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# The role of cellular memory modules in the determination and transdetermination of *Drosophila melanogaster* imaginal disc cells

Gutachter: Prof. Dr. Renato Paro

Prof. Dr. Klaus-Armin Nave

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Finally, I want to thank my parents who always supported me with all their love and courage.

# -Abstract-

Mechanisms of cellular memory allow cells to remember their embryonically established determination state through the many mitotic divisions required to complete development. The Polycomb-group (PcG) and trithorax-group (trxG) proteins ensure this function through their interplay at chromosomal elements, termed Cellular Memory Modules (CMMs), by creating stable and inheritable chromatin structures. By this way, CMMs are able to remember the embryonic state of transcription of homeotic genes and to maintain it throughout development.

I asked whether such CMMs could also control expression of genes involved in patterning imaginal discs during larval development, which expression pattern may be modulated with time. The results demonstrate that expression of one of these genes, *hedgehog*, once activated, is maintained by a CMM even when the initial activator has disappeared. These experiments suggest that the chromatin-based epigenetic inheritance of gene expression involving CMMs may be widely spread. This implies that during imaginal disc patterning, the determination of a morphogenetic field is not only established by the surrounding combinations of signaling molecules but is also dependent from the history of the composing cells.

Transdetermination is a switch of disc identity that occurs in some cells, under specific conditions, when cells are already determined. It was shown to appear in *Drosophila* when manually fragmented imaginal discs were cultivated for a period of time allowing several cell divisions. It is still unclear what factors are involved in the switch to the new identity and how cells are able to inherit it through cell divisions. I investigated the role of PcG and trxG genes in the determination and transdetermination of imaginal disc cells. My results show that reducing the concentration of some PcG members in the cells affects the frequency of transdetermination suggesting that some genes involved in the establishment of the new disc identity are targets of PcG-mediated regulation. Furthermore, PcG proteins may also be required for the accurate inheritance of the new transdetermined state through mitosis. These results suggest that the establishment and the maintenance of the new cell identity is generated through the switching of the activation state of the CMM of developmental genes.

In conclusion, several conditions are defined that may favour or are necessary for transdetermination, in which a transient downregulation of some PcG/trxG proteins as well as several rounds of DNA replication may facilitate the switch of CMMs.

Finally, I discuss these results with a perspective on how the control of CMM switching may have applications for medical research in tissue remodelling.

# -Contents-

# I. Introduction.

# 1. Introduction

## 1.1 Short introduction to the concepts of epigenetics and cell determination

The central question in developmental biology concerns how a small number of undifferentiated embryonic cells, with the same genomic content, can give rise to the diverse complex structures of the adult.

Organisms with different tissues must establish tissue-specific patterns of gene expression early in development and maintain these patterns until adult stages. During early embryogenesis, cells receive information, through molecules called “determinants”, which commit them to specific fates. This leads to the establishment of cell specific transcriptional patterns necessary to activate the developmental pathways which will define the determined identities. Once established, epigenetic processes allow all cells to inherit their own specific transcriptional patterns through cell divisions, in order to maintain the initial determined state, until tissue development is accomplished and cells differentiated. In this way, epigenetic mechanisms allow heritable changes in gene expression that occur without a change in DNA sequence (Wolffe and Matzke, 1999).

Consequently, a determined state is cell heritable (Hadorn, 1965). Understanding cell determination requires understanding both how determined states are established, and by which epigenetic processes they are maintained throughout development.

## 1.2 The establishment of determined states during early embryogenesis in *Drosophila*

Early patterning events are under the control of the *maternal genes*. These are genes coming from the mother genome which must be expressed in the developing oocyte for the subsequent correct development of the embryo. These gene products form gradients in the egg providing the embryo with polarity, and lay down a pre-pattern for subsequent development (Ingham, 1988; St Johnston and Nüsslein-Volhard, 1992). Polarity is established before fertilisation, and results from signaling between the oocyte and the surrounding somatic follicle cells of the mother (Ray and Schüpbach, 1996). The transcription factors encoded by the maternal genes *bicoid* (*bcd*), *hunchback* (*hb*), *nanos* (*nos*) and *caudal* (*cad*) regulate expression of the so-called *gap genes* (because gap gene mutants lack contiguous blocks of segments) (Jäckle, 1987; Driever and Nüsslein-Volhard, 1988; Gaul and Tautz, 1988; Driever and Nüsslein-Volhard, 1989; Struhl et al., 1989; Rivera-Pomar et al., 1995). Later, the periodic pattern of expression of gap genes along the embryo, together with the maternal determinants, acts on the specific enhancers of the *pair-rule genes* to induce their expression

in a spatially restricted pattern of seven or eight stripes along the antero-posterior (A-P) axis of the embryo.

The gap and pair-rule genes, mainly encoding for RNA and DNA-binding proteins, are part of a transcriptional cascade which directly activates gene expression. Their coordinated action culminates by establishing the pattern of expression of *segmentation genes* and *homeotic genes*.

The products of segmentation genes divide the body into a repeated array of 14 parasegments defining cell identity along the antero-posterior axis. The parasegments are units of cell lineage, and the metameric structure of the adult is a direct consequence of the basic organisation of the embryo (Martinez-Arias and Lawrence, 1985). Two groups of founder cells of each embryonic parasegment are allocated to make either an anterior or posterior compartment of each adult segment. All descendents of a group of founder cells will exclusively form one adult compartment, and no other cells contribute to it (Morata and Lawrence, 1975).

Each parasegment expresses a specific set of homeotic genes (or Hox genes) which determine its own identity. These parasegments must maintain their own homeotic gene expression pattern throughout development in order to give rise to the defined adult structure.

The early expression pattern of the gap and pair-rule proteins disappears by about four hours of embryogenesis. Thus, distinct regulators are needed to maintain homeotic gene expression during the rest of development.

## 1.3 Maintenance of embryonically established expression patterns throughout development

### 1.3.1 Introduction to the concept of *cellular memory*

The fate of a cell is determined by its gene expression program. Once this has been embryonically established, some mechanisms must allow its persistence through the required round of cell divisions at the end of which the initial embryonic cell becomes a whole adult structure.

Cellular memory is a term used to define the epigenetic processes allowing the cells to remember and propagate their initial gene expression program through cell divisions when the early patterning factors have disappeared. In the last decade, there has been accumulating evidence that chromatin structure can control the transcriptional state of a gene and be inherited through DNA replication and mitosis to the next generation of cells. How a given chromatin structure can resist to the processes of DNA replication and compaction of

metaphasic chromosomes, and be inherited to the daughter cells is still unknown. In fact, several studies observed that the chromatin structure itself is mainly disrupted at least during mitosis but reassembles in daughter cells to allow the reformation of the transcriptional pattern (Buchenau et al., 1998; Dietzel et al., 1999; Yamamoto et al., 1997). These observations suggest that there could be a discrete mark only that would persist on the DNA during the replication and the mitotic processes, could be copied to newly replicated chromosomes and would re-recruit the appropriate repressing or activating chromatin complexes to the target gene at the end of the affair.

Changing this memory mark at any time during development would lead to a loss of the memory and could switch the transcription of a gene to a new state. It is obvious that the failure of a cell to strictly remember its own gene expression program during development would make it lose its identity and could have disastrous consequences.

### 1.3.2 What is chromatin?

In contrast to prokaryotic organisms, eukaryotes need to package their DNA within a nucleus. The first level of packaging is provided by nucleosomes. They consist of an octamer of core histones, that directly interacts with DNA. The DNA is wrapped around nucleosomes and the repetition of this event produces a primary fiber itself coiled into a secondary fiber ordering the complex into a basic architecture. Beside the need for packaging the enormous eukaryotic DNA in an independent compartment, histones and various other proteins as well as RNAs (Kelley and Kuroda, 2000) act together on DNA to form dynamic higher-ordered structures. They contribute to a system of regulation of gene expression that supports development of multicellular organisms, with extensive specialization of cell types. The overall mixture formed by the DNA interacting with all these proteins and RNAs is called "*chromatin*".

