG1135 mutation and associated phenotype was to find out if the interaction between CG1135 and Mirror discovered in the yeast-two-hybrid screen, was indeed real. Genetic interactions between mirror and the \( l(3)rG166 \) allele were therefore investigated. Heteroallelic combinations of single alleles of CG1135 and mirror did not affect survival or produce any obvious adult phenotypes. The CG1135 mutation was recombined with two mirror alleles to generate double mutant chromosomes. Combinations of these mirror alleles leads to reduced viability and specific bristle phenotypes. When the \( l(3)rG166 \) allele was also present this led to further reductions in viability and to an enhancement of the bristle phenotypes. The \( l(3)rG166 \) allele does not cause any loss or duplication of bristles in combination with a wild type or balancer chromosome (not shown). This indicates that reduction in the levels of CG1135
can affect Mirror levels or function. Interactions between the \( l(3)rG166 \) allele and the phenotypes associated with over-expression of Mirror discussed in chapter 3 were also tested. A deficiency covering CG1135 had been shown to enhance the phenotypes slightly. However, the CG1135 mutation did not lead to any enhancement of the phenotypes. In fact, the presence of the \( l(3)rG166 \) allele seemed to suppress the reduction in viability caused by the general over-expression of Mirror (not shown). The results from the genetic interactions suggest that CG1135 may be required for the normal function of Mirror, but may not important in situations when Mirror is over-expressed.

If CG1135 is affecting Mirror function, then mutation of CG1135 might cause similar phenotypes to loss of Mirror. The affects of the CG1135 mutation \( l(3)rG166 \) have so far only been investigated in the eye. The main function of CG1135 seems to be related to cell proliferation or survival, but the protein could still have other functions which may involve Mirror. The two main characteristics of \textit{mirror} clones in the eye are smooth borders and effects on planar polarity. CG1135 does not seem to be important for cell adhesion as the outline of \( l(3)rG166 \) clones is not smooth. However, the CG1135 mutation was associated with defects photoreceptor cluster polarity. Staining with the Bar antibody in eye discs revealed that some clusters were abnormally rotated in and/or around CG1135 clones. These defects could be an indication of reduced Mirror function earlier in development. These effects might also be linked to an uncharacterised later function of Mirror posterior to the furrow. However, the polarity defects of the CG1135 clones could also be associated with the putative role of CG1135 in differentiation and/or survival. Further investigation of the function of CG1135 in other tissues need to be performed and compared to known Mirror functions in order to establish if CG1135 and \textit{mirror} have similar phenotypes. In addition, the affect of the CG1135 allele on confirmed Mirror targets also needs to be investigated.

To investigate the effect of CG1135 on Mirror function in the eye further, clones of the \( l(3)rG166 \) allele were also stained with Mirror antibodies. Preliminary results from
these experiments indicate that Mirror localisation is different within the clones compared to surrounding wild type tissue. Mirror staining in the CG1135 clones seems less concentrated within the R3/4 photoreceptors. Again, these affects could be a result of a defect in differentiation and/or survival within the CG1135 clones. The effects on Mirror staining need to be studied further by co-staining with neuronal markers such as Elav to eliminate secondary affects. However, these results may suggest that the function of CG1135 is somehow linked to the localisation or stability of Mirror protein posterior to the furrow.

The physical interaction between CG1135 and Mirror has so far not been detected outside the yeast-two-hybrid system. Experiments using a bacterially produced C-terminal Mirror construct lacking the homeodomain and in vitro translated CG1135 were unsuccessful at detecting an interaction (not shown). The interaction domain of CG1135 with Mirror includes the phosphopeptide-binding FHA domain. If the interaction of Mirror and CG1135 is dependent on phosphorylation, the interaction can probably not be detected using bacterially produced proteins.

Mirror has been shown to be phosphorylated in Drosophila tissue culture cells. In order to determine if CG1135 and Mirror can interact in S2 cells, a construct for expressing a HA-tagged version of CG1135 was made and expression of tagged CG1135 protein in S2 cells was confirmed by western blotting. Immunoprecipitation experiments using a FLAG-tagged Mirror construct were initiated, but have so far not conclusively shown that the two proteins interact (but has not disproved it either). The best way of detecting an in vivo interaction would be to IP either Mirror or CG1135 from Drosophila embryos or discs as these interactions would not be affected by ectopic expression artefacts. However, the lack of suitable Mirror and CG1135 antibodies has prevented such experiments so far. Although the results from the genetic interaction and phenotypic studies discussed above suggest that CG1135 and Mirror may interact in vivo, a physical interaction between the two proteins in Drosophila still needs to be demonstrated.
CHAPTER 5

Further characterisation of CHD1

5.1 Introduction

The chromo-helicase/ATPase-DNA-binding protein 1 is named after three signature motifs and is part of a family of proteins containing these domains called the CHD family (Woodage et al. 1997). The CHD family is part a larger family of SNF2 related helicase/ATPases which contains proteins that have functions in transcriptional regulation, recombination and DNA repair (Eisen et al. 1995). CHD1 was first cloned in mouse, but there are now several CHD related proteins in the databases in organisms ranging from yeast to humans. The Drosophila CHD1 was cloned by virtue of its similarity to the mouse protein, but has not been extensively studied (Stokes et al. 1996). The yeast homologue of CHD1, Chd1p, has been found to be an ATP-dependent chromatin modifying factor (Tran et al. 2000) and the closest homologue of CHD1 in Drosophila, dMi-2 (or CHD4) has also been shown to be an ATPase that promotes nucleosome mobilisation (Brehm et al. 2000). It is therefore likely that Drosophila CHD1 is a chromatin remodelling factor which could be involved in regulating transcription. A possible interaction with the transcription factor Mirror is therefore very intriguing. Many transcription factors regulate gene expression by recruiting chromatin modifying proteins...
and it would be very interesting to find out CHD1 is a co-factor for Mirror in transcriptional regulation.

5.1.1 Introduction to Chromo domains and ATPase/Helicase domains

Members of the CHD family of proteins are unique in that they contain both a chromo (chromatin organization modifier) domain and an ATPase/helicase domain. Chromo domains were first recognized as a 50 amino acid motif in *Drosophila* heterochromatin protein 1 (HP1) and the Polycomb protein (Paro and Hogness 1991). Chromo domains have since been identified in a variety of proteins from fungi and plants to mammals that all seem to be connected to chromatin structure and/or function (Eissenberg 2001). The structure of the chromo domain fold consists of three \( \beta \)-strands and an \( \alpha \)-helix and is related to a DNA binding fold found in the archael chromatin proteins (Ball *et al.* 1997). The Chromo domain has been shown to function in protein-protein as well as protein-RNA and protein DNA interactions all of which are related to a general function of targeting chromo domain proteins to specific regions or positions in the nucleus. Protein-protein interactions mediated by chromo domains include interactions with specifically modified histones ie the HP1 chromo domain which interacts with the H3 tail methylated at lysine 9. Chromo domain interactions are also important within protein complexes, such as the Polycomb chromo domains which targets the PcG silencing complex to chromatin (Eissenberg 2001). Protein-RNA interactions are mediated by the MOF and MSL2 chromo domains, two proteins which are part of the dosage compensation complex in *Drosophila* (Akhtar *et al.* 2000). The chromo domain of *Drosophila* dMi-2, which is most closely related to the CHD1 chromo domain (see below), has recently been found to interact with DNA in vitro (Bouazoune *et al.* 2002).
The ATPase domain of CHD1 is part of the SNF2 subfamily of ATPase/helicases (Eisen et al. 1995). Proteins containing these domains are usually found in large protein complexes and function to increase the accessibility of nucleosomal DNA in an ATP-dependent manner (Narlikar et al. 2002). The SNF2 type of ATPase/helicase domain is part of the DEAD/H superfamily of helicases (Bork and Koonin 1993), but none of the SNF2 related ATPase proteins have been found to possess helicase activity. The domain and is made up of seven conserved “helicase” motifs (I-VII) which are highlighted in the CHD1 sequence in figure 5.3. There are three subclasses of ATP-dependent chromatin remodelling complexes which are classified according to the ATPase subunit they contain. Figure 5.1 gives an overview of the complexes that have been studied in yeast, mammals and Drosophila.

The SWI/SNF family of complexes contain members of the SWI/SNF subfamily of ATPases which include SWI2/SNF2 and STH1 from yeast, BRG1 and BRM from mammals and Brahma from Drosophila (Tsukiyama 2002). Proteins in this subclass contain a bromodomain, a motif which interacts with acetylated histone tails, in addition to the ATPase domain. The SWI/SNF ATPases can both change the translational position of nucleosomes on DNA and cause conformational changes that affect the DNA-protein contacts within the nucleosome in an ATP dependent manner (Narlikar et al. 2002). The in vivo functions of the different SWI/SNF complexes varies although they are all involved in regulating transcription and are mainly thought to cause activation of transcription. SWI/SNF complexes have been shown to have specific effects on subsets of genes in yeast, mammals and Drosophila, but have also been suggested to have more general roles in maintaining chromatin structure (Tsukiyama 2002).

The ISWI family of complexes contain ISW1 and 2 in yeast, hSNF2 in humans and the ISWI ATPase in Drosophila. These ATPase subunits contain a SANT domain which is found in many proteins involved in transcriptional control and is predicted to be a DNA binding motif (Aasland et al. 1996). As opposed to the conformational changes...
observed with SWI/SNF, the ISWI ATPases are thought to use sliding of nucleosomes on DNA as their main remodelling activity (Narlikar et al. 2002). In vivo, the ISWI complexes are mainly thought to be involved in the negative regulation of transcription and creating an inaccessible chromatin structure (Tsukiyama 2002).

The CHD or Mi-2 family of complexes is the least well studied subclass of chromatin remodellers. In yeast, the CHD proteins Chdpl and hrp1 have not been isolated as part of a complex to date. In mammals the NuRD complex which contains the Mi-2/CHD3/4 ATPases is the only well characterised complex in this subclass (Xue et al. 1998), (Tong et al. 1998), although a similar complex is thought to exist in Drosophila (Brehm et al. 2000). The known in vitro and in vivo functions of the CHD family are discussed below.
Figure 5.1. ATP-dependent Chromatin remodelling factors

The SWI (switching)/SNF (sucrose non-fermenting) family of complexes include yeast SWI/SFN and RSC (remodels structure of chromatin) which contains the STH1 (Snf two homologue) ATPase. Drosophila SWI/SFN complexes contain BRM (Brahma) and human complexes contain human BRM or BRG1 (Brm/SWI2-related gene 1). The ISWI (Imitation Switch) family of complexes comprises ISW1 and 2 in yeast. In Drosophila, the ISWI ATPase is found in the NURF (nucleosome remodelling factor) complex, CHRAC (chromatin accessibility complex) and ACF (ATP-dependent chromatin assembly and remodelling factor). The human ISWI homologue, SNF2h is found in human CHRAC and ACF as well as RSF (remodelling and spacing factor). Some of the proteins in the CHD family of ATPases are found in the NuRD (nucleosome remodelling and histone deacetylation) complex.
5.1.2 The CHD family of proteins

The CHD family of proteins can be subdivided into two classes according to the domains they contain addition to the chromo and ATPase domains (Woodage et al. 1997). CHD1 and CHD2 contain a C-terminal DNA binding domain whereas CHD3 and CHD4, also called Mi-2α and Mi-2β, contain N-terminal PHD zinc fingers. A comparison between some of the different CHD family members as well as other SNF2-like ATPases from Drosophila is presented in figure 5.2. The CHD3/4 subclass has been much more extensively studied than the CHD1/2 subclass. The CHD1 proteins from yeast, mammals and *Drosophila* will be discussed in detail in section 5.1.3.

The Mi-2 proteins were originally identified as autoantigens in the human disease dermatomyositis (Seelig et al. 1996). The first clues to their function came from the purification of large complexes containing histone deacetylases. Hyperacetylation of histone tail lysines is linked to active transcription whereas hypoacetylation is linked to a repressed chromatin state (Narlikar et al. 2002). Like the ATP-dependent chromatin remodellers, histone acetyltransferases (HATs) and histone deacetylases (HDACs), also reside in large multi-subunit complexes. The HAT and HDAC complexes can be recruited to specific chromatin sites by transcription factors to activate or repress transcription.

When antibodies to human HDAC1 and HDAC2 were used to purify a multi-subunit complex it was found to contain both CHD3 and CHD4 (Tong et al. 1998). In parallel, antibodies raised against CHD4 were used to purify a complex containing HDAC1 and 2 (Xue et al. 1998). One variant of this complex called NuRD or NRD (nucleosome remodelling and deacetylation) can be seen in figure 5.1. CHD3/4 and HDACs have also been found as part of other complexes, such as the PYR complex which share some subunits with the NuRD complex (O'Neill et al. 2000). Amongst the other components of the NuRD complex, the presence of the methylated-DNA-binding protein MDB3 is of special interest. Methylation of DNA is also linked to transcriptional repression and
suggests that these complexes may be recruited to methylated DNA to create a repressed chromatin state (Tsukiyama 2002).

The association of CHD3 and CHD4 with a histone deacetylase suggests that they are involved in transcriptional repression. Indeed, antibodies against the NuRD complex partially relieves repression by thyroid hormone receptor in Xenopus oocytes (Xue et al. 1998). In mammalian cells a CHD3/4 complex interacts with the zinc-finger repressor Ikaros and may be involved T cell differentiation (Tsukiyama 2002). A CHD3/4 – Ikaros complex may also be involved in repression of γ-globin during γ- to β-globin switching (O’Neill et al. 2000). In addition to specific targeting by transcription factors, CHD3/4 complexes may also exhibit a more constitutive association with chromatin which causes chromatin acetylation in a non-targeted fashion (Li et al. 2002).

The *Drosophila* Mi-2 protein is the closest homologue of *Drosophila* CHD1 and was first identified in a yeast two-hybrid screen for proteins that interact with the transcription factor Hunchback (Kehle et al. 1998). The Hunchback protein is involved in the initial repression of HOX genes during embryonic development, but the maintenance of repression requires the Polycomb-group (PcG) proteins. *Drosophila* Mi-2 (or CHD4) was shown to interact with Hunchback in vitro, and to study a possible in vivo interaction, mutant alleles of *dMi-2* were generated. Homozygous *dMi-2* mutants survive until larval stages due to maternal contribution of RNA and/or proteins, but germ-line and somatic clones are cell lethal. Mutants in *dMi-2* were however shown to interact genetically with *hunchback* causing derepression of *Ubx*. Interestingly, *dMi-2* was also shown to interact with PcG mutations suggesting that Mi-2 may serve as a link between the initial repression of Hox genes during embryonic development and the long term maintenance by PcG proteins. *Drosophila* Mi-2 has also been shown to interact physically and genetically with another transcription factor, Tramtrack69 (Murawsky et al. 2001). Like Hunchback, Tramtrack69 is a zinc-finger transcription factor and has been shown to regulate nervous
system development. Tramtrack69 and dMi-2 have been shown to associate in vivo by
immunoprecipitation and they also show overlapping localisation on polytene
chromosomes.

*Drosophila* Mi-2, like its vertebrate homologues, is found in a large complex in
vivo. Gel filtration experiments indicate that the dMi-2 complex is around 1.2 MDa
(Murawsky *et al.* 2001). Using a dMi-2 antibody, the *Drosophila* RPD3 HDAC could be
immunoprecipitated as part of the dMi-2 complex. The complex was shown to have both
ATPase and HDAC activity [Brehm, 2000 #22. A recent report investigated the role of the
chromo domains in dMi-2 function and found that deletion of these motifs leads to loss of
nucleosome binding and remodelling activities (Bouazoune *et al.* 2002). It was found that
the chromo domains did not interact with histone tails like the HP1 chromo domain, but
instead displayed DNA binding activity. This is a novel activity of chromo domains and it
remains to be seen if other CHD chromo domains share this function.