Two different conditions of chromatin co-inhabit the nucleus. *Heterochromatin* mainly consists of silent DNA which contains very few transcribed genes. It is highly packaged and dense. *Euchromatin* contains most of the transcribed genes. Its structure is globally looser.

The structural organisation of chromatin regulates the expression of many genes, presumably by controlling the access of trans-acting factors to the promoter regions, and seems to be a critical parameter for the epigenetic inheritance of gene expression throughout development.

### 1.3.3 Polycomb and trithorax group proteins

Once the expression pattern of homeotic genes is established in each parasegment, the initial transiently expressed patterning factors disappear later in embryogenesis, and a memory system has to take over to maintain the initial transcriptional state of the genes throughout development. Two families of proteins fulfill this role. The Polycomb group (PcG) proteins are known to maintain the silenced state whereas the counteracting trithorax group (trxG) proteins allow inheritance of the active state. Both these families of proteins act as multi-protein complexes thought to be able to remodel the structure of chromatin. The PcG/trxG maintenance system is evolutionarily conserved from *Drosophila* through to mammals (Goodrich et al., 1997; Deschamps et al., 1999).

#### *1.3.3.1 The Polycomb group proteins are needed to maintain repression*

Pc-G mutants were originally identified on the basis of their homeotic phenotype. Mutants been proved to cause expression of homeotic genes in unusual locations, regions in which they normally would not be expressed. This ectopic expression is attributed to the failure of proper gene silencing by Pc-G mutants. The fact that in PcG mutants, homeotic gene expression is normal at early stages of development indicates that the PcG genes do not have a role in the establishment of the repression of these selector genes. They would rather maintain the silenced state throughout development, when the initial repressor proteins encoded by the gap genes have established the initial off-state and are no longer expressed. For this purpose, the products of most PcG genes are required continuously throughout development.

Molecular analysis of the Polycomb protein (PC) gave important insights into the mechanism of PcG repression. A 48 amino acid domain (the chromodomain) was identified which showed significant homology the heterochromatin-associated protein HP1. Since HP1 is involved in heterochromatic silencing, it was proposed that the PcG proteins may silence target genes in a manner comparable to that of heterochromatin (Paro, 1990).

Based on genetic studies, it is estimated that there could be about 40 potential PcG genes, but only about 15 have been molecularly identified and characterised in *Drosophila*. There is no significant sequence similarity among them. However, several conserved protein motifs seen in other nuclear factors are potential domains for protein-protein interactions. Further experiments confirmed the idea that PcG proteins act as multimeric complexes (Franke et al., 1992; DeCamillis et al., 1992). Precisely, at least 2 biochemically distinct complexes can be observed with different roles. One consists of a 600 kD complex containing the product of the

genes *extra sex comb* (*esc*), *Suppressor of zeste 12* (*Su(z)12*) and *Enhancer of zeste* (*E(z)*) which possesses a histone-methyl-transferase (HMT) activity (Jones et al., 1998; Czermin et al., 2002; Müller et al., 2002; Cao et al., 2002; Kuzmichev et al., 2002). This complex is critically required for establishment of PcG-mediated silencing during early embryogenesis. However in contrast with *esc, E(z)* is still required for later stages of development (Simon et al., 1995; Tie et al., 1998; Ng et al., 2000). A second complex, the Polycomb Repressive Complex 1 (PRC1) containing several other PcG proteins such as PC, PH, PSC and SCM, has been purified. PRC1 is able to block remodelling of a nucleosome array by the SWI/SNF chromatin remodelling complex (Shao et al., 1999). Thus it may stabilize and maintain the chromatin in a repressive structure counteracting the action of trans-activating factors during development. Interestingly, in several studies, these complexes have been shown to copurify with other kinds of proteins able to modify histones (like Rpd3, a histone deacetylase), or components of the general transcription factor TFIID (dTAFIIs) indicating a direct physical connection between PcG proteins and histone modifying enzymes or proteins of the general transcription machinery (Tie et al., 2001; Saurin et al., 2001; Breiling et al., 2001).

Polycomb-G proteins act through the binding of cis-regulatory elements called Polycomb Response Elements (PREs) (Simon et al., 1993; Chan et al., 1994). Several PREs have been identified in the bithorax complex (BX-C) (Orlando et al., 1993; Simon et al., 1993; Strutt et al., 1997; Mihaly et al., 1997; Barges et al., 2000). They are necessary to maintain the repressed expression status of corresponding homeotic genes in appropriate segmental domains. So far only one PcG protein, Pleiohomeotic (PHO) has been found to have the capacity to directly bind these PREs in a sequence specific manner (Mihaly et al., 1998). It has been proposed that PHO acts to recruit and anchor PcG protein complexes to DNA (Fritsch et al., 1999). Nevertheless an intact PHO binding site is necessary but not sufficient for PRE activity, indicating that additional sequence elements are required to establish a Pc-repressing complex (Brown et al., 1998). Furthermore, PHO itself when tethered to DNA is not able to recruit the correct Pc-complex and does not silence a reporter gene, unlike Pc (Poux et al., 2001).

| **Gene** | **Complex** | **Protein domains** |
|---|---|---|
| *Polycomb (Pc)* | PRC1 | Carboxy-terminal domain; chromodomain |
| *polyhomeotic (ph)* | PRC1 | SPM, H1, zinc finger |
| *Posterior sex comb (Psc)* | PRC1 | Homology region: RING (zinc finger) + |

| | | |
|---|---|---|
| | | helix-turn-helix |
| *dRing* | PRC1 | RING |
| *Sex comb on midleg (Scm)* | PRC1 (small fraction only) | SPM |
| *Enhancer of zeste (E(z))* | E(z) / Esc | SET (HMT activity) |
| *extra sex combs (esc)* | E(z) / Esc | WD40 repeats |
| *Suppressor of zeste 12 (Su(z)12)* | E(z) / Esc | Zinc finger, VEFS box |
| *Additional sex combs (Asc)* | | WD40 repeats |
| *super sex combs (sxc)* | | |
| *multi sex combs (mxc)* | | |
| *Polycomb-like(Pcl)* | | PHD fingers |
| *cramped (crm)* | | |
| *pleiohomeotic (pho)* | | Zinc fingers |
| *Sex comb extra (Sce)* | | |
| *Enhancer of Polycomb (E(Pc))* | | |
| *Suppressor of zeste 2 (Su(z)2)* | | HR region (shared with Psc) |

**Table 1: Polycomb group genes.** Derived from Francis and Kingston (2001)

In summary, it appears that PcG proteins form large multimeric complexes associated with different kinds of enzyme activities linked to histone modifications or connected with the general transcription machinery. These complexes associate with the DNA at specific sequences (PREs) and may organise and maintain a spatially restricted heterochromatin-like structure, preventing activation of transcription of the packaged gene during development.

*1.3.3.2 The trithorax-Group proteins are needed to maintain activation*

Most of the trxG genes have been identified by their ability to suppress homeotic phenotypes caused by PcG gene mutations. Mutations in any of these genes mimic the homeotic transformations caused by loss of function mutations of homeotic genes such as *Antennapedia* and *Ultrabithorax*. This interaction between trithorax (*trx*) and homeotic genes has lead to observations that *trx* is necessary to sustain homeotic gene expression past the gastrulation phase (Breen, 1991). In fact, they appear to counteract the formation of repressive

PcG-mediated chromatin structures, and to maintain the environment around the target gene in an open, permissive configuration allowing accessibility of the transcription factors to the promoter region. Their role is to maintain the expression of a gene throughout development when its initial expression state was activated by the primary early transcription factors (gap genes, pair rule genes). Like PcG genes, for this purpose, they are required and expressed continuously during development.

Polytene chromosome stainings (Zink and Paro, 1989; Paro and Zink, 1993; Rastelli et al., 1993; Chinwalla et al., 1995; Tripoulas et al., 1996) as well as chromatin immunoprecipitation analysis (Strutt et al., 1997; Orlando et al., 1998) showed that TrxG and PcG proteins mainly overlap on the same regulatory sequences responsible for the maintenance of homeotic gene expression. However, functional dissection of one of these modules showed that the TRE (trithorax response element) and PRE activities can be ascribed to separable DNA elements, even though they are located within 30-40 nucleotides of each other (Tillib et al., 1999). This proximity suggests that there may be some direct interactions between protein complexes formed at these elements. So far, the GAGA factor (GAF), encoded by the Trithorax-like (Trl) gene, and ZESTE are the only trxG proteins shown to directly bind DNA. These proteins bind short consensus sequences in PREs/TREs (Benson and Pirrotta, 1988; Strutt et al., 1997; Cavalli and Paro, 1998). However, the $(GA)_n$ repeats to which the GAF binds are also found at the promoter of many genes like the heat shock genes (O'Brien et al., 1995) and are not implicated in epigenetic phenomena.

There is growing evidence that the trxG represents a heterogeneous family of proteins with diverse functions. Some of them, such as TRX, ASH1, ASH2, GAF, and ZESTE, are associated with particular sites on polytene chromosomes (Rastelli at al., 1993; Kuzin et al., 1994; Chinwalla et al., 1995; Adamson and Shearn, 1996; Tripoulas et al., 1996), while others, such as Brahma (BRM) and SNR1, are found in chromatin remodelling complexes that may not be associated with specific chromosomal regions. There is some evidence that one of the functions of trxG proteins may be to recruit chromatin remodelling complexes to DNA. GAGA factor is required for the function of one chromatin remodelling complex, the Drosophila NURF complex (Tsukiyama and Wu., 1997), and TRX has been shown to physically interact with SNR1, a component of the Drosophila SWI/SNF complex (Rozenblatt-Rosen et al., 1998). However, there is no evidence thus far that these interactions are mediated through particular TREs. In addition, there is evidence that TRX and its human homologue, ALL-1/HRX, may be involved directly in the activation of promoters, since both of these proteins possess transactivation activity in cells (Chang et al., 1995; Prasad et al.,

1995; Zeleznik-Le et al., 1994). Therefore, it is likely that trxG proteins not only can counteract formation of PcG-mediated repressive chromatin structure but may also play a more general role in maintaining transcription. Very recently, it has been demonstrated that the SET domain of ASH1 is associated with an HMT activity, leading to the hypothesis that methylation of histones may have a role in maintaining activation of transcription (Beisel et al., 2002)

| **Gene** | **Complex** | **Protein domains** |
|---|---|---|
| *brahma (brm)* | Brahma complex (SWI/SNF) | SNF2/SWI2 ATPase domain; bromodomain |
| *moira (mor)* | Brahma complex (SWI/SNF) | SANT domain, leucine zipper |
| *osa* | Brahma complex (SWI/SNF) (sub-stochiometric) | ARID |
| *trithorax (trx)* | | SET |
| *trithorax-like (trl)* | | Single zinc-finger DNA-binding motif |
| *ash1* | | SET (HMT activity), PHD |
| *ash2* | | PHD |
| *little imaginal discs* | | ARID; RING/PHD; leucine zipper; PHD (2) |
| *urdur* | | |
| *kismet* | | SNF2/SWI2 ATPase; chromodomain |
| *modifier of (mdg4)* | | |
| *lawc* | | |
| *kohtalo* | Mediator-related co-activator complex? (homolog of human TRAP240) | |

**Table 2: trithorax group genes.** derived from Francis and Kingston (2001)

*1.3.3.3 Proteins with overlapping functions: the enhancer of trithorax and Polycomb (ETP) group*

There is now evidence that some members of the PcG and trxG show overlapping functions. It has been proposed that a third class of genes should be established to encompass genes that enhance both PcG- and trxG-mediated phenotypes (Gildea et al., 2000; Brock and van Lohuizen, 2001). Some proteins could be common sub-units actually involved in both processes of activation or repression through their ability, in different contexts, to allow the recruitment of activating or repressing factors. For this reason, this class of proteins have been named the enhancer of trithorax and Polycomb (ETP). A recent screen for enhancer of trxG mutation phenotypes, indeed, identified six different members of the PcG genes (*Asc*, *E(Pc)*, *E(z)*, *Psc*, *Scm*, *Su(z)2*), suggesting that a subset of PcG genes are required to activate as well as to suppress homeotic gene expression (Gildea et al., 2000). The same applies to some presumptive trxG members, the GAF and Zeste, which both have sequence specific DNA binding activities. Although *Trl* (GAF) mutations show clear trxG phenotypes (Farkas et al., 1994), the GAF can interact with PcG complexes *in vitro* (Farkas et al., 1994) and is localised to PRE sequences *in vivo* (Cavalli and Paro 1998). Furthermore several studies have recently shown that the GAF is required for the correct silencing activity of different PREs (Mishra et al., 2001; Hodgson et al., 2001; Busturia et al., 2001). Genetic data link *Zeste* to both the PcG and trxG indicating that it might function as an activator or as a repressor in different contexts (Pirrotta and Rastelli, 1994; Kal et al., 2000). Zeste was also found to bind directly to the components of the BRM complex to increase transcription (Kal et al., 2000), whereas it is also associated with PRC1 (Saurin et al., 2001) and is necessary to maintain repression of Ubx transgenes (Hur et al., 2002).

In agreement with the apparent overlap of PRE and TRE sequences, ETPs might simply be a link between the PcG-/trxG complexes and the DNA, marking genes as potential targets, whereas other proteins or the state of the chromatin template might determine which complexes should be recruited, and therefore, whether activation or repression is the outcome (Francis and Kingston, 2001).

*1.3.3.4 Targeting of the PcG/trxG proteins to specific genes*

So far very little is known about the mechanisms leading to the targeting of PcG/trxG complex to specific genes. However, as mentioned earlier, PcG/trxG complexes might be attracted to PREs/TREs in a sequence specific manner. In this way, the PcG protein PHO binds a short consensus sequence found in many PREs (Mihaly et al., 1998; Brown et al.,

1998) although it can not by itself induce silencing. Nevertheless, a 138 bp sequence within the MCP PRE (controlling expression of the *Abd-B* gene in the bithorax-complex) containing four PHO-binding sites and two GAGA sites seems sufficient for silencing (Busturia et al., 2001). This confirms that the EPT member, GAF, might mark the target DNA and interact with PcG proteins to recruit the PcG complex at PREs. Furthermore, PREs seem to be continuously required to maintain the PcG-mediated silencing since excision of the MCP PRE from a transgene during larval stages leads to the loss of silencing. This finding suggests that these DNA elements, and/or an epigenetic mark they carry, may be needed to continuously attract the protein complexes in order to maintain at least silencing through cell proliferation (Busturia et al., 1997).

*1.3.3.5 The interplay of PcG and trxG complexes at homeotic PREs conveys transcriptional memory: the cellular memory modules (CMMs)*

In an attempt to isolate and investigate how regulatory sequences can allow inheritance of the initial transcriptional state throughout the whole of development, Cavalli and Paro have set up a transgenic model system showing that PREs can be switched at embryogenesis to an activated state, allowing continuous transcription of a nearby reporter gene through many rounds of mitotic divisions and surprisingly, with a lower frequency, also through meiosis (Cavalli and Paro, 1998).

The model was first established with the *Fab7* PRE/TRE (Fig. 1). This element belongs to the bithorax-complex and is needed for maintaining segment-specific expression of the homeotic *Abdominal-B* gene. The transgenic construct carries *Fab7* upstream of several UAS sites (upstream activating sequence) acting as a specific enhancer for two reporter genes: the *lacZ* gene, and the mini-*white* gene possessing its own minimal promoter.