In addition to CHD1 and dMi-2, there is a third protein in the *Drosophila* genome
with extensive homology to this family. It has been named CHD3, but is as yet only a
predicted protein and has not been studied. Another protein, Kismet, also contains two
chromo domains and an ATPase domain which is highly homologous to the CHD ATPase
domain. However, Kismet does not contain putative DNA binding domain and also
contains a motif found only in the Brahma family of ATPases (Daubresse *et al.* 1999). The
functions of Kismet will be discussed with other *Drosophila* ATPases below.
Figure 5.2. Comparison of *Drosophila* CHD1 with other ATPases

<table>
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<tr>
<th>Protein</th>
<th>Domain Representation</th>
<th>Percentage</th>
</tr>
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</tr>
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<td>HsCHD2 (1739aa)</td>
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</tr>
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<td>DmISWI (1027)</td>
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<td>42%</td>
</tr>
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</table>

**Figure 5.2.** Schematic representation of various ATPase containing proteins in the CHD family and in Drosophila. C= Chromo domain, ATPase = ATPase/helicase domain, DNA = DNA binding domain, P= PHD fingers, B= Bromo domain, S = SANT domain.

Percentages represent the amino acids identify between the domain and the equivalent domain in Drosophila CHD1.

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**Figure 5.3.** Amino acid sequence of Drosophila CHD1 indicating the chromo domains in blue, the ATPase/helicase domain in red and the DNA binding domain in green. Bold residues mark the seven conserved helicase motifs. Underlined residues indicate the part of CHD1 which putatively interacts with Mirror.
5.1.3 CHD1 function in yeast, mammals and Drosophila

One CHD protein has been identified in yeast called Chd1p in Saccharomyces cerevisiae and Hrp1 in Saccharomyces pombe (Jin et al. 1998; Tran et al. 2000). Both these proteins are most closely related to CHD1 and 2 as they contain a C-terminal putative DNA binding domain, and no PHD domains in the N-terminus. Like many other chromatin remodelling factors in yeast, CHD1 is not essential for viability. Fission yeast lacking hrp1 do however show a slight increase in growth rate and slight mitotic chromosome loss and defective anaphase phenotypes (Yoo et al. 2000). Early experiments in budding yeast showed that chdlp mutants were resistant to the effects of 6-azauricil (6AU) which causes increased RNA Polymerase II pausing (Woodage et al. 1997). This result was proposed to suggest that Chd1p functions as a negative regulator of transcription. More recently it has been shown that loss of Chd1p leads to both up and down-regulation of various genes in the genome as determined by DNA microarray experiments (Tran et al. 2000). Mutants in chdlp do show synthetic lethality with other members of the SNF2 family of ATPases. A double mutant for chdlp and swil, which encodes the ATPase subunit of the SWI/SFN complex, is lethal, as is a double mutant for chdlp and swil, another component of the complex (Tran et al. 2000). In addition, chdlp is synthetic lethal with a isw1, isw2 double mutant (Tsukiyama et al. 1999). These interactions seem to suggest that the CHD1, SWI/SNF and ISWI ATPases may have overlapping functions in yeast.

Biochemical studies of the yeast CHD1 proteins have shown that they function as DNA-dependent ATPases which can be stimulated by both free DNA and nucleosomal DNA (Tran et al. 2000; Yoo et al. 2000). When Chd1p was purified from whole cell extracts it was not found to co-purify with other proteins which might be part of a CHD1 complex. The size of purified Chd1p as determined by gel filtration did however indicate that it might form a dimer (Tran et al. 2000).
Although CHD1 was originally cloned in mammals, it has not been studied extensively. CHD1 was initially isolated from a mouse cDNA expression library as a DNA binding protein (Delmas et al. 1993). Further examination of the DNA binding activities of CHD1 found that it was sequence selective, binding preferentially to AT tracts, rather than sequence specific (Stokes and Perry 1995). Electro mobility shift assays (EMSA) were used to localise the DNA binding activity to 229 amino acids in the C-terminal part of the protein (indicated in figure 5.3). An antibody raised against the C-terminal part of the protein showed that CHD1 was localised to the nucleus in human (HeLa) and mouse (NIH 3T3) cells as well as in nuclear extracts from mouse S194 cells (Stokes and Perry 1995). The high salt concentrations necessary to extract CHD1 from nuclei were taken to indicate that the protein is part of bulk chromatin. However, co-staining of cells with CHD1 and Hoechst 33258 indicated that CHD1 was not concentrated to heterochromatin or centromeric regions. The nuclear localisation properties of CHD1 were studied in detail using tagged versions of wild type and mutant constructs (Kelley et al. 1999). The wild type protein has a homogenous, finely granular nuclear distribution in U2OS cells. Deletion of the chromo domains and the nucleotide binding pocket of the ATPase domain caused the protein to be concentrated in punctate bodies whereas deletion of the DNA binding domain did not affect the nuclear distribution.

A yeast-two-hybrid screen for proteins that interact with the N-terminal part of CHD1 identified the HMG domain containing protein SSRP1 (Kelley et al. 1999). The interaction domain of CHD1 with SSRP1 was found to be N-terminal to the chromo domain. CHD1 and SSRP1 co-localise in U2OS cells and the distribution of SSRP1 could be altered by expression of the CHD1 deletion constructs. A two-step purification from HeLa nuclear extracts showed that both CHD1 and SSRP1 are found in the same fraction.
from a Superose 6 column, suggesting that they interact in vivo. In addition this purification indicated that CHD1 in mammals is part of a large complex of about 700kDa.

The *Drosophila* CHD1 protein was isolated from cDNA libraries using a probe which showed strong sequence similarity to the H domain of mouse CHD1 (Stokes *et al.* 1996). The full-length sequence of the *Drosophila* protein highlighting all the conserved domains as well as the putative Mirror interaction domain can be found in figure 5.3. An antibody raised against a C-terminal fragment of *Drosophila* CHD1 recognises a protein of about 200kDa on western blots of nuclear extracts from embryos and Schneider cells. This antibody was used to study the localisation of CHD1 on polytene chromosomes. Costaining with propidium iodine and CHD1 showed that CHD1 was localised to regions of the chromosomes with less compacted DNA ie the interbands and puff regions. Some puff regions were identified as developmental puffs that occur in response to pulses of ecdysone. CHD1 was also localised to heat shock puffs. However, CHD1 was not depleted from the developmental puffs upon heat shock as is the case for proteins like RNA polymerase II (Stokes *et al.* 1996). There have been no studies of the in vivo function of CHD1 in *Drosophila* as there are no mutants available, nor have any biochemical studies been performed in vitro.

### 5.1.4 Functions of other ATPase chromatin remodelling factors in *Drosophila*

In addition to CHD1 and dMi-2, three other ATPase containing chromatin remodelling factors have been studied in *Drosophila*, Brahma, Kismet and ISWI. *brahma* was first identified as a trithorax group gene (trxG) because it is a dominant suppressor of Polycombe mutations (Kennison and Tamkun 1988). Due to the suppressor effect of *brahma* on *polycomb* or *antennapedia* mutations it was thought that Brahma would be
involved in activation of transcription (Brizuela et al. 1994). Indeed, it has been shown that the Brahma complex is essential for activation of transcription by the trxG protein Zeste in vitro (Kal et al. 2000). But Brahma may not be exclusively involved in activation as it has also been shown that a Brahma complex is required to repress Wingless target genes (Collins and Treisman 2000). A role in both activation and repression of expression has also been shown for the Swi2/Snf2 and Sth1 proteins in yeast (Tsukiyama 2002). Brahma may have a more general role in transcription than that indicated by studying specific targets. This is suggested by the fact that loss of Brahma function causes reduced viability. Embryos carrying strong brahma mutations die as late embryos and germline clones of brahma do not develop (Brizuela et al. 1994). Somatic brahma clones generated at larval stages cause a reduction in viability of the clones and expression of a dominant negative Brahma protein also leads to reduced viability.

The Brahma protein is expressed ubiquitously in nuclei during embryonic and larval development, but does show enhanced expression in the ventral nerve cord and brain of the embryo (Elfring et al. 1998). Like its yeast and mammalian homologues, Brahma can be found in a large multi-subunit complex of about 2Mda (Dingwall et al. 1995). Several of the subunits of the Brahma complex have been identified and found to be homologous to proteins found in the equivalent yeast and mammalian complexes (Papoulas et al. 1998). The function of some of the subunits, such as Snrl and Osa, has been studied in vitro and in vivo confirming both physical and genetic interactions with Brahma (Collins et al. 1999; Dingwall et al. 1995).

The mechanism of targeting the Brahma complex to chromatin is still not understood. For example, removing the DNA binding protein Osa from the Brahma complex does not change its localisation on polytene chromosomes indicating that direct interactions with DNA via Osa is not responsible for targeting (Collins et al. 1999). In vitro studies of protein interactions suggest that Zeste may be involved in targeting, but this has not been confirmed (Kal et al. 2000). Recent studies on the localisation of Brahma
on polytene chromosomes has however shown that at least in the salivary glands, Brahma is associated with nearly all sites of active transcription (Armstrong et al. 2002). Surprisingly, expression of the dominant negative Brahma, which is an ATPase mutant, resulted in reduced levels of RNA Polymerase II associated with polytene chromosomes. These results may indicate that Brahma has a much more general role in transcriptional regulation that first anticipated.

The *Drosophila* Kismet protein contains two chromo domains and an ATPase/helicase domain (Daubresse et al. 1999). As the combination of chromo and ATPase domains is unique to the CHD family, it could be proposed that Kismet should be included as a member of this family. However, Kismet also contains a so called BRK motif, a 41-amino acid segment conserved in Brahma related chromatin remodellers. Indeed, the *kismet* gene was isolated in the same screen as *brahma* for dominant suppressors of *polycomb* and has phenotypes consistent with it being atrxG gene (Kennison and Tamkun 1988). *kismet* has been shown to activate expression of *sex combs reduced* in leg discs and loss of kismet causes homeotic transformations in the fifth abdominal segment (Daubresse et al. 1999). Loss of both maternal and zygotic *kismet* causes strong segmentation defects. However, unlike Brahma, Kismet is not an essential protein and somatic clones of *kismet* in many areas do not display any obvious phenotypes. Also, Kismet is not associated with the Brahma complex in *Drosophila* (Daubresse et al. 1999).

The ISWI protein in *Drosophila* was identified due to its similarity to Brahma within the ATPase domain and was found to be highly homologous to the human hSNF2L protein (Elfring et al. 1994). The function of ISWI was first studied biochemically as it was found to be part of several chromatin remodelling complexes purified from *Drosophila* embryos; the nucleosome remodelling factor (NURF), the chromatin
accessibility complex (CHRAC), and the ATP-dependent chromatin assembly and
remodelling factor (ACF) (Varga-Weisz and Becker 1998) (see also figure 5.1). These
three complexes were identified using different remodelling assays and even though they
contain the same ATPase subunit seem to cause nucleosome remodelling using different
mechanisms. All three complexes increase access to nucleosomal DNA, but NURF seems
to destabilise nucleosomal arrays whereas, CHRAC and ACF can catalyse the creation of
regular arrays from irregular chromatin. Both NURF and ACF can also facilitate
chromatin remodelling mediated by transcription factors such as GAGA and Gal4-VP16
in vitro which suggested that ISWI complexes may be involved in transcriptional
activation.

In vivo studies of ISWI function have been performed using both ISWI null alleles
and a dominant negative ISWI protein, which has the same mutation in the ATPase
domain as dominant negative Brahma. Loss of ISWI, like loss of Brahma leads to a
reduction in viability (Deuring et al. 2000). Individuals homozygous for ISWI mutations
die late in larval or early in pupal development, whereas germline clones of ISWI do not
develop. Somatic clones of ISWI show reduced viability, and expression of dominant
negative ISWI in imaginal discs causes death or reduction in size of adult structures. In
addition, the expression of both Engrailed and Ubx is reduced in ISWI mutants. The most
interesting phenotype of loss of ISWI is the effects seen on the male X chromosome. The
X chromosome in Drosophila males is transcribed at twice the level compared to the two
female X chromosomes. This process, called dosage compensation, is characterised by the
acetylation of lysine 16 of histone H4 on the male X. Loss of ISWI causes the male X to
become much less compacted compared to normal suggesting that ISWI is important for
proper chromatin compaction. Recent studies found that acetylation of H4K16 reduces the
ability of ISWI to interact productively with nucleosomes (Corona et al. 2002).

Although ISWI containing complexes seem to be able to cause activation of
transcription in vitro, detailed studies of the localisation of ISWI on polytene
chromosomes found that ISWI is enriched in regions of heterochromatin. In addition, co-
staining with ISWI and RNA Polymerase II showed that ISWI is preferentially associated
with regions that are not actively transcribed. The discrepancy between these results and
the fact that loss of ISWI causes reduced expression of certain genes may be explained by
the fact that ISWI is part of three different complexes in vivo, which may have different
functions. No reports of mutations in any of the subunits of the three complexes have yet
been reported.

5.1.5 Could CHD1 be a co-factor for Mirror?

Mirror is a transcription factor which may be involved in repression or activation
of target genes. It is not known how Mirror functions to regulate transcription, but many
transcription factors activate or repress gene expression by recruiting co-activators or co-
repressors to enhancer elements. These co-factors have often been shown to be chromatin
modifying complexes which cause activation or repression by changing the state of the
chromatin around the promoter region of the gene. The identification of CHD1, which is
homologous to chromatin remodelling factors, as a putative Mirror-binding protein is
therefore very interesting. A protein which is highly homologous to CHD1, dMi-2
(CHD4), has been shown to interact with two transcription factors, Hunchback and
Tramtrack69. It has been proposed that these transcription factors recruit the dMi-2
complex to specific genes to repress transcription. If the interaction between CHD1 and
Mirror occurs in vivo, it could be proposed that CHD1 is a co-factor for Mirror in
controlling the expression of some or all of the Mirror target genes.

As with the other ATPase chromatin remodelling factors in Drosophila, CHD1 is
expected to be ubiquitously expressed. In situ hybridisation experiments in chapter 3
indicated that this was indeed the case in the embryo. Affinity purified CHD1 antibody
was obtained from Robert Perry and used to stain imaginal discs to confirm that the protein is ubiquitous. Figure 5.4 shows the distribution of CHD1 protein in the wing and eye discs. Preliminary results described in chapter 3 also indicated that there might be a genetic interaction between mirror and a deficiency covering the CHD1 locus.

The function of Drosophila CHD1 has not been studied in vitro or in vivo. Although homologues of CHD1 in yeast have been shown to be ATP-dependent nucleosome remodelling factors, the activity of the Drosophila protein is not known. In addition, there is nothing known about the type of complex CHD1 might be part of. In vivo studies of CHD1 function have not been performed as there is no mutant available. In order to determine if CHD1 could be a co-factor for Mirror, it was decided to try to characterise Drosophila CHD1 further. This chapter presents attempts to generate a dominant negative CHD1 protein and the analysis of phenotypes associated with expression of wild type and mutant versions. Preliminary results comparing the putative loss of CHD1 function to mirror loss of function as well as attempts to establish a genetic interaction, will also be presented. To find out more about the role of CHD1 with respect to chromatin, the localisation of CHD1 on polytene chromosomes was also analysed in detail.
Figure 5.4 Expression of CHD1 in the eye and wing disc

Figure 5.4. Eye disc (left panel) and wing disc (right panel) stained with CHD1 antibodies (green). CHD1 protein is found in all nuclei. The pictures are single confocal sections. Variations in the intensity of staining is therefore likely due to the fact that specimens are not flat. For example, the lack of staining in the morphogenetic furrow (arrow) in this picture is due to the indentation in the disc.
Chapter 5

5.2 Generation and analysis of a putative dominant negative CHD1

5.2.1 Generation of a putative dominant negative CHD1

In order to identify and analyse phenotypes associated with the loss of CHD1 function as well as investigate any genetic interactions with mirror, it was decided to try and generate a dominant negative CHD1 protein. A dominant negative (DN) protein is a non-functional protein which will cause a loss of function phenotype when over-expressed in a wild type background. The DN protein will typically loose a particular activity through a specific mutation, but will still be localised normally and be able to interact with its protein partners and/or targets. The dominant effect and loss of function phenotype is caused by the replacement of endogenous protein with a non-functional molecule. Mutations which cause SNF2-like ATPase/helicase proteins to exert dominant negative effects have already been identified. Mutations of a conserved lysine (K798) in the A box of the ATP binding domain of the SWI2 ATPase caused reduced ATPase activity and loss of transcriptional activation (Khavari et al. 1993). However, this mutation did not affect the ability of SWI2 to be part of the SWI/SNF complex (Cote et al. 1994; Peterson et al. 1994). In addition, the mutated SWI2 showed a dominant negative effect in the presence of wild type protein (Khavari et al. 1993). The conserved lysine lies within the GxGKT box of the nucleotide binding site and is conserved in all the SNF2-related ATPases (figure 5.3). Mutation of this lysine to an arginine in the Drosophila Brahma protein generates a dominant negative protein which when over-expressed gives the same phenotypes as a mutation in the brahma gene (Elfring et al. 1998). The same mutation in Drosophila ISWI has also been shown to create a proteins which exerts dominant negative effects (Deuring et al. 2000). If CHD1 acts like the other SNF2-like chromatin remodelling factors in an ATP-dependent manner, it would be expected that the same mutation in the CHD1 ATPase domain would generate a dominant negative protein.