When no activation of transcription is initiated during embryogenesis, the *Fab7* PRE acts as a repressing element since mini-*white* gene expression is silenced in adult eyes. This repressive effect seems to be the default state for a PRE, confirming that PcG-mediated silencing is preferentially established on inactive genes. A short embryonic GAL4 pulse (acting as an early embryonic transcription factor) can activate transcription of the reporter genes through the binding to the UAS sites, as expected. However it appears that the transcription of the reporter genes is maintained throughout the whole of development as indicated by the transcription of the mini-*white* gene in the eyes of adult flies in spite of the absence of the initial transactivator (Cavalli and Paro, 1999). Conclusively, the *Fab7* element can be “switched” from a silenced to an active state, and this state could be inherited throughout the

whole development. For this reason, the *Fab7* element has been termed a *Cellular Memory Module* (CMM).

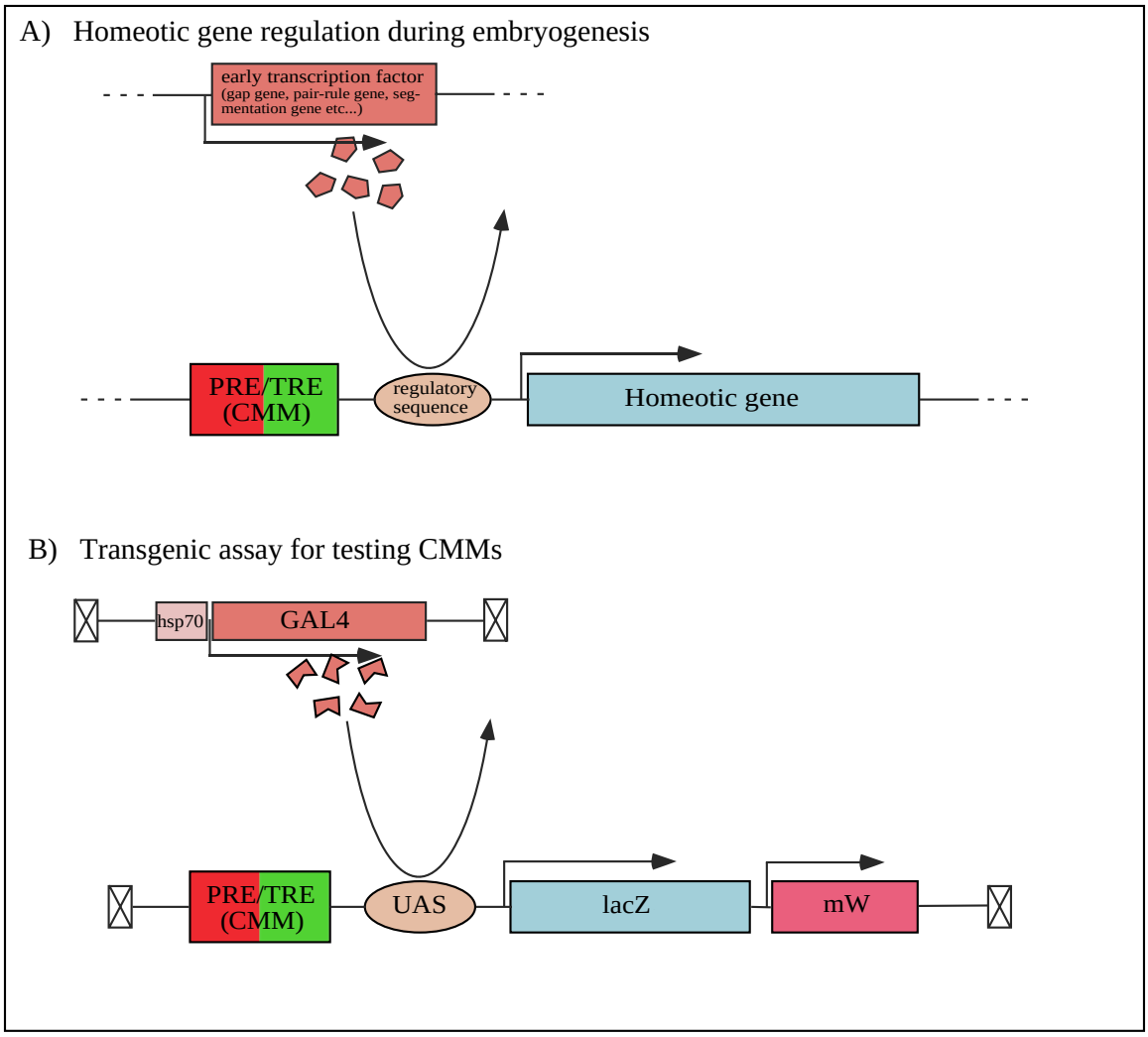



**Figure 1: The transgenic assay allows to test whether a chromosomal element can maintain the embryonically established state of transcription through the whole of development.** A) In the endogenous situation, early transcription factors are transiently expressed in the embryo, establishing a specific pattern of transcription of the homeotic genes. Later on, the early transcription factors disappear, and the PRE/TRE chromosomal element is able to maintain the initial state of transcription throughout development. B) In the transgenic assay, an embryonic heat-shock induces a transient expression of GAL4 which can activate transcription of the reporter genes through the binding of the UAS sites, mimicking the action of the early transcription factors. In this way, it is possible to test whether a specific chromosomal element is able to maintain the initial state of transcription throughout development when placed upstream of the reporter genes.

Activity is dependent on the PcG and trxG proteins (Cavalli and Paro, 1998; 1999). The interplay of PcG and trxG proteins at elements such as *Fab7* allows transcriptional memory presumably by setting and maintaining epigenetic marks during DNA replication and mitosis. Surprisingly, the same kind of GAL4 pulse, when provided in larval stages, was only able to transiently activate transcription of the reporter gene, but no switching of the *Fab7* CMM was observed since transcription was lost as soon as the transactivator (GAL4) was down-

regulated. These observations led to the hypothesis that Pc-mediated silencing might be more stable in larval stages than in embryonic stages and CMMs can not be switched to mitotically heritable activity at these later stages.

This experiment gave insights into how PcG and trxG proteins are targeted at PREs/TREs (or CMMs) to maintain the initial silent/active state of expression of homeotic genes, which was decided by the regulatory cascade of the early transcription factors. This assay also allows to determine whether a regulatory element is able to function as a CMM.

## 1.4 Molecular mechanisms of cellular memory: What is the epigenetic mark?

### 1.4.1 The *histone code* hypothesis

In the last years, the discovery that histones could be subjected to different kind of covalent modifications (Fig. 2) affecting the gene transcription state raised the possibility that a "*histone code*" might operate for the longer-term maintenance and modulation of patterns of gene expression (Turner, 2000; Jenuwein and Allis, 2001). The *histone code hypothesis*, indeed, predicts that the modification marks on the histone should provide binding sites for effector proteins. The main modifications found so far are the acetylation, methylation and phosporylation of specific residues of the N-terminus of histones called histone tails. Proteins responsible for these modifications are often associated with chromatin remodelling complexes. Some histone deacetylases (HDAC) like Rpd3, whose role is to deacetylate histones, and which are generally connected with silencing activities, have been found associated with PcG complexes and genetically interact with some PcG mutants (van der Vlag et al., 1999; Tie et al., 2001; Breiling et al., 2001). Conversely some histone acetyl-transferases (HAT), which are generally connected with activation of gene expression, have been found in complexes containing trxG proteins (Petruk et al., 2001). Furthermore, the SET domain of Su(var)3-9 has been shown to methylate histone H3 at lysine 9 (Rea et al., 2000) and to affect the structure of heterochromatin. The SET domain is found as well in the PcG member E(z) and is able *in vitro* to methylate histone H3 at lysines 9 and 27 (Czermin et al., 2002; Müller et al., 2002; Cao et al., 2002; Kuzmichev et al., 2002). The methylation pattern of lysine 9 correlates well with the Pc-binding sites on polytene chromosomes (Czermin et al., 2002). Likewise, the SET domain of the member of the trxG protein ASH1 is able to methylate H3 at lysine 4 and 9 which is necessary for the maintenance of homeotic gene expression (Beisel et al., 2002). Moreover, a trithorax-group complex purified from *Saccharomyces cerevisiae* was recently demonstrated to be required for methylation of

histone H3 at lysine 4 (Nagy et al., 2002). The fact that all these histone-modifiers are found associated with heterochromatin protein as well as with PcG/trxG activities suggests that PcG/trxG activities may use the histone code to generate and/or maintain higher-ordered chromatin structure.

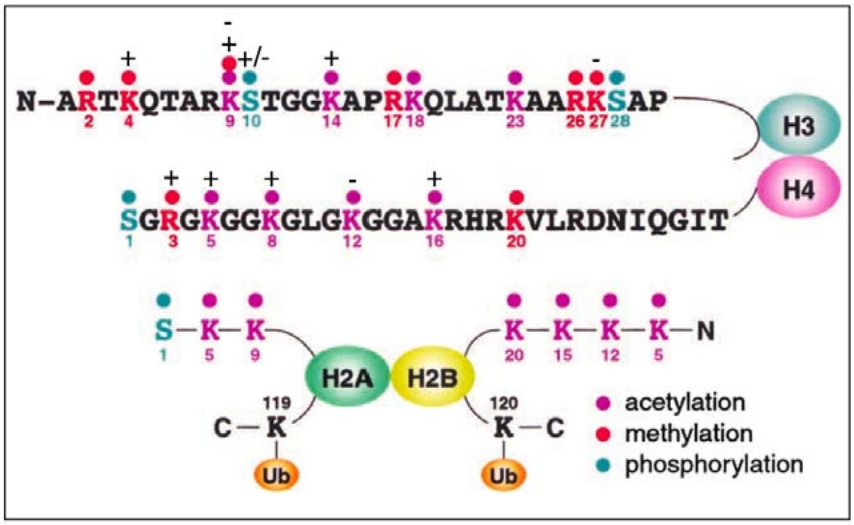



**Figure 2: Known modifications of histone tails**. + suggests that this modification is involved in activation of transcription whereas – suggests that the modification is involved in repressing transcription. *(derived from Zhang and Reinberg, 2001)*

The tremendous number of various combinations that these modifications can offer suggests that the histone code may considerably extend the information potential of the genetic (DNA) code. Since it has now been demonstrated that the entire PcG complex does not remain associated at its target genes throught mitosis (Yamamoto et al., 1997; Buchenau et al., 1998; Dietzel et al., 1999), the semi-conservative nucleosome distribution on DNA after replication and their persistence on the DNA during mitosis make these nucleosomes good candidates to carry the epigenetic mark through cell division. The combination of histone marks could provide, after mitosis, the anchor for specific protein complexes to recapitulate their initial formation on the target DNA. In this way, several protein domains have been found to be able to read the histone code and selectively bind specific combination of modifications. The bromodomain has been the first protein module to be shown to bind acetylated histone tails (Dhalluin et al., 1999; Winston and Allis, 1999; Owen et al., 2000; Jacobson et al., 2000). It is found in TAFII250 (Jacobson et al., 2000) as well as in the human *ash1* gene (Nakamura et al., 2000). The chromodomain of HP1 was recently shown to bind the Su(var)3-9 methylated histone 3 at lysine 9 (Bannister et al., 2001; Lachner et al., 2001). Interestingly, other chromodomain proteins exist (PC itself has one) that may recognize other methylated residues although this has not been shown so far.

### 1.4.2 The *marker* proteins in the PcG / trxG complexes

A recent study proposed that, in the PcG complex, the maintenance of the repression and the repression itself are performed by different proteins (Beuchle et al., 2001). Based on an inducible "knock-out" strategy, the authors reported that the silencing was lost at different rates depending on which PcG gene was knocked out during development. Furthermore, when resupplying the protein after several cell divisions, they observed that, in some genes, the silencing could be restored suggesting that in these cases, the epigenetic mark was still present on the DNA and could still attract the repressing complex. They concluded that PcG proteins could mainly be separated in two groups: the *repressor* (or *effector*) proteins (PSC-SU(Z)2, PH), and the *marker* proteins (PC, PCL, SCM, SCE). In this model, the repressors are responsible for the silencing by compacting the chromatin for example, whereas the markers establish the epigenetic mark on the DNA allowing maintenance of the transcriptional state through cell division.

Such a model could also be extrapolated to the trxG proteins, and we could imagine that a subset of proteins in the trxG complex are responsible for marking the CMMs as being active, whereas others are responsible for activating transcription by having a direct effect on the transcriptional machinery or by attracting some coactivators.

Consistent with the existence of a histone code, marker proteins in the PcG / trxG may actually modify histone tails in a very specific manner. The fact that some of these proteins contain domains involved in histone modifications (like the SET domain in *E(z)* and *ash1*) supports this hypothesis. Furthermore, the idea that a specific epigenetic mark could specify the activation state of a CMM was first proposed by Cavalli and Paro (1999), who reported that acetylation of histone H4 is characteristic for active *Fab7* CMMs (Cavalli and Paro, 1999). However, it is still not known whether histone H4 acetylation is the cause of the activation of the CMM or only a consequence of the transcription allowed by the active CMM.

To summarize, it is one major focus of current studies to find the epigenetic marks defining the activity of CMMs and it is suspected that these marks may lie in the histone code (Ringrose and Paro, 2001).

## 1.5 Cellular memory modules besides homeotic genes?

Immunostainings on polytene chromosomes from salivary glands revealed that PC and other PcG proteins are localised at about 100 different sites, including homeotic gene complexes (bithorax, antennapedia...). However, for most of the PC-binding sites, the actual

target of PC regulation is not known (Zink and Paro, 1989, Paro and Zink, 1993). The same kind of pattern has also been shown for some trxG proteins like ASH1, TRX or Z, often colocalising with the PcG proteins (Rastelli et al., 1993; Chinwalla et al., 1995; Tripoulas et al., 1996). Other studies showed that mutations in some PcG genes interfere with regulation of some segmentation genes although for most of them it was not clear whether the effect is direct or indirect (McKeon et al., 1994). However, interestingly, *en* itself is directly controlled by PcG and trxG proteins in embryos (Moazed and O'Farrell, 1992; Breen et al., 1995) and imaginal discs (Randsholt et al., 2000; Americo et al., 2002), opening the possibility that the maintenance of expression of this selector gene is supported by cellular memory processes. It suggests that besides homeotic genes, an important number of genes involved in determining cell identity and in patterning imaginal disc could be controlled by PcG and trxG proteins during development and could show cellular memory processes.

## 1.6 The determination and transdetermination of imaginal disc cells

### 1.6.1 The development of imaginal discs

As the identity of each parasegment along the A-P axis is being defined by the combination of homeotic genes in the embryo, in the mean time small clusters of 10-40 cells deriving from these parasegments are set aside which will give rise to the imaginal disc anlagen. These imaginal discs are found in larvae and are the undifferentiated precursors of the adult appendages. Later, during the pupal stages, each imaginal disc undergoes metamorphosis to form one of the specific adult appendages (Fig. 3).