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To generate a dominant negative CHD1 protein, the conserved lysine 559 was changed by site-directed mutagenesis to an arginine (CHD1 K559R). A scheme for the mutagenesis can be seen in figure 5.5. The CHD1 cDNA was obtained from Robert Perry (Stokes et al. 1996) as a clone in pBluescript. To minimise the risk of secondary mutations introduced by the mutagenesis PCR, a 860 bp StuI-EcoRI fragment was subcloned into the pLitmus28 plasmid before the mutagenesis was performed. The presence of the mutation as well as the integrity of the rest of the sequence was confirmed by sequencing before the fragment was cloned back into the CHD1 cDNA.
Figure 5.4. Scheme for mutagenesis of CHD1. CHD1 cDNA was obtained from Robert Perry. A StuI-EcoRI fragment (860bp) was sub-cloned into pLitmus38 and site directed mutagenesis performed using the Clontech Site-Directed Mutagenesis Kit. The primers used are indicated in green, mutated base pairs and amino acids in red. Putative mutants were sequenced as described to confirm the presence of the point mutation and to confirm that the rest of the fragment had not been affected. The mutated StuI-EcoRI fragment was subcloned back into the full length CHD1 cDNA in pBluescript.
Figure 5.6 pExPress UAS CHD1

Figure 5.6 Schematic representation of the pExPress UAS CHD1 constructs generated for the production of transgenic flies that express CHD1 under the control of the Gal4 UAS.
Chapter 5

5.2.2 Expression of CHD1 constructs using the UAS-GAL4 system

In order to be able to express the mutant and wild type CHD1 proteins in *Drosophila* it was decided to use the UAS-Gal4 system for directed expression of transgenes (Brand and Perrimon 1993). This system places the Gal4 responsive UAS elements upstream of the transgene and uses flies which express the Gal4 protein in the desired pattern to activate transcription. To generate UAS-CHD1 WT and K559R transgenes, these constructs were cloned into the pExPress UAS P element vector. As there were no convenient sites to clone the CHD1 constructs directly into pExPress UAS, they were first subloned into pQBI-AdBN (Qbiogene) before being cloned into pExPress. The resulting pExPress construct is illustrated in figure 5.6.

To generate flies carrying the UAS CHD1 WT and K559R P elements, the pExPress constructs were injected into *Drosophila* embryos by Sheena Pinchin. Flies carrying the transgenes were identified by eye colour after injected flies had been crossed to a *yw* stock. Insertions were mapped to chromosomes I, II and III by crossing to appropriate balancers. A list of the various P element insertions obtained can be seen in table 5.1 below.

<table>
<thead>
<tr>
<th>Construct</th>
<th>Chromosomes with inserts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
</tr>
<tr>
<td>pExPress UAS CHD1 WT</td>
<td>Lines 40 and 65</td>
</tr>
<tr>
<td>pExPress UAS CHD1 K559R</td>
<td>Line 140</td>
</tr>
</tbody>
</table>

To analyse the phenotypes caused by over-expression of WT and K559R CHD1, flies carrying the UAS CHD1 transgenes were crossed to flies expressing Gal4 under the control of various enhancers. Table 5.2 contains a list of the Gal4 drivers used in this...
study including their expression patterns. The UAS-Gal4 system is temperature sensitive and the expression levels of the Gal4-driven transgene increases with increasing temperature (Morimura et al. 1996). At 18°C expression levels are usually quite low, whereas at 25°C expression levels are relatively high.

Table 5.2. GAL4 lines used in this study

<table>
<thead>
<tr>
<th>GAL4 Line</th>
<th>Expression pattern</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eyeless Gal4</td>
<td>Larva: Eye part of eye-antennal disc</td>
<td>Hazelett et al. 1998</td>
</tr>
<tr>
<td>GMR Gal4</td>
<td>Larva: All cells posterior to the morphogenetic furrow in eye disc</td>
<td>Freeman 1997</td>
</tr>
<tr>
<td>Fringe Gal4</td>
<td><em>fringe</em> expression pattern Importantly in the ventral half of the developing eye</td>
<td>Ken Irvine, personal communication</td>
</tr>
<tr>
<td>69B Gal4</td>
<td>Embryo: Embryonic ectoderm (stripes) from stage 11 Larva: Eye-antennal, haltere, leg and wing imaginal discs</td>
<td>Brand and Perrimon 1993; Perrimon 1997</td>
</tr>
<tr>
<td>30A Gal4</td>
<td>Embryo: embryonic salivary glands; muscle attachment sites. Larva: dorsal sector of the leg disc; wing hinge region of the wing disc; dorsal and ventral edges of the eye disc; single, central dot in antennal disc.</td>
<td>Brand and Perrimon 1993; Perrimon 1997</td>
</tr>
<tr>
<td>PNR Gal4</td>
<td><em>pannier</em> expression pattern Importantly in the dorsal anterior part of the eye disc, dorsal region of the notum part of the wing disc and in the medial notum</td>
<td>Calleja et al. 1996</td>
</tr>
<tr>
<td>756 Gal4</td>
<td>Larva: patchy expression in notum part of wing disc and strong expression in wing pouch.</td>
<td>Jennings et al. 1999, Gomez-Skarmeta et al. 1996</td>
</tr>
<tr>
<td>Prd Gal4</td>
<td>paired expression pattern In seven epidermal stripes from stage 8 to stage 13 in the embryo</td>
<td>Yoffe et al. 1995</td>
</tr>
<tr>
<td>VP16-Gal4</td>
<td>Maternally contributed VP16-Gal4 fusion.</td>
<td>Arno Muller, personal communication.</td>
</tr>
</tbody>
</table>
The effects of expressing of UAS CHD1 WT and K559R constructs in the eye and wing discs will be discussed below. Expression of the constructs in the embryo using the Prd Gal4 and VP16 drivers at 25°C produced viable adult flies without any obvious phenotypes. A lack of effects in the embryo has also been reported for the expression of DN versions of other chromatin remodelling factors in flies and is thought to be due to a large maternal contribution of RNA and/or protein.

5.2.3 Expression of WT and K559R CHD1 in the eye disc

The effect of over-expressing the CHD1 constructs was first tested in the eye disc. In order to ensure that the GAL4 drivers were working as expected, the UAS-Mirror transgene was used as a control (not shown). Two or three different CHD1 transgenes were tested for each construct, most of which were on the third chromosome. Overall, the effect caused by each construct varied only slightly between different insertions. Expression of WT (lines 88, 113 and 126) and K559R (lines 60, 88 and 101) CHD1 using the Eyeless and Fringe Gal4 drivers did not cause any obvious external phenotypes in the adult eye at 25°C (not shown). The 69B Gal4 driver is expressed in most imaginal discs and expression of UAS-Mirror using this driver is lethal. Expression of the CHD1 transgenes (WT lines 113 and 126, K559R lines 60 and 101) using the 69B driver led to a slight roughness of the eye at 25°C, but no obvious phenotype at room temperature (21-22°C). However there were phenotypes associated with the expression in the wing disc (see below). The most interesting phenotypes were observed by over-expression of the CHD1 constructs using the GMR Gal4 driver (figure 5.7). At 25°C the CHD1 K559R construct (lines 101 and 60) caused moderate to severe eye roughness, whereas the CHD1 WT construct (lines 88 and 126) caused moderate to mild eye roughness. At room
temperature (21-22°C) both phenotypes were milder and the difference between the WT and K559R constructs were less obvious. Figure 5.7 shows examples of the phenotypes observed.

In order to study the phenotypes generated by expression of the CHD1 constructs in the eye in more detail, adult eyes were embedded and sectioned to reveal the internal structure of the eye. Sections of eyes from flies expressing CHD1 WT and K599R under the control of Eyeless Gal4 did not show any defects in the structure of ommatidia or in planar polarity (not shown). Expression of the CHD1 constructs (WT line 126, K559R line 60) using 69B Gal4 led to a slight roughness in the eye. Sections of these eyes show subtle defects in the structure of the ommatidia, however, the overall structure of the eye and the array of ommatidia seems normal (not shown). Figure 5.8 shows sections of eyes expressing WT (line 88) and K559R (line 101) CHD1 using GMR Gal4 at 25°C. Expression of the putative dominant negative construct causes severe phenotypes which are characterised by a complete disorganisation of the ommatidial array as well as the structure of individual ommatidia. Disorganised clusters of rhabdomere producing cells can be distinguished, but not distinct photoreceptor subtypes. There also seems to be a massive overproduction of pigment cells. GMR driven expression of WT CHD1 also leads to a disorganised ommatidial array. However, the photoreceptor clusters seem to consist of more or less the right number of cells although the morphology is abnormal. Surprisingly, expression of the CHD1 (WT line 126, K559R line 60) constructs at room temperature using GMR Gal4 leads to much more similar and subtle phenotypes (figure 5.9). The ommatidial array is slightly disrupted, and the morphology of the pigment and cone cells seems abnormal. The photoreceptor clusters are mostly normal with some showing slightly abnormal morphology.
Figure 5.7 Expression of WT and K599R UAS CHD1 constructs using GMR Gal4

Figure 5.7 Phenotypes associated with the expression of UAS CHD1 WT and UAS CHD1 K599R constructs at 25°C. Lateral views of adult eyes. A) Wild type. B) GMR Gal4/++;+. C) GMR Gal4/++;UAS CHD1 WT 88. D) GMR Gal4/++; UAS CHD1 K599R 101. One copy of the GMR Gal4 construct causes a slight roughness of the eye at 25°C (B). Expression of the WT CHD1 protein at 25°C leads to mild roughness of the eye whereas expression of the K599R construct leads to a moderate roughness.
Figure 5.8 Sections of eyes expressing UAS CHD1 WT and K599R with GMR Gal4

Figure 5.9 Sections of eyes expressing UAS CHD1 WT and K559R with GMR Gal4

Figure 5.9 Sections of eyes from GMR Gal4; UAS CHD1 WT and K559R flies raised at room temperature (21-22°C). A) GMR Gal4/+; UAS CHD1 K559R 101
B) GMR Gal4/+; UAS CHD1 WT 126. Expression of the WT and CHD K559R constructs at room temperature leads to much less severe effects than expression at 25°C (compare to figure 5.8). Most ommatidia contain the correct number of photoreceptors, but the ommatidial array seems disturbed and there are defects in cone cells and pigment cells. The phenotypes caused by the WT and K559R constructs are similar.
The lack of phenotypes caused by expression of WT and K559R CHD1 constructs using the Eyeless, Fringe and 69B drivers was surprising. It was therefore decided to check the expression of the proteins by staining eye discs from flies expressing WT (line 88 and 126) and K559R (line 60 and 101) CHD1 under the control of Eyeless and GMR Gal4 drivers. The expression levels of CHD1 in the Eyeless Gal4 discs could not be distinguished from endogenous expression levels (not shown). However, as seen in figure 5.10, the GMR-Gal4 driver produced very high levels of expression posterior to the morphogenetic furrow at 25°C. As the phenotypes observed by over-expression of the WT and K599R constructs were more similar at lower temperatures, it could be that the differences seen between the two constructs are due to differences in levels of expression. However, the expression levels of the WT lines 88 and 126 are very similar to those of the K599R lines 60 and 101. Staining of the developing photoreceptors using the CHD1 antibody also showed that the development of discs over-expressing the K559R constructs seems disrupted compared to over-expression of the WT constructs. This suggests that the defects associated with the expression of the K559R construct may be due to effects on early photoreceptor development.
Figure 5.10 Eye discs expressing UAS CHD1 constructs with GMR Gal4

Figure 5.10. Eye discs expressing UAS CHD1 K599R 101 (left panel) and UAS CHD1WT 88 (right panel) under the control of GMR Gal4 at 25°C. Discs are stained with CHD1 antibody (green). Both pictures are single confocal sections which were taken using the same settings. The expression levels of the UAS constructs are similar and very high compared to endogenous protein levels. Similar results were obtained with the lines K559R 60 and WT 126. Note that the structure of the developing photoreceptor clusters seems to be disrupted in the disc over-expressing the K559R construct.
5.2.4 Expression of WT and K559R CHD1 in the wing disc.

Phenotypes caused by the expression of WT and K559R CHD1 constructs were also investigated in the wing disc. As for the over-expression experiments in the eye, several insertion lines for each construct were tested. In addition, expression of Gal4 in each driver line was verified by crossing in the UAS-Mirror transgene. Expression of the CHD1 constructs using the 756 and 30A Gal4 lines did not produce obvious phenotypes (not shown). As mentioned above, expression of WT and K559R CHD1 using the 69B Gal4 driver produced a subtle eye phenotype. In addition, expression of the CHD1 transgenes (WT lines 113 and 126, K559R lines 60 and 101) also produced a phenotype in the wing. As seen in figure 5.11, expression of both WT and K559R constructs led to a slight reduction in the size of the wing as well as overproduction of wing vein material. Ectopic vein material is mostly associated with L2, the more proximal parts of L4 and the posterior cross vein. In addition, ectopic veins can sometimes be seen forming at the distal margin between L3 and L4 (arrow in figure 5.11B). The most severe phenotypes include the formation of blisters in the wing (figure 5.11D). The WT and K599R constructs seem to induce various degrees of the same phenotype, the differences between the two constructs being less marked at room temperature than at 25°C.

Expression of the CHD1 WT (lines 65, 88 and 126) and K559R (lines 3, 60 and 101) constructs using the PNR Gal4 driver generated phenotypes in the medial part of the notum and the scutellum. As with the 69B driver, the phenotypes generated by the WT and K559R constructs were similar, although the K559 constructs generally produced more severe effects. Figure 5.12 shows the defects caused by over-expression using PNR Gal4. The notum of these flies has an abnormal morphology characterised by the formation of a groove in the middle of the thorax. The notopleural suture also develops into a more marked groove. The size of the scutellum is affected and in the more extreme
cases is severely reduced. However, the most of the bristles still seem to be present and in
the correct positions.