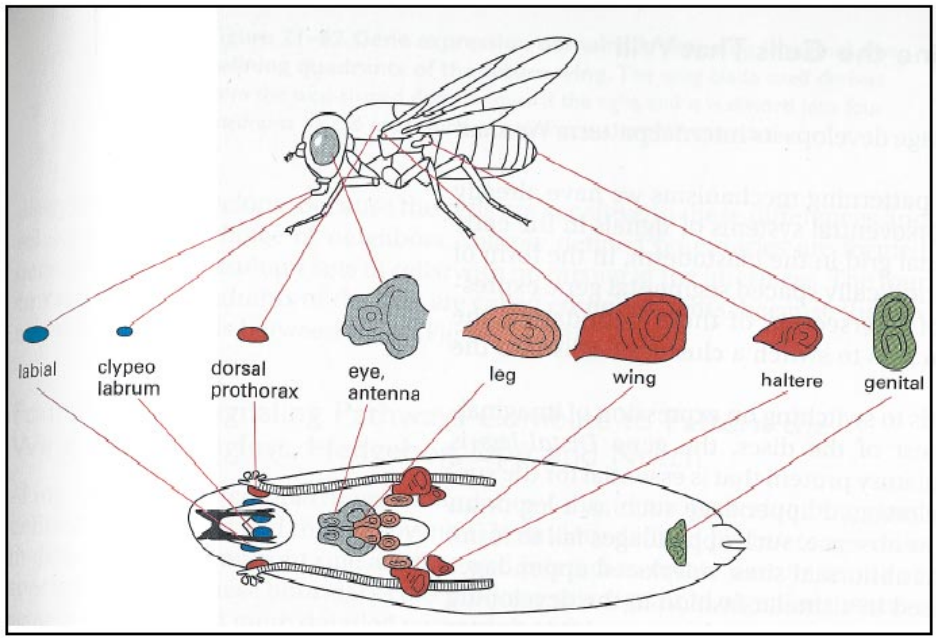



**Figure 3: The imaginal discs in the drosophila larvae and the adult structures they give rise to.** (After Fristrom et al., 1969).

These cells do not participate in larval life, but instead divide during the larval instars to form large epithelial sacs, each containing tens of thousands of cells. Therefore, imaginal disc cells acquire a disc-specific (such as leg, wing, or eye) determined state during embryogenesis and heritably maintain that determined state as they proliferate throughout larval life. Furthermore, proliferating cells must decide between alternative fates during the growth of the appendage anlage. In the wing and leg discs, an early arising cell lineage restriction subdivides the discs into anterior (A) and posterior (P) compartments. The A-P restriction is present at the earlier stages of leg and wing development resulting from the inheritance of selector gene expression, like *en*, from the embryo.

In the wing disc, a second lineage restriction appears during the middle stages of disc growth subdividing the disc into dorsal and ventral compartments. The borders of these compartments form distinct signaling centers which organise the patterning and the growth of the developing disc. The understanding of the morphogenesis of *Drosophila* appendages is important for the understanding of vertebrate limb development since it follows similar processes.

The PcG and trxG genes are required for the proper development of these imaginal discs. Mutations in some PcG genes can lead to several kind of transformations such as antenna to mesothoracic leg and mesothoracic leg to prothoracic leg, that mimic the effect of gain of function homeotic gene mutation (Lewis, 1978; Struhl, 1981). Likewise, loss of trxG gene function causes loss or reduction of homeotic gene expression and induces transformations mimicking loss of homeotic gene function, such as haltere to wing (Ingham, 1985; Adamson and Shearn, 1996; Brizuela and Kennison, 1997).

Furthermore, experiments have shown that the imaginal disc cells are able to maintain their determination for many rounds of division. When specific fragments of imaginal discs are isolated and transplanted into a larval host, they will continue to develop according to their prospective fate, and produce specific adult structures (Hadorn, 1963). Thus imaginal disc cells maintain their determined state through larval life.

### 1.6.2 *In vivo* culture of imaginal disc fragments

To more rigorously test the ability of imaginal disc cells to maintain their determined states, Ernst Hadorn's group developed, in the 1960's, an *in vivo* culture system for imaginal discs. When imaginal discs or disc fragments are transplanted into adult female abdomens, the hormonal conditions are such that the disc cells will still proliferate but do not differentiate.

The state of determination of the implant can be tested afterwards by reisolating the disc fragment and transplanting it into a larval host to allow differentiation. Studies of imaginal disc fragments that are given extra time to grow in culture demonstrate that they do, for most of the part, maintain their determined states.

It was initially observed that transplanted fragments of imaginal discs could either duplicate the patterns of the fate map already present in the fragment, or regenerate patterns of the map missing in the fragment (Schubiger, 1971). Regeneration of imaginal disc tissue has been observed in other insects (Gehring and Nöthiger, 1973), revealing that this pattern regulation response is not unique to *Drosophila*.

Only cells at the cut edges of imaginal disc fragments appear to participate in pattern regulation. Typically, cells of the cut edges of a cultured disc fragment form a blastema in that they divide more frequently than other cells of the fragment and can give rise to structures that differ from their normal fate (Abbott et al., 1981; Kiehle and Schubiger, 1985). Even after extensive growth in culture, imaginal disc fragments can still maintain their disc-specific determined states (Hadorn, 1966). These observations provide strong evidence that disc-specific determined state are propagated over hundreds of cell divisions and are thus cell heritable.

### 1.6.3 Imaginal disc transdetermination

*1.6.3.1 The discovery of imaginal disc transdetermination*

Unexpected discoveries were made in Hadorn's laboratory when Schläpfer (1963) observed, in rare cases, wing tissue derived from eye-antennal disc fragments and when Hadorn (1963) observed that genital disc fragments could give rise to leg and antennal tissue. These observations were the first evidence that the disc-specific determined states of imaginal cells could be altered after they are established. The process by which imaginal disc cell determination switches to that of another disc type is called transdetermination (Hadorn, 1965).

Further studies of cultured imaginal disc fragments revealed that all of the imaginal discs can transdetermine, but transdetermination events do not occur randomly: each disc transdetermines in a particular, reproducible direction and with a particular probability. For example, leg disc cells are able to transdetermine to wing, whereas wing disc cells rarely transdetermine to leg (Schubiger, 1968) (Fig. 4).

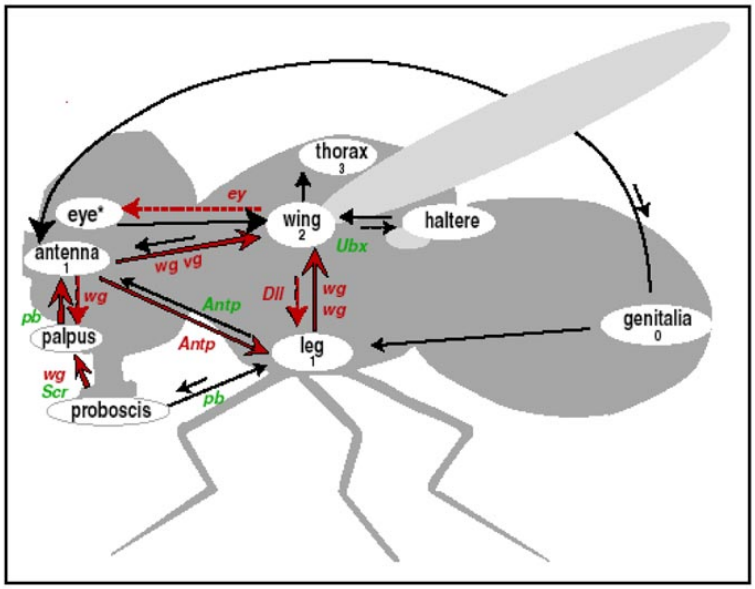



**Figure 4: Sequence of transdetermination events and effect of homeotic mutations and ectopic gene expression on *Drosophila* imaginal discs (from Wei et al. 2000).**

Transdetermination is often considered to be a rare event that occurs only after many extra cell divisions. However, specific regions within discs have been identified as particularly susceptible to fragmentation-induced transdetermination, and cuts through such regions, which have been termed “weak points” (Hadorn, 1978), can induce transdetermination at high frequency. Thus, leg disc fragments with cuts through this proximal-dorsal region can transdetermine after only a few extra cell divisions in culture (Schubiger, 1971). Moreover, Gehring showed that transdetermination is a polyclonal event, arguing against somatic mutations causing the switch (Gehring, 1967).

Taken together, these observations suggest that cells in specific regions of a disc tend to give rise, under certain conditions, to particular cell types of another disc. This localized directionality may provide insight into how transdetermination occurs, and thus, may help understanding mechanisms of disc cell determination.

*1.6.3.2 Inducing transdetermination in situ*

Interestingly, many transdeterminations resemble mutant phenotypes of homeotic genes. By misexpressing the homeotic gene *Antennapedia* (*Antp*) in the eye-antennal disc of third instar larvae, Schneuwly et al. (1987) induced antenna-to-leg transdetermination, without wounding, fragmentation or transplantation. The effect of *Antp* is typically restricted to particular appendages, and a leg appeared only in the antenna at the same weak point identified in the fragmentation/regeneration experiment.

However, the homeotic genes are not the only genes that control appendage identity in *Drosophila*. Therefore, ectopic expression of *eyeless*, normally restricted to the eye imaginal disc, induces eye structures in some specific regions of the wings, halteres, legs and antennae

(Halder et al., 1995). Likewise, ectopic expression of *vestigial* (*vg*), normally restricted to wing and haltere discs, induces wing structures in some regions of the eye, head, antennae, leg and gennitalia (Kim et al., 1996). These results suggest that Vg regulates the expression or activity of genes that are critical for the determination of dorsal appendage (wing or haltere) identity. In the same way, Distal-less (Dll) was shown to promote ventral appendage (leg, antenna) identity when expressed in eyes or wings (Gorfinkiel et al., 1997).

These results demonstrate that the proper expression of homeotic genes and other regulatory factors is crucial for the determination of imaginal disc identity and therefore must be tightly controlled all along development. Understanding how the expression of these genes is established and maintained in the appropriate imaginal disc cells is central to understand disc determination. We can expect these genes to be misexpressed in the fragmentation-induced transdetermination experiment.

*1.6.3.3 The disc fragmentation experiment induces transdetermination by creating new combinations of cell signaling molecules.*

In 1998, Maves and Schubiger showed that transdetermination may be the consequence of cell signaling molecules interacting out of their proper context. They produced ectopic clones of cells expressing *wingless* (*wg*) in leg imaginal disc and observed that a leg-to-wing transdetermination event could occur in the same region previously described as being a weak point for transdetermination (dorsal leg disc cells). They further demonstrated that this change of cell identity is due to the fact that the induction of *wg* clones in, or close, to the dorsal leg region, expressing *decapentaplegic* (*dpp*), establishes a new combination of cell signaling molecules. Forcing this new interaction in dorsal leg disc may mimick the situation occurring in ventral wing disc cells and promote the expression of the wing hinge marker: *vestigial*.

Therefore, an interaction between Wg and Dpp signaling may also initiate transdetermination in fragmented leg discs. Leg disc fragments that have the ability to transdetermine are those with cuts through dorsal as well as through ventral leg disc cells (Schubiger, 1971). During wound healing in culture, the fragmented disc cut edges heal together, thereby effectively juxtaposing *wg*-expressing (ventral) leg cells with dpp-expressing (dorsal) leg cells. Intercellular signaling interactions can thus explain how the expression of nuclear regulatory factors that play critical roles in the specification of segmental identity and imaginal disc cell fate, such as Vg, could become activated following a wounding event.

In some cases, sublines of transdetermined cells could be established and maintained in culture indicating that once these cells have transdetermined and changed their identity, upon the action of new combinations of cell signaling molecules, they could stably maintain their new cell identity through hundreds of rounds of cell divisions, even when cell signaling molecules have disappeared (Gehring, 1967). These cells are thus able to maintain their new gene expression program in a clonally inheritable manner.

In recent years, substantial progress has been made in the understanding of how the switch from a determined identity to a new one is established in the leg discs. However, very little is know about how the new gene expression program is then maintained through cell divisions.

*1.6.3.4 Vertebrate parallels to imaginal disc transdetermination*

Transdetermination-like events have been observed in vertebrates. One example is the ability of retinoids to induce homeotic transformations of regenerating amphibian tails to hindlimbs (Mohanty-Hejmadi et al., 1992). In such experiments, tadpole tails are amputated, and then the tadpoles are bathed in a solution containing retinoids. The tail-to-hindlimb transformation that is induce by this procedure resembles imaginal disc transdetermination in many ways: it occurs in already determined tail cells; it is dependant on tail regeneration (Maden and Corcoran, 1996), it is induced by signaling molecules (retinoids), and it is likely mediated by changes in Hox gene expression. Retinoic acid treatment can also induce homeotic transformations, accompanied by changes in Hox gene expression, along the A-P embryonic body axis in mice (Kessel and Gruss, 1991; Marshall et al., 1992). Other studies suggest that homeotic transformations induced by retinoic acid signaling may be mediated by interactions with signaling pathways (such as TGF-ß/Dpp) that are used in imaginal disc transdetermination.

In humans, metaplasias represent another parallel to imaginal disc transdetermination. Metaplasias are transformations in tissue type that occur postnatally and are often precursors to cancer. These metaplasias are changes in the determined states of epithelial states. They usually occur in response to damage and subsequent regeneration. Metaplasias are throught to be polyclonal and a way for local stem or progenitor cells to adapt to a changed environment by producing cells appropriate for the new conditions. The transformations are directional and Kauffman (1993) has argued that the directions of human metaplasias, like imaginal disc transdetermination, represent “neighbouring” developmental programs. These analogies

predict that the directions of metaplasias are controlled by nuclear regulatory factor switches such as those used in imaginal disc transdetermination.
In a courageous comparison, Wei et al. (2000) tried to find similarities between stem cell plasticity in mammals and transdetermination in *Drosophila*. They suggest that observations like neural stem cell and muscle stem cell reconstitution of the hematopoietic system represent stem cell fate changes equivalent to transdetermination events. They postulated that transdetermination in *Drosophila* may serve as a paradigm for the better understanding of mammalian stem cell biology.

### 1.7 Aim of the thesis

PcG and trxG proteins are able to maintain the established transcriptional state of homeotic genes during the whole of development through their binding to Cellular Memory Modules (CMMs). Polytene chromosome immunostainings reveal that PC could regulate the expression of about 100 different genes, the identity of most of them being unknown. This suggests that PcG/trxG genes potentially control the expression of all these genes during development allowing the maintenance of their initial transcriptional state until adult stages. Therefore it is possible to imagine that genes responsible for the determination of imaginal disc identity may be controlled by such CMMs.
In transdetermination events, as a consequence of the appearance of new combinations of signaling molecules in the disc, new gene expression programs are established in the transdetermined cells and maintained through cell divisions.
In this thesis, I ask the question whether transdetermination results in the switching of the CMMs of developmental genes to a new state of activation. This switching would establish a new gene expression program in the cells, which would, by this way, be inherited through mitosis in order to maintain the new transdetermined identity.

To answer this question, in the first part, I will demonstrate that the function of CMMs during development is not restricted to the control of homeotic gene expression. It may instead be a much more widely spread mecanism.
Furthermore, I will prove that the state of activation of CMMs can be switched to a new one in embryonic as well as larval stages.

The next part will investigate the role of PcG and trxG members in the determination and transdetermination of imaginal discs. It will be shown that the establishment of transdetermination events is influenced by some PcG members.

Furthermore, it will be suggested that PcG as well as trxG proteins may be needed for the inheritance of the new transdetermined identity through their interplay at CMMs.

This study may help in understanding how gene expression patterns are established and maintained, or possibly switched, from embryogenesis to adulthood.

# II. Results.

## *Part 1*

A Cellular Memory Module conveys epigenetic inheritance of *hedgehog* expression during *Drosophila* wing imaginal disc development.