In order to determine if there was a genuine difference between the phenotypes
produced by over-expression of the WT and K559 constructs, progeny from crosses of
homozygous UAS CHD1 flies to the PNR Gal4/TM3 and 69B Gal4 lines were scored
according to the severity of the phenotypes. The crosses were performed at room
temperature (21-22°C), 25°C and 29°C and the phenotypes were rated as very mild, mild,
moderate, and severe in each case. In figures 5.11 and 5.12 these phenotypic variations are
represented in panels A, B, C and D respectively. The results are listed in table 5.3 below
and show that at 25°C expression of the K559R construct with PNR Gal4 produces more
severe phenotypes overall, whereas at room temperature, the differences between the WT
and K559R constructs are less distinct. At 29°C expression of the K559R construct is
lethal and flies expressing the WT CHD1 with PNR Gal4 show reduced viability. No
survivors were seen when expressing either construct using 69B Gal4 at 29°C. Expression
of the K559R construct leads to a reduction in viability compared to expression of the WT
construct at both 20 and 25°C. However, the phenotypes of the flies that survive are
similar for both CHD1 constructs.
Figure 5.11 Wings from flies expressing UAS CHD1 WT and K599R with 69B Gal4

A) UAS CHD1 WT 126/69B Gal4 at room temperature. B) UAS CHD1 K559R 60/69B Gal4 at room temperature. C) UAS CHD1 WT 126/69B Gal4 at 25°C. D) UAS CHD1 K559R 60/69B Gal4 at 25°C. E) wild type. Expression of WT and K559T constructs leads to the production of ectopic wing vein material associated with L2 and L4 as well as the posterior cross vein. Occasionally ectopic veins appear at the distal margin (arrow in B). The severity of the phenotypes depends on temperature. The most severe phenotype includes the formation of bristles in the wing.
Figure 5.12 Expression of WT and K559R CHD1 using PNR Gal4

Figure 5.12 Pictures of the thorax from flies expressing UAS CHD1 constructs using PNR Gal4. A) UAS CHD1 WT 126/ PNR Gal4 at room temperature. B) UAS CHD1 WT 126/ PNR Gal4 at 25°C. C) UAS CHD1 K559R 60/ PNR Gal4 at room temperature. D) UAS CHD1 K559R 60/ PNR Gal4 at 25°C. E) wild type. Expression of WT and K559R CHD1 leads to similar defects in the notum, but the K559R construct generally leads to more severe phenotypes. Note the groove forming in the medial notum and the reduction in size of the scutellum. There seems to be no major effects on bristle development.
Table 5.3 Comparison of phenotypes caused by the expression of UAS CHD1 WT and K559R constructs

<table>
<thead>
<tr>
<th>Gal4 line</th>
<th>CHD1 construct</th>
<th>Temp.</th>
<th>% of phenotypes that were</th>
<th>n=</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>very mild</td>
<td>mild</td>
</tr>
<tr>
<td>PNR Gal4</td>
<td>WT</td>
<td>RT</td>
<td>43%</td>
<td>34%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>25°C</td>
<td>30%</td>
<td>53%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>29°C</td>
<td>19%</td>
<td>81%</td>
</tr>
<tr>
<td></td>
<td>K559R</td>
<td>RT</td>
<td>32%</td>
<td>42%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>25°C</td>
<td>10%</td>
<td>47%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>29°C</td>
<td>100%</td>
<td></td>
</tr>
<tr>
<td>69B Gal4</td>
<td>WT</td>
<td>RT</td>
<td>18%</td>
<td>58%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>25°C</td>
<td>10%</td>
<td>33%</td>
</tr>
<tr>
<td></td>
<td>K559R</td>
<td>RT</td>
<td>40%</td>
<td>54%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>25°C</td>
<td>20%</td>
<td>8%</td>
</tr>
</tbody>
</table>

Table 5.3. RT : room temperature (21°C-22°C). The crosses were UAS CHD1 WT (or K559R) x PNR Gal4/TM3 or UAS CHD1 WT (or K559R) x 69B Gal4. n= number of flies scored ie number of flies of the genotypes UAS CHD1/PNR Gal4 or UAS CHD1/69B Gal4 form each cross (total number of progeny in brackets).

5.2.5 Attempting to establish if CHD1 K559R is acting as a dominant negative

The experiments above indicate that expression of the K559R CHD1 mutant does produce a more severe phenotype than expression of the wild type protein. However, the phenotypes generated are still very similar, especially at lower temperatures. In order to try and establish if the K559R CHD1 construct is acting as dominant negative, it was decided repeat the over-expression experiments in a heterozygous CHD1 background. A dominant negative protein would be expected to produce a more severe phenotype in a background with reduced amounts of endogenous protein. However, overexpression of the WT construct might be expected to produce a less severe phenotype. The UAS CHD1 WT and K559R lines were therefore combined with the deficiency covering CHD1 (Df(2L)JS17) and then crossed to the 69B and PNR Gal4 drivers. Removing one copy of...
CHD1 did not cause an enhancement of the severity of the CHD1 K559R phenotypes at 25°C, but there did seem to be a slight suppression of the phenotype caused by the CHD1 WT construct. Figure 5.13 shows some examples of expression of the CHD1 transgenes in the background of the CHD1 deficiency. Higher levels of expression may be necessary to see enhancing effects, but as expression of the CHD1 constructs affects viability it was not possible to test the effects of the deficiency at 29°C. To quantify the effects of overexpression of the CHD1 transgenes in the background of the deficiency, the phenotypes observed at 25°C were scored as described above (table 5.4).

Table 5.4 Comparison of phenotypes caused by the expression of UAS CHD1 WT and K559R constructs in the background of a CHD1 deficiency

<table>
<thead>
<tr>
<th>Gal4 line</th>
<th>CHD1 construct</th>
<th>Temp.</th>
<th>Phenotypes of Df, UAS CDH1 flies</th>
<th>n=</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>mild</td>
<td>moderate</td>
</tr>
<tr>
<td>PNR Gal4</td>
<td>WT</td>
<td>25°C</td>
<td>55%</td>
<td>45%</td>
</tr>
<tr>
<td></td>
<td>K559R</td>
<td>25°C</td>
<td>35%</td>
<td>50%</td>
</tr>
<tr>
<td>69B Gal4</td>
<td>WT</td>
<td>25°C</td>
<td>54%</td>
<td>30%</td>
</tr>
<tr>
<td></td>
<td>K559R</td>
<td>25°C</td>
<td>62%</td>
<td>38%</td>
</tr>
</tbody>
</table>

Table 5.4. Crosses were Df(2L)JS17/Cyo; UAS CHD1 (WT or K599R) x +/++; PNR Gal4/TM3 and Df(2L)JS17/Cyo; UAS CHD1 (WT or K599R)/TM3 x +/++; 69B Gal4. n= number of flies of the genotype 5.4/+; UAS CHD1 (WT or K599R)/PNR Gal4 or Df(2L)JS17/+; UAS CHD1 (WT or K599R)/69B Gal4 scored from each cross (total number of progeny in brackets).
Figure 5.13 Effect of the CHD1 deficiency on the PNR Gal4 UAS CHD1 phenotypes

Figure 5.13 Pictures of the thorax of adult flies of the genotypes A) CyO/+;UAS CHD1 WT 126/PNR Gal4, B) Df(2L)JS17/+;UAS CHD1 WT126/PNR Gal4, C) CyO/+;UAS CHD1 DN 60/PNR Gal4, B) Df(2L)JS17/+;UAS CHD1 DN 60/PNR Gal4. All flies raised at 25°C. Expression of the WT CHD1 construct, with the PNR Gal4 driver, in the background of the CHD1 deficiency leads to slightly less severe thorax phenotypes. There does not seem to be any effect on the phenotype generated by the expression of the K559R construct. See also table 5.4.
5.2.6 Attempting to detect genetic interactions between chd1 and mirror

Although the CHD1 K559R mutant had not been definitively shown to act as a dominant negative protein, it was decided to test if the CHD1 constructs would interact genetically with mirror. The deficiency covering CHD1 had been shown to lead to a slight suppression of the phenotype generated by expression of UAS-Mirror using the GMR Gal4 driver (figure 3.8). To test if over-expression of the UAS CHD1 constructs using the same driver could affect the Mirror phenotype, the GMR Mirror flies (GMR Gal4/Cyo; UAS Mirror) were crossed to flies homozygous for the UAS CHD1 constructs (WT lines 113 and 126, K559R lines 60 and 101). Flies co-expressing either WT or K559R CHD1 with Mirror had very similar phenotypes to the over-expression of Mirror alone (not shown). Expression of UAS Mirror using the Eyeless Gal4 driver leads to a reduction in the size or complete loss of the eye. Co-expression of either of the two CHD1 constructs with Mirror using Eyeless Gal4 did not have any obvious effects on this phenotype (not shown).

5.3 Detailed studies of the localisation of CHD1 on polytene chromosomes

5.3.1. Localisation of CHD1 on polytene chromosomes

Localisation on the polytene chromosomes of the salivary glands has been used to gain information about the distribution of various proteins on chromosomes. The localisation of CHD1 on polytene was reported to be mainly in interbands, ie regions of less compacted DNA, and in transcriptional puffs which are regions in which high levels of transcription are proposed to take place (Stokes et al. 1996). In order to gain more
information about the function of CHD1 and to determine if it might be a co-factor for Mirror, it was decided to study the distribution of CHD1 on polytene chromosomes in detail. These experiments were performed with help and advice from Jennifer Armstrong in the laboratory of John Tamkun at the University of Santa Cruz, California. Co-staining of salivary gland squashes with CHD1 antibody and DAPI confirmed the localisation of CHD1 to interbands and puffs (figure 5.14). The CHD1 staining does not seem to overlap with regions of highly compacted DNA.

To study the localisation properties of the WT and K559R CHD1 constructs, UAS CHD1 lines (WT 126 and K559R 60) were crossed to a hs Gal4 line which expressed Gal4 in the salivary glands at 18°C. Over-expression of most genes in these situations leads to a reduction in the size of the salivary glands and makes it difficult to produce good quality squashes for staining (J. Armstrong, personal communication). In addition, as the constructs are not tagged, detection of the heterologous proteins cannot be distinguished from the endogenous CHD1. However, staining with the CHD1 antibody of polytenes from larvae expressing the WT or the K559 construct showed a more intense staining, about 7-8 times stronger, than in the non-overexpressing condition. Due to the poor quality of the squashes it was difficult to judge if the staining appeared in the same pattern, but both the WT and K559R CHD1 proteins were certainly found in distinct bands and in puffs (figure 5.14). Therefore it seems that the K559R mutation in CHD1 does not have a dramatic affect on its distribution on polytene chromosomes.
Figure 5.14 Localisation of CHD1 on polytene chromosomes

**Figure 5.14** Drosophila polytene chromosomes stained with anti-CHD1 antibodies (green) and DAPI (blue). A) Wild type, B) UAS CHD1 K559R 60/ hs Gal4, C) UAS CHD1 WT 126/ hs Gal4. CHD1 is localised to discreet bands that contain less compacted DNA and to puffs (arrows). Overexpression of the UAS CHD1 K559R or WT constructs in salivary glands does not seem to affect the localisation of the protein dramatically. The images in B and C were captured using gain settings 8x lower than the image in A.
5.3.2 Comparison of the localisation of CHD1 and RNA polymerase

As described in the introduction, the CHD1 proteins have been suggested to have roles in both activation and repression of transcription. In order to find out more about the role of CHD1 in the regulation of transcription, it was decided to compare the localisation of CHD1 on polytenes to RNA Polymerase II. The carboxy-terminal domain (CTD) of RNA Polymerase II is differentially phosphorylated according to where it is found within the transcription unit (Armstrong et al. 2002; Komarnitsky et al. 2000). Initiating and promoter paused forms are hypophosphorylated (Pol IIA), whereas elongating forms are hyperphosphorylated (Pol IIo). Co-staining of polytenes using CHD1 and antibodies specific for Pol IIA and Pol IIo allowed the detailed investigation of the association of CHD1 with actively transcribed regions of the chromosomes. Figure 5.15 shows the localisation of CHD1 and Pol IIA on polytenes. The initiating or paused polymerase, Pol IIA, has a much more widespread distribution than CHD1 and therefore there are many loci that contain Pol IIA and not CHD1. Of the bands that are staining for CHD, about half are co-staining with Pol IIA. This indicates that CHD1 is associated with initiating or paused RNA polymerase at a specific subset of sites. The comparison of the localisation of CHD1 and the elongating form of RNA polymerase, Pol IIo, gave a somewhat surprising result. Figure 5.16 shows that CHD1 and Pol IIo have almost identical staining patterns. CHD1 distribution overlaps nearly perfectly with the distribution of the hyperphosphorylated polymerase indicating that CHD1 is somehow associated with elongation.

It has recently been shown that expression of a dominant negative version of Brahma in the salivary glands can lead to a loss of RNA Polymerase from the polytenes. Salivary gland squashes from larvae expressing WT and K559R CHD1 constructs were also co-stained for Pol IIo. However, due to the poor quality of the squashes it was
difficult to make any conclusions about the presence and distribution of Pol IIo, although it seemed mostly normal (not shown). Further staining of polytenes to determine if either CHD1 or RNA polymerase distribution is affected would be necessary to confirm these results.

5.3.3 Comparison of the localisation of CHD1 and other chromatin remodelling factors

The localisation of *Drosophila* chromatin remodelling factors such as Brahma and ISWI has been important for the understanding of their in vivo functions. Brahma localises with both forms of RNA polymerase at most sites and this reflects the predominant role of Brahma in activation of transcription (Armstrong *et al.* 2002). ISWI distribution on the other hand does not overlap extensively with RNA polymerase reflecting its role in repression of transcription and maintenance of chromatin structure (Deuring *et al.* 2000). The localisation of the Kismet protein on polytenes is currently being investigated (J. Tamkun, personal communication). Using antibodies generated in the Tamkun laboratory, the distribution of CHD1 was compared to that of Brahma and Kismet to look for co-localisation. Figure 5.17 shows the distribution of Brahma and CHD1. As Brahma is already know to co-localise with both initiating and elongating Polymerase, CHD1 would be expected to co-localise extensively with Brahma. This is indeed the case, although there are also some loci which contain only CHD1 or only Brahma. The co-localisation between CHD1 and Kismet was less extensive (figure 5.17). The two proteins seem to overlap at about 50% of the CHD1 sites and there were many sites that contained Kismet, but not CHD1.
Figure 5.15 Co-localisation of CHD1 and Pol IIa on polytene chromosomes

Figure 5.15. Polytene chromosomes stained with anti-CHD1 (green) and anti-Pol IIa (red). A-C are pictures of the same polytene squash visualising the CHD1 pattern (A) and the Pol IIa pattern (B) as well as the merged image (C). CHD1 and Pol IIa co-localise in some bands and puffs (arrow head), but there are also many sites at which only Pol IIa or CHD1 is localised (green arrow head CHD1, red arrow head Pol IIa).
Figure 5.16 Co-localisation of CHD1 and Pol IIo on polytene chromosomes

Figure 5.16 Polytene chromosomes stained with anti-CHD1 antibodies in green and anti-Pol IIo antibodies in red. A-C are pictures of the same polytene squash showing the pattern of CHD1 staining (A), Pol IIo staining (B) and the merged image (C). The patterns of localisation of CHD1 and Pol IIo look almost identical. D shows the co-localisation in several bands and puffs. E and F are close ups of chromosome arms showing a split view of the CHD (green) and Pol IIo (red) staining. The intensity of staining with CHD1 and Pol IIo varies at different loci, but split views confirm that the two proteins overlap at about 90-95% of sites.
Figure 5.17 Co-localisation of CHD1 and Brahma and CHD1 and Kismet

Figure 5.17 Polytene chromosomes stained with anti-CHD1 antibodies in green and anti-Brahma antibodies in red (A) or anti-Kismet antibodies in red (B). CHD1 and Brahma overlap at many sites on the chromosome, especially in puffs. CHD1 and Kismet only overlap at about 50% of sites.
5.4 Discussion

5.4.1 Generation of a putative dominant negative CHD1

CHD1 contains a SNF2-like ATPase domain and is expected to function as an ATP dependent nucleosome remodelling factor. Mutation of the conserved lysine in the GxGKT motif of ATPases has been shown to generate proteins that have reduced ATPase function and that show dominant negative effects (Elfring et al. 1998; Khavari et al. 1993). The lysine residue 559 of CHD1 was changed to arginine by site directed mutagenesis and transgenic flies containing both UAS CHD1WT and K559R constructs were made. The presence of the K599R mutation was confirmed by amplification and sequencing of the transgenes in lines 60 and 101 (WT lines 113 and 126 were also sequenced, not shown). Expression of the CHD1 constructs using a variety of Gal4 drivers led to phenotypes in the eye, wing and notum (discussed below). However, the phenotypes associated with over-expression of the WT construct were very similar those resulting from over-expression of the K559R construct. The K559R constructs did cause more severe effects, especially at higher temperatures. The increase in severity did not seem to be due to a large difference in levels of expression. Two lines carrying both the WT and K559R UAS CHD1 constructs showed comparable expression at least in the eye.

The reason for the very similar effects of over-expression could be that the levels of CHD1 are critical for its proper function. Expression of either a dominant negative or wild type protein would influence the levels of functioning CHD1 protein and this could have the same deleterious effects. On the other hand, the entire length of the CHD1 cDNA obtained from Robert Perry was not sequenced before it was cloned into the pExPress vector. Therefore, it is possible that both the WT and K599R constructs contain an additional mutation which is responsible for the observed phenotypes. It is also possible that the K559R mutation in CHD1 does not generate a dominant negative protein. Similar
mutations in two other *Drosophila* proteins with ATPase domains have been reported (Deuring *et al.* 2000; Elfring *et al.* 1998). In the case of the ISWI protein, targeted expression of both wild type and mutant constructs have been performed and only the dominant negative protein generates specific phenotypes (J. Tamkun, personal communication). On the other hand, targeted expression of wild type Brahma has not been compared to the expression of the dominant negative Brahma.

In addition to generating similar phenotypes when over-expressed, the WT and K559R CHD1 proteins also appear to localise normally on polytene chromosomes. This is somewhat surprising as it has been shown that dominant negative Brahma shows abnormal localisation when expressed in salivary glands (J. Armstrong, personal communication). Studies on the mouse CHD1 protein had found that deletion of the first helicase motif (which includes the GxGKT sequence) caused the protein to be abnormally localised in U2OS cells (Stokes and Perry 1995).

In order to try to determine if the CHD1 K559R construct is acting as a dominant negative, one copy of the CHD1 locus was removed to reduce the levels of endogenous protein. In this background it would be expected that the expression of a dominant negative protein has an enhanced effect. No enhancement of the phenotype was seen at 25°C. Similar experiments using the dominant negative ISWI protein had shown that the enhancement caused by reduction of endogenous protein levels could only be detected at higher temperatures (J. Armstrong, personal communication). At 29°C expression of the K559R CHD1 construct was lethal. It could therefore not be definitively concluded if the CHD1 K559R is acting like a dominant negative protein.

The expression of a dominant negative protein allows the investigation of phenotypes in specific tissues and as the level of expression can be controlled, varying degrees of phenotypes can also be studied. However, a null allele is necessary for the study of true loss of function phenotypes as well as for detailed investigations of genetic interactions. A screen for a mutant in the CHD1 locus has therefore been undertaken with
the help of Sophia Pinto. So far 21 lethal lines have been identified that do not complement a deficiency covering CHD1 (not shown). Further complementation tests are being performed using overlapping deficiencies in order to identify mutations within a ~100Kb region that covers the CHD1 locus. Final identification of mutants should be possible using the WT UAS CHD1 transgene as a rescue construct.

### 5.4.2 Phenotypes associated with the over-expression of CHD1 constructs

The aim of generating a dominant negative CHD1 constructs was to characterise CHD1 function further and to find out if CHD1 interacts genetically with *mirror*. Although the K559R construct has not been proven to be a dominant negative protein, a discussion of the observed over-expression phenotypes is still valid. Most surprising was the lack of phenotypes observed with some Gal4 lines and the relatively mild phenotypes generated by others. The lack of phenotypes observed in the embryo after over-expression of CHD1 constructs is not surprising as over-expression of both ISWI and Brahma dominant negative constructs does not affect embryonic development due to maternal contribution. The Eyeless Gal4 line drives expression in the entire eye discs early in eye development. Expression of proteins such as Mirror and ISWI using this driver, leads to dramatic reduction or loss of the eye field. However, expression of UAS CHD1 constructs at 25°C does not seem to affect eye development. When eye discs from larvae expressing UAS CHD1 WT and K559R under the control of Eyeless Gal4 were stained with CHD1 antibodies, expression levels did not seem elevated above normal (not shown). The low expression levels observed could be due to heterologous mRNA and/or protein instability, although expression from drivers such as GMR Gal4 does produce very high levels of over-expression (figure 5.10). It is also possible that the expression of Gal4 is somehow affected by the expression of the CHD1 constructs in some lines. Other Gal4 lines which do not produce any phenotypes in combination with UAS CHD1 lines are Fringe and PNR
Gal4 in the eye and 765 and 30A Gal4 in the notum and wing. It could be that these drivers do not generate high enough levels of expression for the CHD1 constructs to have any effect at 25°C. Expression of the WT and K559R constructs at 29°C may reveal new phenotypes.

Expression of the CHD1 constructs using some Gal4 drivers did produce interesting phenotypes. Expression of WT and K559R CHD1 using the 69B Gal4 line produced mild to moderate phenotypes in the eye and wing at lower temperatures, but was lethal at 29°C. Expression of both dominant negative ISWI and Brahma using this driver is lethal (Deuring et al. 2000; Elfring et al. 1998). In the eye, both WT and K559R constructs were associated with a mild roughness and subtle defects in the ommatidial array. In the wing, expression of CHD1 using 69B Gal4 led to the production of ectopic wing vein material indicating defects in patterning. Expression of the CHD1 transgenes using the PNR Gal4 line produced a quite severe thorax phenotype, especially at high temperatures and affected viability at 29°C. The phenotype was characterised by the formation of a groove in the middle of the notum and could be due to defects in the fusion between the two wing discs that make up the notum. The phenotypes associated with the expression of dominant negative ISWI or Brahma using PNR Gal4 have not been reported. However, over-expression of Osa, a subunit of the Brahma complex, leads to very similar phenotypes (Collins and Treisman 2000). Mitotic clones of Brahma in the notum show bristle defects (Elfring et al. 1998). However, the formation of bristles did not seem affected by the over-expression of CHD1 WT or K559R. The GMR Gal4 driven expression of CHD1 WT and K559R led to serious defects in eye development at 25°C, including complete disorganisation of ommatidia. The abnormalities associated with the GMR driven expression of the K599R construct could also be detected in the eye disc (figure 5.10). At room temperature however, the defects were more subtle consisting of occasional photoreceptor defects and disruption of the ommatidial array. This phenotype
is very similar to the one observed when expressing a UAS-dMi-2 construct using GMR Gal4 (Hirose et al. 2002).

Although it has not been shown that the K559R mutant is a dominant negative, it might have been expected that the phenotypes associated with over-expression of a mutant chromatin remodelling protein would be more severe. The phenotypes described for the over-expression of CHD1 constructs are quite specific. This could indicate that although CHD1 is expressed ubiquitously, it only has very specific developmental functions.

It is also possible that CHD1 has overlapping or redundant functions with other ATPase containing proteins. The closest homologue of CHD1, dMi-2, could be replacing CHD1 function. In addition there is another uncharacterised CHD protein present in the genome. As will be described below, the localisation of Brahma on polytene chromosomes overlaps extensively with CHD1 and therefore it is conceivable that Brahma could replace CHD1 function in some circumstances.

5.4.3 Localisation of CHD1 on polytene chromosomes

The localisation of CHD1 to regions of less compacted DNA ie interbands, and transcriptional puffs was confirmed by co-staining of polytene chromosomes with the CHD1 antibodies and DAPI. The distribution of the heterologous CHD1 proteins was also investigated by expression of the UAS WT and K559R constructs in salivary glands. Like for most other constructs, over-expression of the CHD1 transgenes leads to a reduction in the size of the glands and reduces the quality of the polytene squashes. This makes the interpretation of the antibody stainings more difficult. However, as mentioned above, the localisation of both the WT and K559R was not strikingly different to that of the endogenous protein.
In order to investigate the role of CHD1 in transcription further, the distribution of CHD1 was compared with that of the initiating and elongating forms of RNA polymerase, Pol IIa and Pol IIo (Armstrong et al. 2002). CHD1 was found co-localise with Pol IIa about 50% of the time. This indicates that CHD1 is not associated with all initiation complexes, but may be important for the initiation at a subset of loci. On the other hand, CHD1 was found to overlap nearly perfectly with Pol IIo. Only a few sites were found that contained CHD1, but not Pol IIo.

The association of CHD1 with elongation is intriguing as the yeast Chd1p proteins has recently been found to interact genetically and physically with the yeast elongation factor Spt5 (G. Hartzog, personal communication). Spt5 and its partner Spt4 are involved in regulation of elongation in both yeast and humans together with other elongation complexes such as PafI and FACT (Squazzo et al. 2002). Chd1p has also been shown to interact with the PafI complex and to modulate interactions within the yeast FACT complex (G. Hartzog, personal communication). Subunits within these complexes have previously been shown to interact genetically with the Swi-Snf complex and mutations in histones. This indicates that the regulation of elongation is linked to the regulation of chromatin structure. In Drosophila, the Spt5 and Spt 6 homologues also co-localise with Pol IIo on polytene chromosomes. To study the role of CHD1 in regulation of elongation in Drosophila, genetic interactions with Drosophila elongation factors could be investigated.

The Drosophila Brahma protein is also associated with both initiating and elongating polymerase (Armstrong et al. 2002). A comparison of the distribution of CHD1 and Brahma reveals extensive overlaps, but also some loci that only contain CHD1 or Brahma. A recent report suggests that Brahma has a general role in the control of transcription which is linked to the association of polymerase with chromatin. Expression of dominant negative Brahma leads to a loss of RNA polymerase from polytene chromosomes. The effect of expression of CHD1 on the distribution of Pol IIo was
investigated briefly, but appeared to be minor. However, the poor quality of the data made the interpretation difficult. Further analysis of the over-expression of WT and K559R constructs in salivary glands should reveal any affect on the distribution of polymerase.

The localisation of CHD1 on chromosomes was also compared to the localisation of Kismet. Kismet is found in two isoforms, one which includes the chromo domains and the ATPase domains, and one which only contains the C-terminal BRK motif (Daubresse et al. 1999). Nothing is known about the type of complexes Kismet or CHD1 may be part of, so it was thought that investigation of their localisation on polytenes might reveal if they were somehow associated. However, CHD1 and Kismet were not found to co-localise extensively. Only about 50% of the CHD loci also contained Kismet. This indicates that CHD1 and Kismet are probably not part of a stable complex.

5.4.4 Could CHD1 be a co-factor for Mirror?

In order to try and determine if CHD1 could be a co-factor for Mirror, it was decided to try and generate a dominant negative CHD1 construct. This would allow the characterisation of the loss of function phenotype for CHD1, comparisons with mirror phenotypes and the investigation of genetic interactions. If Mirror controls gene expression through CHD, it would first of all be expected that reduction or loss of CHD1 function would affect Mirror target genes. Also reducing the levels of Mirror and CHD1 might be expected to have synergistic effects. Phenotypes associated with ectopic expression of Mirror may also be affected if CHD1 is a general co-factor for Mirror.

In the eye, loss of Mirror function leads to a reduction of the eye field and defects in planar polarity. However, expression of UAS CHD1 K559R using Eyeless Gal4 does not affect eye size or ommatidial polarity. The only phenotypes observed in the eye from
expression of the UAS CHD1 constructs were associated with expression after the morphogenetic furrow. As the function of Mirror after the furrow is not yet known, a comparison between phenotypes can not be performed. In the notum, loss or reduction of Mirror leads to loss of lateral bristles. Expression of UAS CHD1 K559R in the wing disc using various drivers did not seem to affect bristle formation or patterning. However, the formation of wing veins was affected by over-expression of both WT and K559R CHD1. The phenotype produced is similar to an over-expression phenotype of Ara in the wing (Gomez-Skarmeta et al, 1996). In addition to comparisons of phenotypes, genetic interactions were investigated between over-expression of Mirror and CHD1 constructs. It was reasoned that if CHD1 is a co-factor for Mirror, reducing the amount of functional CHD1 may suppress the Mirror over-expression phenotypes. However, no effect was detected when CHD1 WT and K559R constructs were co-expressed with Mirror using GMR and Eyeless Gal 4 drivers.

However, the K559R mutation in CHD1 has not been proved conclusively to produce a dominant negative protein. This makes the analysis of the phenotypes generated and interactions with Mirror difficult. As mentioned above, a screen for alleles of CHD1 has been initiated. Hopefully the identification of a null allele for CHD1 may make the investigation of an in vivo interaction between Mirror and CHD1 more conclusive. It would allow the investigation of interactions between mirror and CHD1 alleles possible and would allow the effect of loss of CHD1 on over-expression of Mirror be properly analysed.

In order to find out more about the function of CHD1 in transcription, the localisation of CHD1 on polytene chromosomes was studied in detail. Initially, the distribution of Mirror protein was also investigated with a view to look for co-localisation with CHD1. However, staining of polytenes using Mirror antibodies was inconclusive (not shown). CHD1 was shown to associate with many sites of initiation of transcription indicating that it may have a positive role in gene regulation. However, the association of
CHD1 with elongating polymerase may indicate that CHD1 has a more general role as a co-factor for elongation. Until direct Mirror targets have been identified, it will be difficult to assess if CHD1 is important for the regulation of transcription by Mirror.

The confirmation of a physical interaction between CHD1 and Mirror was attempted by immunoprecipitation. As described in the previous chapter a FLAG-tagged Mirror construct can be immunoprecipitated from *Drosophila* S2 cells. As CHD1 is expressed endogenously in S2 cells, the co-precipitation of CHD1 with Mirror was investigated, but could not be detected (not shown). Releasing CHD1 protein from chromatin requires high salt concentrations (Stokes and Perry 1995) and these conditions may not allow weak or transient interactions to be detected. Other methods for detecting in vivo interactions include co-fractionation from cell and embryo extracts (Kelley *et al.* 1999; Murawsky *et al.* 2001). In collaboration with John Tamkun, an investigation into the presence of a CHD1 construct was initiated. Extracts from embryos were fractionated using a Suparose 6 gel filtration column and probed with the CHD1 antibody. Initial results indicate that CHD1 elutes in fractions of 600-1000KDa indicating that it is found in a complex (I. Dahlsveen, D. Corona and J. Tamkun, not shown). It is interesting to note that the size of the putative *Drosophila* CHD1 complex is very similar to that of the putative mouse complex (Kelley *et al.* 1999). The presence of Mirror and other proteins in this complex will be investigated further.

The investigations into the function of CHD1 in *Drosophila* have not yet been able to establish if CHD1 could be a co-factor for Mirror. However, it has revealed interesting and previously unknown features of CHD1 function in vivo. Further investigations using new alleles of CHD1 and specific Mirror target genes may still reveal an in vivo interaction. In addition experiments investigating the CHD1 complex in vivo, may help in dissecting any physical interactions between CHD1 and Mirror.
CHAPTER 6

Materials and Methods

In general, chemicals were obtained from BDH, Fisons or Sigma. Commonly used buffers and media were made by the Cancer research UK media services according to Sambrook et al, 1989. Other materials are described within each section.

6.1 General DNA Manipulation

6.1.1 DNA preparation, purification and analysis

DNA was prepared from bacterial cultures grown in LB or Terrific broth (Sambrook et al, 1989) using Qiagen Mini, Midi, Maxi, Mega, or Giga prep kits according to the instruction manuals. Digested DNA and PCR products were purified either by using the QIAquick Gel Purification or PCR Purification kits (Qiagen) according to manufacturer’s instructions. Alternatively, DNA was purified by phenol:chlorofrom extraction and ethanol precipitation. Briefly, an equal volume of phenol:chlorofrom was added to the DNA sample, mixed and centrifuged to separate the phases. To the aqueous phase was added 1/10 volume 3M NaOAc (pH 5.2) and 2.5x volume 100% cold ethanol. DNA was precipitated at -20°C for 30 min, collected by centrifugation (13000 rpm, 20-30 min), washed with 70% ethanol and resuspended in EB buffer (10mMTris-HCl, pH7.5). Preparative and analytical gel electrohoresis was performed using 1-1.5% agarose (GIBCO BRL) gels in TAE (Sambrook et al, 1989).
6.1.2 Cloning

General cloning vectors used:

<table>
<thead>
<tr>
<th>Vector</th>
<th>Source</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBluescript</td>
<td>Stratagene</td>
<td>Contains T7 and T3 promoters for generating RNA probes</td>
</tr>
<tr>
<td>pCRBlunt 2.1</td>
<td>Invitrogen</td>
<td>Used for cloning blunt PCR products</td>
</tr>
<tr>
<td>pCR 2.1 TOPO</td>
<td>Invitrogen</td>
<td>Used for cloning PCR products with TA overhangs</td>
</tr>
<tr>
<td>pLitmus 28</td>
<td>New England Biolabs</td>
<td></td>
</tr>
</tbody>
</table>

DNA was digested with restriction endonucleases (New England Biolabs) using the suggested buffers and conditions for single and double digests according to the New England Biolabs catalog. Ligations were performed using T4 ligase (Promega) for periods of 3-6 hours at room temperature or 16 hours at 16°C. Ligations were either transformed into Invitrogen One Shot® Chemically Competent TOP10 cells (F mcrAΔ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 recA1 deoR araD139 Δ(ara-leu)7697 galU galK rpsL (Str*) endA1 nupG) according to manufacturer’s instructions or TOP10 cells that had been made competent using the Calcium Chloride method from Short Protocols in Molecular Biology (Ausubel et al., 1992). Transformed bacteria were plated on LB agar plates containing the appropriate antibiotics and incubated at 37°C for 16-24 hours.

PCR products were cloned using the pCR®-Blunt or TOPO TA cloning kits from Invitrogen according to the instruction manuals. PCR products were generated using pfu DNA polymerase (see below) and either cloned directly into the pCR®-Blunt vector or incubated for 10 min. with Taq Polymerase at 72°C and cloned into the pCR®2.1-TOPO® vector. All clones were analysed by preparation of DNA and restriction digests.
6.1.3 Polymerase Chain Reaction

All primers were synthesized by the ICRF/Cancer Research UK oligo services and were either pre-purified or ethanol precipitated as suggested.

Primers used for the amplification of Mirror constructs:

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mrr1_5'</td>
<td>TATTCATGGCAGTGACAGTGAATGAAACC</td>
<td>\textbf{NcoI} site at ORF start</td>
</tr>
<tr>
<td>Mrr2_5'</td>
<td>GTGGCCATGGCATGGCCTTGAGCCATATCCA</td>
<td>\textbf{NcoI} site at bp 579</td>
</tr>
<tr>
<td>Mrr3_3'</td>
<td>CCAGATCTGCTTTGAGCCGTACCTCCGTGAACC</td>
<td>\textbf{BglII} site at bp 994</td>
</tr>
<tr>
<td>Mrr4_3'</td>
<td>GGAGATCTTGGCTTTGAGCCGTACCTCCGTGAACC</td>
<td>\textbf{BglII} site at ORF end</td>
</tr>
<tr>
<td>Mrr5_5'</td>
<td>GCCATGGTGACCCGAGGCTCCGGGACC</td>
<td>\textbf{NcoI} site at bp 1013</td>
</tr>
</tbody>
</table>

Primers used for the amplification of CGI 135:

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>lGaEco5'</td>
<td>GAATTCATGGACAGTGAATGAAACC</td>
<td>\textbf{EcoRI} site at ORF start</td>
</tr>
<tr>
<td>lGaXba3'</td>
<td>CGAATCCCCTCAACTAGTCTAGATC</td>
<td>\textbf{XbaI} site at ORF end after stop codon</td>
</tr>
</tbody>
</table>

Primers used for amplification from yeast plasmids:

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Vector</th>
</tr>
</thead>
<tbody>
<tr>
<td>pACT 3'</td>
<td>GTGAACCTTGCGGGGTTTTTCAGTATCTACGA</td>
<td>pACT 2</td>
</tr>
<tr>
<td>pACT 5'</td>
<td>TGTCACTGCGGGTTCAGTGACGACGACGAACCTGCGTAT</td>
<td>pACT 2</td>
</tr>
</tbody>
</table>

Primers used for the amplification of the CGI 135 locus:

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>CGI 135 genomic 5'</td>
<td>CAGTAATTCAATTCAACGTCGG</td>
</tr>
<tr>
<td>GC1135 genomic 3'</td>
<td>CTGCACAGCCAGGCTCTGACC</td>
</tr>
</tbody>
</table>
PCR was performed using a Primus 96plus PCR machine from MWG-BIOTECH. Amplification of DNA for cloning was done using pfu or pfu turbo DNA Polymerase and buffers from Stratagene. Template DNA was generally used at 10-50ng, primers at 125ng and dNTPs (Roche) at a final concentration of 2.5mM each per reaction. Reaction components (except DNA polymerase) were mixed on ice before denaturation at 98°C for 2min. The reactions were kept at 96°C whilst polymerase was added followed by 25-30 cycles of denaturation at 95-97°C for 1min, anneling at 45-57°C (according to primer melting temperature) for 1min. and elongation at 72°C for 3-6min (according to the length of the resulting DNA fragment).

PCR was carried out on yeast plasmid DNA using Taq polymerase (in house) with 2.5µl of template (see 2.5.7 Yeast DNA Isolation), 250ng each of primers and dNTPs at 2.5mM each per reaction. Conditions were 95°C for 3 min. followed by 5 cycles of 95°C for 45 sec., 55°C for 1 min., 72°C for 4 min.; 25 cycles of 95°C for 45 sec., 52°C for 1 min., 72°C for 4 min.; 72°C for 10 min.

Single fly analytical PCR was performed using the Taq Master Mix from Qiagen. Templates were prepared by placing single adult flies were in 0.2 ml tubes and freezing at -80°C for 15 min. To each tube, 50 µl of fly extraction buffer (10mM Tris-Cl, pH 8.2, 1mM EDTA, 25mM NaCl, 10ug Proteinase K) was added and the fly homogenized using a 20-200µl pipette until the solution was cloudy. Samples were incubated at 30°C for 25 min. before inactivation of Proteinase K at 95°C for 2min, and cooling to 4°C. Template was used at 2µl per each reaction with 50-125ng of the appropriate primers and 1/2 reaction volume Master Mix. Amplification conditions were 5 min. at 95°C, 30 cycles of 95°C for 30 sec, 52-55°C for 1 min, 72°C for 2min, and 10 min at 72°C.
6.1.4 Sequencing

General sequencing primers:

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Vectors:</th>
</tr>
</thead>
<tbody>
<tr>
<td>M13Fwd</td>
<td>GTAAAACGACGGCCAGT</td>
<td>pCR®-Blunt, pCR®2.1-TOPO, pBluescript</td>
</tr>
<tr>
<td>M13Rev</td>
<td>CAGGAAACAGCTATGAC</td>
<td>pCR®-Blunt, pCR®2.1-TOPO, pBluescript</td>
</tr>
<tr>
<td>GAL4 AD</td>
<td>CATCCATTGTAGTGTA</td>
<td>pACT2</td>
</tr>
</tbody>
</table>

DNA sequencing was carried out by the Cancer Research UK in house facilities using the ABI PRISM Dye terminator cycle sequencing kit. Each reaction contained 8μl of the Dye Terminator Ready Reaction Mix (Perkin Elmer), 500ng-1μg of plasmid DNA template or ~200ng PCR product and 10pmol of primer. For sequencing of the mirror constructs, 1μl DMSO was added to the sequencing reactions. PCR cycle sequencing and purification of the products was performed as described in the ABI Cycle Sequencing Kit instructions. The reactions were loaded on an automated sequencing machine (ABI PRISM 377, Perkin Elmer) and analysed using ABI PRISM software (Sequencing Analysis v 3.0).

6.1.5 Site Directed Mutagenesis

Primers used for mutagenesis of mirror and CHD1:

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Comments:</th>
</tr>
</thead>
<tbody>
<tr>
<td>MrrIRO_5′</td>
<td>CCGCAGTGGGGGCAACCGCAGTGGCCTCCAAGGACAGC</td>
<td>Bind each side of IRO box.</td>
</tr>
<tr>
<td>MrrIRO_3′</td>
<td>GCTGTCCTTGACCCGGATCGCGCCTGCGGCCCCTCCAAGGACAGC</td>
<td>Changes K559 to R</td>
</tr>
<tr>
<td>ChdKtoR_5′</td>
<td>CGAGATGGCCCTCGCAGAACCATCCAAACTATTTGC</td>
<td></td>
</tr>
<tr>
<td>ChdKtoR_3′</td>
<td>GCAAATAGTTGATGACATCGGCCCGAGCAGCAGCCATCTCG</td>
<td></td>
</tr>
</tbody>
</table>

Primers for mutagenesis were synthesized by the ICRF/ Cancer Research UK oligo services and purified by HPLC. Mutagenesis was performed by PCR as described in the
manual for the Stratagene QuikChange™ Site-Directed Mutagenesis Kit (cat. no. 200518, revision no. 108005b). Template DNA was digested using DpnI and the PCR products transformed into supercompetent TOPP 10 cells (as described). Resulting clones were analysed by preparation of DNA, restriction enzyme digest and sequencing.

6.2 General Protein manipulation

6.2.1 SDS PAGE

Polyacrylamide gel electrophoresis was performed using the BioRad Protean systems II and III. Resolving gels were made to contain 7.5-12% polyacrylamide (37.5:1 acrylamide/Bis, BioRad) in 375mM Tris pH 8.8, 0.1% SDS and stacking gels contained 4% polyacrylamide in 125mM Tris pH 6.8, 0.1% SDS. Protein samples were mixed with 4X sample buffer (125mM Tris pH 6.8, 20% glycerol, 4% SDS, 10% β-mercaptoethanol, 0.008% Bromophenol Blue) and boiled for ~ 5min. before loading with appropriate molecular weight markers. Gels were run in SDS-PAGE running buffer (25mM Tris pH 8.3, 192mM Glycine, 0.1% SDS). Electrophoresis conditions were 200V constant voltage for small gels and 50mA (stacking gel) and 20-70mA (resolving gels) constant current for large gels.
### 6.2.2 Western Blots

Primary antibodies used for detection of Western blots:

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
<th>Dilutions</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rb α-Mirror</td>
<td>Helen McNeill, antigen = GST-Mirror C-term fusion</td>
<td>1:1000-1:2000</td>
<td>rabbit polyclonal</td>
</tr>
<tr>
<td>α-HA 11</td>
<td>Babco</td>
<td>1:1000</td>
<td>mouse monoclonal</td>
</tr>
</tbody>
</table>

Secondary antibodies used for detection of Western blots:

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-mouse Ig horseradish peroxidase conjugate</td>
<td>Amersham Lifescience</td>
<td>1:1000</td>
</tr>
<tr>
<td>α-rabbit Ig horseradish peroxidase conjugate</td>
<td>Amersham Lifescience</td>
<td>1:1000</td>
</tr>
</tbody>
</table>

Western blots were performed using the Trans Blot Electrophoretic Transfer Cell or the Semi Dry Electrophoretic Transfer Cell, both from BioRad, according to the instruction manual. Polyacrylamide gels were equilibrated in transfer buffer (48mM Tris pH8.3, 192 mM Glycine, 20% methanol, 0.05% SDS) and proteins transferred onto ECL Hybond nitrocellulose membranes (Amersham Pharmacia) at 10V for 60-90 min. using the Semi Dry system or 10V for 5-16 hours using the Trans Blot system. Membranes were blocked in PBSTween (PBS, 0.1% Tween 20) with 5% milk for 1 hour at room temperature or overnight at 16°C. Primary antibodies were diluted in PBSTween, 0.2% milk and incubated with the membrane for 1-2 hours at room temperature. Following two rinses and 3x 10 min washes in PBSTween, the membrane was incubated with the appropriate horseradish peroxidase conjugated secondary antibody (diluted in PBSTween, 0.2% milk) for 1 hour. The membrane was washed as before and the HRP detected using the ECL Western
Blotting chemiluminescent detection agent (Amersham Pharmacia) according to the manufactures instructions.

6.3 Yeast-two-hybrid Techniques

All Yeast-Two-Hybrid experiments were done using components from the Clontech MATCHMAKER Two-Hybrid System 2 (Clontech 1998). Most protocols used, unless otherwise indicated are taken from the MATCHMAKER GAL4 Two-Hybrid User Manual (Protocol no. PT3061-1, version PR82033) or The Yeast Protocols Handbook (Protocol no. PT3024-1, version PR91200), both from Clontech. These protocols also contain additional references.

Vectors used:

<table>
<thead>
<tr>
<th>Vector</th>
<th>Description</th>
<th>Size</th>
<th>GenBank #</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pAS2-1</td>
<td>GAL4 DNA Binding domain (aa 1-147) Yeast selection: TRP1, CYH2 Bacterial selection: amp'</td>
<td>8.4 kb</td>
<td>U30497</td>
<td>Harper et al., 1993</td>
</tr>
</tbody>
</table>

Yeast strains used:

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reporters</th>
<th>Markers</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>HF7c</td>
<td>MATa, ura3-52, his3-200, ade2-101, lys2-801, trp 1-901, leu2-3, 112, gal4-542, gal80-538, LYS2::GAL1 UAS::GAL1 TATA-HIS3, URA3::GAL1 17::MBS(x3)·CYC1 TATA·lacZ</td>
<td>HIS3, lacZ</td>
<td>trp1, leu2</td>
<td>Feilotter et al, 1994</td>
</tr>
<tr>
<td>Y190</td>
<td>MATa, ura3-52, his3-200, ade2-101, lys2-801, trp 1-901, leu2-3, 112, gal4Δ, gal80Δ, cyh2, LYS2::GAL1 UAS·HIS3 TATA·HIS3, URA3::GAL1 UAS·GAL1 TATA·lacZ</td>
<td>HIS3, lacZ</td>
<td>trp1, leu2, cyh2</td>
<td>Harper et al, 1993; Flick and Johnston, 1990.</td>
</tr>
</tbody>
</table>
6.3.1 Yeast culture conditions

Yeast were grown in non-selective conditions in YPD medium (20g/L Difco peptone, 10g/L Yeast extract, 2% glucose, pH 5.8) or YPDA medium (YPD, 0.003% adenine hemisulfate) at 30 °C shaking at 200rpm or on YPD/YPDA agar plates (20g/L BactoAgar) at 30°C. For selective medium (SD), minimal medium lacking uracil, leucine, tryptophan and histidine was prepared (1.7g/L Nitrogen Base (Difco), 0.6g/L Amino Acid Mix, CSM-HIS-LEU-TRP-URA (BIO101)) to which the appropriate amino acids were added as required at the following final concentrations: L-histidine HCl monohydrate, 20 mg/L; L-leucine, 100mg/L; L-tryptophan, 20mg/L. 3-amino-1,2,4-triazole (3AT, Sigma) was added to SD medium from a 1M stock to give final concentrations from 2-30mM.

6.3.2 Yeast Transformation

Small and library scale transformations were performed according to the Yeast Transformation Protocols in the MATCHMAKER GAL4 Two-Hybrid User Manual. To make competent yeast cells, an overnight yeast culture was used to inoculate 300ml [1L] of YPD to give a starting OD600 of 0.2-0.3. This culture was grown until the OD600 reached 0.5-0.8 and cells harvested by centrifugation for 5 min. at 1000 x g. The cells were resuspended in 300ml [800ml] sterile H2O, centrifuged as before and resuspended in 1.5ml [8ml] TEL (10mM Tris-HCl, pH7.5, 1mM EDTA, 100mM LiAc) prepared fresh from 10X stocks of TE and 1M LiAc (pH7.5).

For the small scale transformations, DNA (either pAS2-1 constructs, pACT2 constructs or both) at a total concentration of 1-2μg, was mixed with 100μg Herring testes carrier DNA (Clontech) and added to 100μl of competent yeast cells. To each
transformation was added 0.6ml freshly prepared PEG/TEL solution {40% PEG 4000 (Sigma) in TEL} and the tube vortexed to mix. The transformations were incubated for 30 min. at 30°C with shaking at 200rpm before addition of 70 μl DMSO. The tubes were inverted to mix and heatshocked for 15min. at 42°C. Following a 1-2min. incubation on ice, the transformations were centrifuged for a few seconds at 14000 rpm and resuspended in 0.5ml TE. Volumes of 50-200μl of the transformations were plated on agar plates with the appropriate selective media and incubated at 30°C for at least 72 hours.

For library transformations, 8ml of competent yeast cells were mixed with ~ 500μg of library DNA, 1mg pAS2-1 bait construct, 20mg Herring testes carrier DNA, and 60ml PEG/TEL. The mixture was vortexed and incubated at 30° in a 250ml flask, shaking at 200rpm. After the addition of 7 ml DMSO, the flask was heatshocked for 15 min. at 42°C with occasional swirling. The cells were chilled on ice, centrifuged in two 50 ml tubes for 5 min. at 1000x g and resuspended in a total volume of 9.5ml TE. The transformation was plated on 150mm plates with the appropriate medium in 200μl aliquots. Serial dilutions of the transformation was plated on agar plates of SD medium lacking Tryptophan and Leucine to determine transformation efficiency.

6.3.3 Protein Extraction

Protein extractions were done from 50ml YPD cultures grown to an OD₆₀₀ of about 0.550. Cells were harvested by centrifugation for 5 min. at 1000x g, 4°C in presence of ice, washed in ice cold H₂O, and centrifuged again as before. Cell pellets were frozen on dry ice and stored at -70°C. To lyse cells, the frozen pellets were thawed by the addition of 100μl/7.5 OD₆₀₀ units pre-warmed cracking buffer {40 mM Tris-HCl, pH 6.8, 8M Urea, 0.1mM EDTA, 5% SDS, 0.4 mg/ml Bromophenol blue, 125mMβ-mercaptoethanol, 4 mM PMSF, 1.5X Protease Inhibitor cocktail (Complete Protease Inhibitor Tablets from Roche)}. Glass beads (425-600μm, Sigma) were added to give 80μl/7.5 OD₆₀₀ units and
after heating at 70°C for 10 min., the cell walls were disrupted by vortexing for 1 min. Following centrifugation for 5 min. at 14000 rpm, the protein extracts (supernatant) were transferred to new tubes, and the pellet boiled (100°C) for 5 min., re-vortexed and centrifuged and this second supernatant combined with the first to give the total protein extract. Extracts were boiled for 5 min. before SDS-PAGE (as described) or storage at -70°C.

6.3.4 Library Amplification

The *Drosophila melanogaster* embryo cDNA library (MATCHMAKER, Clontech) was titered by plating serial dilutions of the original culture on LB/amp plates. To amplify the library, the culture was diluted and plated on 270 150mm LB/amp plates to give about 4.0 x 10^4 cfu/plate according to the titre. The plates were incubated at 30°C for 36 hours. The colonies were scraped off the plate using 5 ml LB containing 25% glycerol, pooled and stored at 4°C for 1-5 days. Two thirds of the pooled “culture” was used to prepare DNA using two GIGA-prep columns from Qiagen according to manufacturers instructions.

6.3.6 β-Galactosidase assays

Solid phase β-galactosidase assays were performed by lifting fresh (3-5 days old) yeast colonies from agar plates with sterile Whatman filter paper (no.1). The yeast cells were permeabilized by 2 cycles of freezing the filter papers in liquid nitrogen for 10-15 sec. and thawing at room temperature. The filter papers were then placed colony side up in a petri dish containing another filter paper which had been soaked in fresh X-gal staining solution {60mM Na$_2$HPO$_4$·7H$_2$O, 40mM NaH$_2$PO$_4$, 10mM KCl, 1mM MgSO$_4$, 270µl/L β-mercaptoethanol, 0.33mg/ml X-Gal (5-brom-4-chloro-3-indolyl-β-D-galactopyranoside, (Sigma)). The filter assays were incubated at 37°C for 5-24 hours.
Liquid β-galactosidase assays were performed using the Luminescent β-galactosidase Detection Kit II from Clontech according to the manufacturer’s instructions. Small cultures (8-15 ml) of transformed yeast cells were grown to an OD₀₀₀ of 0.45-0.8 and 1.5 ml of cells harvested by centrifugation at 14000 rpm for 30 sec. The pellet was resuspended in 1.5 ml Z-buffer (60 mM Na₂HPO₄·7H₂O, 40 mM NaH₂PO₄, 10 mM KCl, 1 mM MgSO₄), centrifuged as before and resuspended in 200-400 μl Z-buffer. The cell suspension was subjected to two freeze/thaw cycles in liquid nitrogen and a 37°C waterbath before centrifugation at 14000 rpm for 5 min. Different volumes of the supernatant were mixed with the reaction buffer from the kit in 96-well assay plates and incubated at room temperature for 60 min. Chemiluminescence was detected in a plate luminometer (DYNEX MLX technology) as average readings over 5 sec using the Glow Endpoint reader mode and RLU (Recorded Light Units)/cell density in OD₀₀₀ units calculated.

6.3.7 Yeast DNA Isolation and Recovery

Yeast Plasmid DNA was isolated from liquid cultures using the YEASTMAKER™ Yeast Plasmid Isolation Kit from Clontech according to the user manual (PT3049-1, version PR89820). Usually, liquid cultures of ~2 ml were grown for 16-24 hours and 1 ml of these harvested and resuspended in 50 μl TE for DNA isolation following the protocol.

Yeast plasmid DNA was used to transform Chemically Competent KC8 cells (hsdR, leuB600, trpC9830, pyrF::Tn5, hisB463, lacΔX74, strA, galU,K) from Clontech according to their recommendations. To select for transformants with the pACT2 plasmid, the transformations were either plated directly onto agar plates of supplemented M9 minimal medium (Sambrook et al., 1989) lacking leucine or first plated on LB/amp plates and then streaked onto selective medium. DNA was prepared from the KC8 transformants using the Qiagen Mini-preps according to the instructions for isolation of DNA from endA⁺ bacteria and re-transformed into TOP10 cells as described.
## 6.4 Drosophila melanogaster techniques

### 6.4.1 Fly stocks and other materials

Mutant alleles:

<table>
<thead>
<tr>
<th>Stock name</th>
<th>Detailed genotype</th>
<th>Source/comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>mrr \textsuperscript{P1}</td>
<td>mrr \textsuperscript{P1}/mrr \textsuperscript{P1}</td>
<td>Helen McNeill</td>
</tr>
<tr>
<td>mrr \textsuperscript{P2}</td>
<td>mrr \textsuperscript{P2}/TM6</td>
<td>Mike Brodsky</td>
</tr>
<tr>
<td>mrr \textsuperscript{48}</td>
<td>mrr \textsuperscript{48}/TM3</td>
<td>Helen McNeill</td>
</tr>
<tr>
<td>iro \textsuperscript{DFM3}</td>
<td>mvh, iro \textsuperscript{DFM3} 80FRT/TM6</td>
<td>J. Modolell</td>
</tr>
<tr>
<td>dichaete \textsuperscript{513}</td>
<td>dichaete \textsuperscript{513}/TM3</td>
<td>Steve Russell (EMS allele)</td>
</tr>
<tr>
<td>dichaete \textsuperscript{72}</td>
<td>dichaete \textsuperscript{72}/TM3</td>
<td>Steve Russell (EMS allele)</td>
</tr>
<tr>
<td>ming \textsuperscript{24}</td>
<td>ming \textsuperscript{24}/TM3</td>
<td>Chris Doe</td>
</tr>
<tr>
<td>l(3)rG166</td>
<td>P{ry\textsuperscript{m17.2}=PZ}l(3)rG166\textsuperscript{G166}ry\textsuperscript{506}/TM3, Sb1</td>
<td>Bloomington</td>
</tr>
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</table>

Deficiencies:

<table>
<thead>
<tr>
<th>Stock name</th>
<th>Detailed genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Df(1)su(s)\textsuperscript{83}</td>
<td>Df(1)su(s)\textsuperscript{83}, y\textsuperscript{1} cho\textsuperscript{1} ras\textsuperscript{1} v\textsuperscript{1}/Dp(1;Y)y\textsuperscript{sc/C(1)DX, y\textsuperscript{1} fl}</td>
<td>Bloomington</td>
</tr>
<tr>
<td>Df(2L)JS17</td>
<td>Df(2L)JS17, dpp\textsuperscript{630}/CyO, P{ry\textsuperscript{m17.2}=enl}wg\textsuperscript{en11}</td>
<td>Bloomington</td>
</tr>
<tr>
<td>CHD1 Deficiency</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Df(2L)TW2</td>
<td>Df(2L)TW2, Tft\textsuperscript{1} l(2)74i\textsuperscript{1}/CyO</td>
<td>Bloomington</td>
</tr>
<tr>
<td>Df(2R)Jp4</td>
<td>w\textsuperscript{a} N\textsuperscript{var}; Df(2R)Jp4/CyO</td>
<td>Bloomington</td>
</tr>
<tr>
<td>Df(2R)Jp6</td>
<td>w\textsuperscript{a} N\textsuperscript{var}; Df(2R)Jp6/CyO</td>
<td>Bloomington</td>
</tr>
<tr>
<td>Df(2R)X1</td>
<td>Df(2R)X1, Mef2\textsuperscript{X1}/CyO, Adh\textsuperscript{nb}</td>
<td>Bloomington</td>
</tr>
<tr>
<td>Df(2R)stanl</td>
<td>Df(2R)stanl, P{ry\textsuperscript{m17.2}=neoFRT}42D cn\textsuperscript{1} sp\textsuperscript{1}/CyO</td>
<td>Bloomington</td>
</tr>
<tr>
<td>Df(3L)GN24</td>
<td>Df(3L)GN24/TM8, l(3)DTS41</td>
<td>Bloomington</td>
</tr>
<tr>
<td>Df(3R)e-GC3</td>
<td>Df(3R)e-GC3/TM6B, Tb\textsuperscript{1}</td>
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Chapter 6

FRT and FLP lines:

<table>
<thead>
<tr>
<th>Stock name</th>
<th>Detailed genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>eyFLP, pMyc 80FRT</td>
<td>y{w, P{ry+t7.2=ey-FLP.N}6; P[w+,pMyc], P{ry+t7.2=neoFRT}80B}</td>
<td>H.McNeill and Bloomington</td>
</tr>
<tr>
<td>hsFLP, Pw+ 80 FRT</td>
<td>y{w, P{ry+t7.2=hsFLP}22, P[w+,pMyc], P{ry+t7.2=neoFRT}80B}</td>
<td>H.McNeill and Bloomington</td>
</tr>
<tr>
<td>eyFLP, GFP 80 FRT</td>
<td>y{w, P{ry+t7.2=ey-FLP.N}6; P{w+mC=Ubi-GFP}69, P{ry+t7.2=neoFRT}80B}</td>
<td>H.McNeill and Bloomington</td>
</tr>
<tr>
<td>GMR-hid 80 FRT</td>
<td>y{1 w*; P{w+m*=GAL4-ey.H}3-8, P{w+mC=UAS-FLP1.D}JD1; P{ry+t7.2=neoFRT}80B P{w+mC=GMR-hid}SS4, l(3)CL-R1/TM2}</td>
<td>Bloomington</td>
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Gal4 lines:

<table>
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<th>Source</th>
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</thead>
<tbody>
<tr>
<td>69B GAL4</td>
<td>w*; P{w+mW.hs=GawB}69B</td>
<td>Bloomington No. 1774</td>
</tr>
<tr>
<td>30A GAL4</td>
<td>w*; P{w+mW.hs=GawB}30A/CyO</td>
<td>Bloomington No. 1795</td>
</tr>
<tr>
<td>PNR Gal4</td>
<td>y{1 w^118,P{w+mW.hs=GawB}pnRMD237 /TM3}</td>
<td>Ish-Horowicz lab or 3039</td>
</tr>
<tr>
<td>GMR-Gal4</td>
<td>w*; P{w+mC=GAL4-ninaE.GMR}12</td>
<td>Ish-Horowicz lab or 1104</td>
</tr>
<tr>
<td>Prd Gal4</td>
<td>w*; P{w+mW.hs=GAL4-prd.F}RG1/TM3, Sb1</td>
<td>Bloom 1947</td>
</tr>
<tr>
<td>Fng Gal4</td>
<td>w; P{w+mW.hs=GawB} FNG/TM3</td>
<td>Ken Irvine</td>
</tr>
<tr>
<td>Eyeless Gal4</td>
<td>w*; P{w+m*=GAL4-ey.H}4-8/CyO</td>
<td>Bloomington</td>
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<tr>
<td>756 Gal4</td>
<td>w; P{w+mW.hs=GawB}756</td>
<td>Barbara Jennings</td>
</tr>
<tr>
<td>VP16 Gal4</td>
<td>w; VP16-Gal4</td>
<td>Arno Muller</td>
</tr>
<tr>
<td>Hs Gal4</td>
<td>w; P[w +mCGAL4-Hsp70.PB]89-2-1 GAL4-Hsp70.PB]89-2-1</td>
<td>Tamkun lab</td>
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</table>

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Other stocks:

<table>
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<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Balancer</td>
<td>Scu/SM6a, Cy</td>
<td>Ish-Horowicz lab</td>
</tr>
<tr>
<td>Balancer</td>
<td>w; TM3, Sb, Ser/TM6 Tb</td>
<td>Ish-Horowicz lab</td>
</tr>
<tr>
<td>Balancer</td>
<td>Sb/TM3, Ser</td>
<td>Ish-Horowicz lab</td>
</tr>
<tr>
<td>Balancer</td>
<td>Upd/FM7</td>
<td>Ish-Horowicz lab</td>
</tr>
<tr>
<td>Rosy balancer</td>
<td></td>
<td>Bloomington 120</td>
</tr>
<tr>
<td>Double balancer</td>
<td>w; Scu/Cyo;MKRS Sb/TM3 Tb</td>
<td>H. McNeill</td>
</tr>
<tr>
<td>Transposase stock</td>
<td>w;/Sp/Cyo;Sb,ry[506],p[ry+D2-3]99B/TM6B,Tb</td>
<td>Bloomington</td>
</tr>
<tr>
<td>Wild Type</td>
<td>Oregon R</td>
<td>Ish-Horowicz lab</td>
</tr>
<tr>
<td>yw</td>
<td>y,w</td>
<td>Ish-Horowicz lab</td>
</tr>
</tbody>
</table>

Drosophila melanogaster were cultured on a yeast-cornmeal-molasses-malt extract agar (sometimes including propionic acid or Nipagen) at 18°C, 25°C, 29°C or room temperature (20-22°C). Pictures of adult flies were taken using a Kodak DCS420 digital camera attached to a Leica M2 APO microscope. Wings were mounted in DPX mountant for microscopy (Electron Microscopy Sciences, EMS) and pictures using a Nikon DXM1200 digital camera attached to a Nikon eclipse E800 microscope.

6.4.2 Generation of mitotic clones

Clones of mirror and iro alleles were made using the FRT lines used are listed above. To generate mitotic clones of the l(3)rG166 allele, this was recombined onto the 80BFRT chromosome. Flies containing the FRT insert were selected on food containing about 1.2 mg/ml G418 (neomycin, Gibco LifeTech) and backcrossed to the l(3)rG166 allele to confirm the presence of the mutation. For generation of large numbers of clones in the
eye, males from the FRT alleles were crossed to ey FLP; 80pMyc FRT or ey FLP; 80B, 69
GFP virgins. For generation of clones by heatshock, mutant FRT males were crossed to hs
FLP; 80B Pw' FRT virgins. Embryos collected for 12-24 hours at room temperature, aged
for 24 hours and heatshocked in a 37°C waterbath for 2-3 hours, or twice for 2 hours with 5
hours at room temperature in between heatshocks. Clones were analysed in resulting adults
or in discs dissected from wandering third instar larvae.

6.4.3 P element transformation

For the generation of P element transgenes, the CHD1 cDNAs (WT and the K559R
mutant) were cloned into the pExPressUAS vector from Exelexis. No sites would allow
direct subcloning so the CHD1 cDNAs were first cloned into the pQBI-AdBN vector
(Qbiogene) using XhoI-NotI and then subcloned into pExPress using Ascl-NotI. The P
element plasmids pExPressUAS CHD1WT and pExPressUAS CHD1K559R were co-
precipitated with the pTurbo helper plasmid in injection buffer (0.1mM Sodium Phosphate
buffer (Sambrook et al., 1989) 5mMKcl) to a final concentration of 50 μg/ml. DNA was
injected into yw embryos by Shena Pinchin. Surviving adults were crossed to yw and
transformed flies were selected by eye colour in the next generation. Single males were
then used to set up crosses to balancer stocks for chromosomes I, II and III (Upd/FM7,
Scu/SM6a, and Sb/TM3 respectively). The resulting siblings carrying the transgene and
balancer chromosome were mated and the progeny scored to find out which chromosome
carried the insertion.
6.4.4 P element excision

Excision of the l(3)rG166 P element was performed by mating males carrying the transposase, \( \Delta 2-3 Sb /TM6 Tb \) (complete genotype above) to \( P\{r^{y+7.2}\}l(3)rG166 r^{y06}/TM3 \) virgins. Single male progeny of the \( P\{r^{y+7.2}\} r^{y06}/\Delta 2-3 Sb \) genotype were outcrossed to \( TM3 r^{yk}/TM6 \) virgins. Rosy eyed male progeny were selected as excision alleles and crossed individually back to the \( TM3 r^{yk}/TM6 \) stock to generate stable lines (\( l(3)rG166 \) excision \( r^{y06}/TM3 \) or TM6). These lines were checked for homozygous viability and also backcrossed to the original l(3)rG166 P element stock to check for complementation.

6.5 Protein expression in Drosophila S2 cells and immunoprecipitation

Vectors used for expression of proteins in Drosophila tissue culture cells:

<table>
<thead>
<tr>
<th>Vector</th>
<th>Source</th>
<th>Insert and comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>pMT/V5-HisC</td>
<td>Invitrogen</td>
<td>FLAG-Mirror (made by A. Bilioni)</td>
</tr>
<tr>
<td>pMT HA</td>
<td>Helmut Kramer</td>
<td>Full length and truncated CG1135</td>
</tr>
</tbody>
</table>

6.5.1 Drosophila tissue culture and transfection

Drosophila S2 cells (Invitrogen) were cultured at 21-24°C in Schneider's Insect Medium (Sigma) supplemented with 10% foetal bovine serum (Gibco Life Technologies) and Antibiotic + Antimycotic solution (Sigma). Transfection of S2 cells was performed by A. Bilioni using a Calcium-phosphate protocol adapted from the Drosophila Expression System Protocol from Invitrogen (version D). In general, 3 to 4.5 x 10^6 cells were seeded into each well of a 6 well plate and left for 24 hours. A total of 7\( \mu \)g of DNA was added to a solution of 248mM CaCl\(_2\) in a final volume of 125\( \mu \)l which was then mixed with 125\( \mu \)l phosphate buffer (50mM HEPES, 1.5mM Na\(_2\)HPO\(_4\), 280mM NaCl, pH 7.1). After 20 min.
200μl of the resulting precipitate was dropped slowly into each well of S2 cells. After 16 hours the transfection medium was replaced by fresh medium. Expression from the MT promoter was induced by the addition of CuSO4 to a final concentration of 500μM 24 hours prior to harvest.

6.5.2 Immunoprecipitation of proteins from S2 cells

Transfected cells were harvested by scraping and pooled before being counted. Harvested cells were centrifuged for 5 min. at 1000xg, washed in PBS (Sambrook et al, 1989) and re-centrifuged. About 1 x 10^7 cells were used for each immunoprecipitation. Cells were lysed by resuspension in 1 ml ice-cold lysis buffer [25mM Tris pH 8.0, 300mM NaCl, 1 mM MgCl2, 1mM EDTA, 0.4% NP40, and 1 protease inhibitor tablet (EDTA-free from Roche)/10 ml] and centrifuged at 4500 rpm for 10 min. Immunoprecipitations were performed using either Anti-FLAG M2 Affinity Gel (Sigma) or Anti-HA11 Affinity Matrix (BAbCO) which had been pre-equilibrated in lysis buffer. Cell lysates were incubated with 25-40μl of affinity beads for 3-4 hours rotating at 4°C. The affinity beads were harvested by centrifugation at 13000 rpm for 5 sec. and washed 3 times with 1 ml lysis buffer. Proteins were eluted by boiling the beads for 5 min. in 2X sample buffer without BME. Following centrifugation at 13000rpm for 5 sec., the supernatant was taken off the beads, BME added to a final concentration of 5% and the samples boiled again for 5 min. before loading onto SDS-PAGE gels.
6.6 In Situ hybridisation

6.6.1 Generation of probes

Templates for the probes used for in situ hybridisation were either pBluescript clones or Drosophila ESTs (ResGen/Invitrogen) in pOT2. Digoxigenin-labelled probes were made from linearized DNA templates (digested with the appropriate restriction enzymes and purified) using the DIG RNA Labeling Kit (Roche). Reactions were performed as suggested by the manufactures instructions with SP6 (pOT2), T3 or T7 (pBluescript) RNA polymerase and the DNA template digested with DnaseI. The RNA was purified using the Qiaquick PCR purification kit and an aliquot analysed by agarose gel electrophoresis. The remaining RNA was ethanol precipitated with 10µg tRNA as carrier (as described for DNA), resuspended in 20µl hydrolysis buffer (40mM NaHCO3, 60mM Na2CO3, pH 10), and incubated at 60°C for x min., where x = Lo-Lf/(0.11)LoLf. Lo = original length of RNA in kb, Lf = final length in kb. The desired final length of fragments was 50-100 bases. After hydrolysis, 300µl of hybridization buffer (see below) was added and the probes stored at -20°C.

6.6.2 Collection and fixation of embryos

Embryos were collected on apple juice plates for either 2-5 hours and aged to reach the desired stage(s), or overnight. The embryos were dechorionated in 100% bleach for 2-3min, rinsed thoroughly in 0.1% Triton X-100, and transferred into a vial containing equal amounts of fixing solution {0.1M Heps, 2mM MgSo4, 1mM EGTA, 4% paraformaldehyde (EMS from 32% stock)} and heptane. After 20 min. incubation (on rocker), the lower phase (fix) was removed completely and replaced by 100% methanol and the vial vortexed for 20 sec. The de-vitellinised embryos which settle to the bottom of the
vial were collected using a cut off blue tip into a fresh tube and washed twice with 100% methanol. The embryos were stored at -20°C in methanol.

6.6.3 Hybridisation and detection

Before hybridisation the embryos were re-hydrated by sequential 5 min washes in 100%, 70%, 50%, and 30% ethanol and 3 x 5 min. washes in PTw (PBS (Sambrook et al) +0.1% Tween 20). The embryos were then washed for 10 min. in a 1:1 mix of PTw and hybridisation solution { 50% formamide (molecular biology grade, Sigma) 5x SSC (Sambrook et al), 0.1% Tween 20, 100 µg/ml sonicated salmon sperm DNA (Stratagene), 50 µg/ml heparin}, and 10 min. in hybridisation solution. Pre-hybridisation was for 1 hour at 45°C in denatured hybridisation solution (boiled 5 min in water).

The RNA probe was heated to 68°C for 10 min. and used straight or at a 1:10 dilution (with hybridisation buffer). Hybridisation was at 45°C overnight without mixing. After removal of the probe, the embryos were washed by rotating at 45°C for 20 min. each in pre-warmed hybridisation solution, 3:1, 1:1 and 1:3 mixes of hybridisation solution and PTw, and 2 x 20 min. in prewarmed PTw.

For detection of the DIG probe, the embryos were first washed twice for 20 min. at room temperature in PBT (PBS, 0.1% BSA, 0.2% Triton X-100) and then incubated for 1 hour in PBT with a 1:2000 dilution of alkaline phosphatase-conjugated anti-digoxigenin Fab fragments (Roche). After washing 4 x 20 min. in PBT and 3 x 5 min. in AP buffer (100 mM Tris, pH9.5, 100 mM NaCl, 50 mM MgCl2, 0.1% Tween) the alkaline phosphatase was detected using NBT/BCIP solution (Roche, 200 µl in 10 ml AP buffer).

Development of colour was monitored and the reactions stopped by washing embryos in PTw several times. Embryos were incubated overnight in 50% glycerol and mounted on slides in 80% glycerol. Pictures of mounted embryos were taken using a Nikon DXM1200 digital camera attached to a Nikon eclipse E800 microscope.
### 6.7 Immunohistochemistry

Primary Antibodies used for immunohistochemistry:

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
<th>Dilution used</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-BarH1</td>
<td>T. Kojima</td>
<td>1:100 (D)</td>
<td>Rb polyclonal</td>
</tr>
<tr>
<td>α-βGal (Rb)</td>
<td>ICN/Cappel</td>
<td>1:1000 (D and E)</td>
<td>Rb polyclonal</td>
</tr>
<tr>
<td>α-BRM</td>
<td>J. Tamkun</td>
<td>1:25 (P)</td>
<td>rat polyclonal</td>
</tr>
<tr>
<td>α-Castor (Rb)</td>
<td>Dr. Ward Odenwald, NIH</td>
<td>1:2000 (E)</td>
<td>Rb polyclonal, pre-absorbed 1:50 on embryos overnight</td>
</tr>
<tr>
<td>α-CHD1 (Rb)</td>
<td>Dr. Robert Perry, Fox Chase Center</td>
<td>1:100-1:200 (D)</td>
<td>Affinity purified Ab.</td>
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<tr>
<td>α-Dachshund</td>
<td>DHSB mAbdac2-3</td>
<td>1:5 (D)</td>
<td>Ms monoclonal</td>
</tr>
<tr>
<td>α-GFP (Ms)</td>
<td>Molecular probes mAb 3E6</td>
<td>1:1000 (D)</td>
<td>Ms monoclonal</td>
</tr>
<tr>
<td>α-GFP (Rb)</td>
<td>Molecular probes</td>
<td>1:1000 (D)</td>
<td>Rabbit polyclonal</td>
</tr>
<tr>
<td>α-Kismet</td>
<td>J. Tamkun</td>
<td>1:25</td>
<td>Rat polyclonal</td>
</tr>
<tr>
<td>α-Mirror (Rb)</td>
<td>Helen McNeill</td>
<td>1:1000-1:2000 (E)</td>
<td>Rb polyclonal</td>
</tr>
<tr>
<td>α-Mirror (Rat)</td>
<td>Helen McNeill</td>
<td>1:200-1:300 (D)</td>
<td>Included 0.05% DOC in Ab incubation.</td>
</tr>
<tr>
<td>α-Myc</td>
<td>in house, 9E10</td>
<td>1:10 (D)</td>
<td>Ms monoclonal</td>
</tr>
<tr>
<td>α-Pol IIa</td>
<td>J. Tamkun</td>
<td>1:50 (P)</td>
<td>goat polyclonal</td>
</tr>
<tr>
<td>α-Pol IIo</td>
<td>J. Tamkun</td>
<td>1:50 (P)</td>
<td>Ms monoclonal</td>
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<tr>
<td>α-Prospero</td>
<td>DHSB</td>
<td>1:10 (D)</td>
<td>Ms monoclonal</td>
</tr>
<tr>
<td>α-Spalt</td>
<td>Rosa Barrio</td>
<td>1:500 (D)</td>
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</tbody>
</table>

Abbreviations: Ms: mouse, Rb: rabbit, E: embryos, D: discs. P: polytenes
Secondary antibodies used for fluorescent labelling:

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<th>Source</th>
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<tr>
<td>Biotin-SP-conjugated α-Rb Ig</td>
<td>Jackson ImmunoResearch Labs</td>
<td>1:500 (D)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1:250 (P)</td>
</tr>
<tr>
<td>Biotin-SP-conjugated α-rat Ig</td>
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<td>1:500 (D)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1:250 (P)</td>
</tr>
<tr>
<td>FITC conjugated α-Rb Ig</td>
<td>Jackson ImmunoResearch Labs</td>
<td>1:500 (D)</td>
</tr>
<tr>
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<td>1:250 (P)</td>
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</tr>
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<td></td>
<td>1:250 (P)</td>
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<tr>
<td>FITC conjugated α-rat Ig</td>
<td>Jackson ImmunoResearch Labs</td>
<td>1:500 (D)</td>
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<tr>
<td></td>
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<td>1:250 (P)</td>
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<td>Cy3 conjugated α-goat Ig</td>
<td>Jackson ImmunoResearch Labs</td>
<td>1:500 (D)</td>
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<tr>
<td></td>
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<td>1:250 (P)</td>
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<tr>
<td>Cy3 conjugated α-Ms Ig</td>
<td>Jackson ImmunoResearch Labs</td>
<td>1:500 (D)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1:250 (P)</td>
</tr>
<tr>
<td>Cy3 conjugated α-rat Ig</td>
<td>Jackson ImmunoResearch Labs</td>
<td>1:500 (D)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1:250 (P)</td>
</tr>
<tr>
<td>Alexa Flour 488 conjugated α-rat Ig</td>
<td>Molecular probes</td>
<td>1:500 (D)</td>
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<tr>
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<td>1:250 (P)</td>
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<tr>
<td>Alexa Flour 488 conjugated α-Ms Ig</td>
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<td>1:250 (P)</td>
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<td>Alexa Flour 546 conjugated α-Rb Ig</td>
<td>Molecular probes</td>
<td>1:500 (D)</td>
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<td>1:250 (P)</td>
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<td>Alexa Flour 546 conjugated α-rat Ig</td>
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<tr>
<td>Streptavidin conjugated Cy3</td>
<td>Molecular probes</td>
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</tr>
</tbody>
</table>

Abbreviations: Ms: Ms, Rb: Rb, D: discs. P: polytene

Fluorescent antibody staining of embryos, discs and polytene chromosomes were analysed and images captured using a ZEISS LSM 510 confocal microscope.
Chapter 6

6.7.1 Staining of Embryos

Embryos of the appropriate stages were collected on apple juice plates, dechorionated and fixed as described for in situ hybridisation using a PBS-paraformaldehyde fixing solution (4% paraformaldehyde in PBS). Fixed embryos were stored at -20°C in methanol. Upon re-hydration (as described for in situ hybridisation) embryos were washed twice in PBS + 0.1% Triton for 5-10 min. and twice in PBT (PBS, 0.1% Triton X-100, 1% BSA) for 10-15 min. The embryos were then blocked in PBT + 5% normal goat serum (Vector) for 1 hour at room temperature. Primary antibodies were diluted in blocking solution and embryos incubated overnight at 4°C. Following two rinses and three washes of about 20 min. each in PBT, the embryos were blocked again in PBT + 5% goat serum for 10-15 min. Secondary antibodies were diluted in blocking solution and embryos incubated for 2 hours at room temperature. The embryos were then washed as before and if biotinylated secondary antibodies had been used, incubated with labeled streptavidin for 1 hour at room temperature. Embryos stained with fluorescent antibodies were mounted in Vectasheild (Vector Laboratories) following an overnight incubation in 50% glycerol.

6.7.2 Staining of Imaginal Discs

Imaginal discs were dissected from 3rd instar larvae and fixed either in PBS + 2% paraformaldehyde or Brower fix made by mixing 3 parts Brower buffer (0.15M PIPES, pH 6.9, 3mM MgSO₄, 1.5mM EGTA, 1.5% NP-40) and 1 part 8% paraformaldehyde (EMS, diluted from 32% stock). Following fixation for 40-50 min. on ice, discs were rinsed several times in PBS, 0.1% Triton and washed twice in PBT (see above) for 10-15 min. each. Discs were blocked in PBT + 5% goat serum for 1 hour. Staining of discs was performed as described for embryos, but washing and incubation times were usually
increased by about 50%. Following the final wash, discs were incubated in 50% glycerol overnight and mounted in Vectasheild.

6.7.3 Staining of Polytene chromosomes

These experiments were performed with help, advice and materials from Jennifer Armstrong in the laboratory of John Tamkun at the University of Santa Cruz, California. Salivary glands were dissected from 3rd instar larvae in 0.7% NaCl and fixed in 45% acetic acid, 1.85% formaldehyde. Squashes were performed as described in *Drosophila Protocols* (Sullivan et al. 2000). Following freezing of the slide and removal of the coverslip, the slides were washed in PBS for 5 min., PBS, 1% Triton X-100 for 10 min. and blocked in PBT (see above) for 30 min. Polytenes were incubated with primary antibodies diluted in PBT under coverslips overnight at 4°C. The slides were first rinsed in PBS and then washed 3 x 5 min. in PBS and 2 x 15 min. in PBT followed by incubation with diluted secondary antibody for 1 hour at room temperature under coverslips. After rinsing in PBS and washing 2 x 10 min. in PBT, the polytenes were incubated in streptavidin for 1 hour if biotinylated secondary antibodies had been used. After washing in PBS, polytenes were stained for 4 min. with DAPI if required, washed in PBS again before mounting in Vectasheild.

6.8 Embedding and Sectioning of Drosophila Eyes

The protocol for fixation and embedding of adult Drosophila eyes was adapted from Richard Carthew (Rubin Lab manual). Adult heads were dissected and one eye cut away using no. 11 scalpel blades. The heads were placed in 2% gluteraldehyde, 0.1M PO₄ (made by mixing 72 parts Na₂HPO₄: 28 parts NaH₂PO₄) on ice for 10-30 min. before an equal volume of 2% OsO₄ (EMS) in 0.1M PO₄ was added to the tube and the heads incubated for
30 min. on ice. The OsO₄, gluteraldehyde mix was then replaced with 2% OsO₄ in 0.1M PO₄
and incubation on ice continued for 1-2 hours. The heads were then dehydrated by
sequential 10 min. washes in ice cold 30%, 50%, 70% and 90% ethanol followed by two
washes in 100% ethanol at room temperature. Following two incubations for 10 min. in
propylene oxide (EMS), the heads were incubated overnight in a 1:1 mix of propylene
oxide and Durcapan resin (Drucapan ACM from Fluka mixed according to recipe for soft
resin). The propylene oxide:resin mix was replaced by pure resin and the heads incubated
for about 5-6 hours before being placed in moulds filled with resin and baked overnight at
70°C. The embedded eyes were sectioned in to 1 micron sections by the Cancer Research
UK electron microscopy service using a microtome. Pictures of eye sections were taken
using the Nikon DXM1200 digital camera attached to the Nikon eclipse E800 microscope.
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