### 2.1.1 Introduction

Although several PREs regulating developmentally important genes have been identified (*en*, *ph*, as well as from the bithorax and Antennapedia complexes) (Zink et al., 1991; Simon et al., 1993; Fauvarque et al., 1995; Brown et al., 1998) and many more candidates exist, only a few PREs from the bithorax complex have been tested and characterized as CMMs (Cavalli and Paro, 1998; Rank et al., 2002). It is not known whether the concept of epigenetic maintenance of gene expression states is restricted to genes involved in long term decisions, such as the homeotic genes (i.e. to restrict embryonic patterns) or may be a more general phenomenon used at different times of development. Segmentation genes are used to pattern the body at various stages during development and their expression pattern may be modulated with time. Therefore, they constitute interesting targets to investigate the potential role of CMMs in controlling their expression. Importantly, the knowledge of how selector genes and segmentation genes are transcriptionally regulated is of fundamental importance to understand how stem cells established at later stages of development can maintain their identity throughout cell divisions.

The product of one of these segmentation genes, *hedgehog (hh)*, known to act as a morphogen (Heemskerk and DiNardo, 1994), is essential for many crucial developmental pathways involved in the regulation of growth and patterning in both invertebrate and vertebrate species. In humans, mis-activation of the hh pathways leads to congenital diseases (for example prosencephaly) (Villavicencio et al., 2000), and is associated with many kind of tumors and cancers such as basal cell carcinomas and primitive neuroectodermal tumors (Toftgard, 2000; Taipale and Beachy, 2001). In *Drosophila*, one of its roles is to pattern leg and wing imaginal discs through the activation of *decapentaplegic* and *wingless* expression (Basler and Struhl, 1994). In these discs, *hh* is initially activated in the posterior (P) compartment by Engrailed (En) (Tabata et al., 1992; Zecca et al., 1995) which plays the key role in specifying the posterior identity (Kornberg et al., 1985; Simmonds et al., 1995). In late third instar wing discs, Hh induces expression of *en* in the anterior compartment in a thin stripe along the antero-posterior (A-P) boundary (Blair, 1992; Strigini and Cohen, 1997). Several mechanisms seem to prevent *hh* and *en* expression from spreading into the anterior (A) compartment. For example, Polyhomeotic (PH) probably directly or indirectly maintains the repression of *hh* in the anterior cells abutting the A-P boundary (Maschat et al., 1998), whereas Groucho represses both *hh* and *en* in anterior cells (de Celis and Ruiz-Gomez, 1995; Apidianakis et al., 2001).

How cells building compartments can maintain their determined identity until the completion of development is still unclear. The trxG and PcG proteins are known to control *en* expression (Busturia and Morata, 1988; Moazed and O'Farrell, 1992; Breen et al., 1995; Brizuela and Kennison, 1997; Strutt and Paro, 1997; Maschat et al., 1998). Previous studies found indications that *hh* expression itself might also be regulated by the trxG and PcG proteins (Felsenfeld and Kennison, 1995; Randsholt et al., 2000). In this first part of the thesis, I present evidence that *hh* expression is indeed directly controlled by the action of trxG and PcG proteins. I demonstrate that a 3,4 kb fragment situated upstream of the *hh* transcription start site exhibits CMM activity and I show that during larval stages, *hh* expression can be activated by En in wing imaginal disc, and inherited through mitosis to daughter cells, even after En has ceased to act. The maintenance of *hh* expression is not due to any kind of positive feedback loop but is dependent on the trxG and PcG proteins. It can be concluded that, during development, *hh* transcription is controlled by a CMM. In this respect, CMM switching may be a mechanism widely used at any time during development to maintain transcriptional states of genes with diverse functions. Moreover, I could show that the state of histone-acetylation at CMMs do not play a crucial role in inducing CMM switching.

## 2..1.2 Results

*2.1.2.1 hedgehog transcription is directly controlled by PcG and trxG proteins*

The immunoprecipitation technique using cross-linked chromatin (XChIP) allows the mapping of *in vivo* DNA target sites of chromatin proteins (Strutt and Paro, 1999). Since one Polycomb (PC, a member of the PcG) binding site on polytene chromosomes coincides with the cytological position of *hh* at 94E, this method was used to ask whether there are PC and GAGA factor (GAF/Trl; a member of the trxG) binding sites in the *hh* genomic region. These two factors had previously been found to be hallmarks of CMMs (Strutt et al., 1997; Tillib et al., 1999) and the GAF has been shown to be associated with some PcG complexes and necessary for the silencing function of PREs (Horard et al., 2000; Busturia et al., 2001). Initially, the immunoprecipitated material was hybridised to a genomic stretch of 45 kbp, deriving from a BAC (BACR03N12; Genbank: AC008365) containing an insert with the *hh* gene region (Fig. 5). This led to the identification of PC/GAF binding sites in regions close and into the transcription unit.

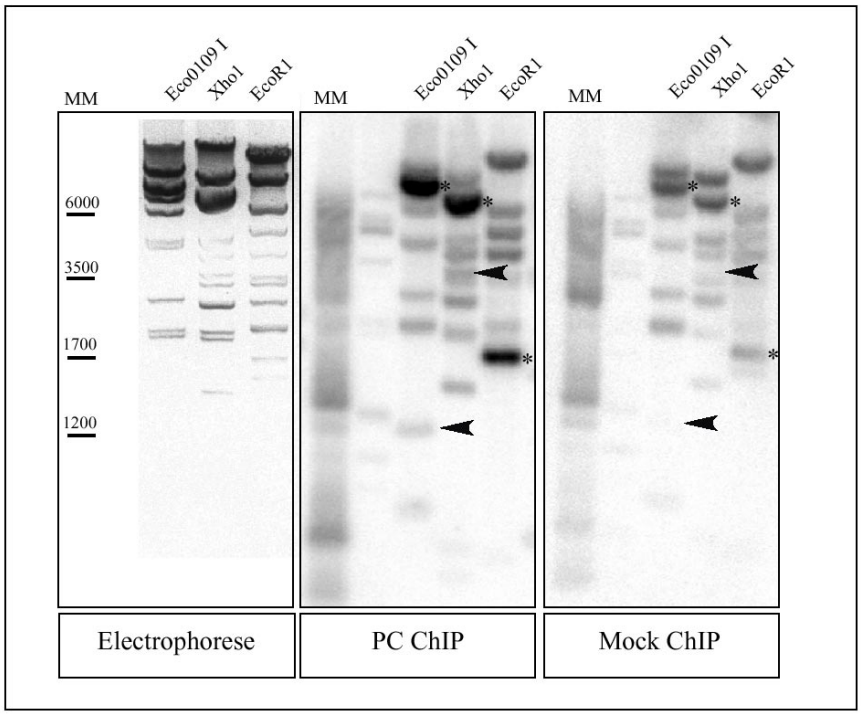



**Figure 5: Identification of Pc-binding sites in the hh gene region.** The immunoprecipitated material was hybridised to a genomic stretch of 45 kbp encompassing the *hh* gene. The hybridisation reveals two new bands in the immunoprecipitated material that correspond to the promoter region (arrowhead at 1200) and a region located between 7 to 11 kb after the transcription start site (arrowhead at 3500 ). The stars depict a DNA region located more than 20 kb upstream of the *hh* gene in the region of another gene.

To further fine-map the location of the PC/GAF binding sites, the region around the *hh* gene was subdivided into 1 kb sized PCR fragments (from 4 kb upstream of the *hh* transcription start site according to the transcript *CG4637* from *Flybase*, to 13.4 kb

downstream to the end of the gene (Fig. 6). Slot-blot hybridizations of immunoprecipitated material (Fig. 6A) revealed two main sites where PC and GAF are strongly enriched (Fig. 6B). The first site (A) is located in a region between 0,07 to 1,06 kb upstream of the transcription start site, whereas the second binding site (B) is found in a region spanning the second exon of the *hh* gene and spreading about 0,4 kb on both sides of the exon. At both peaks, PC and GAF binding sites substantially overlap. The presence of this particular arrangement of PC and GAF binding sites in the *hh* genomic region suggests that these PcG and trxG proteins directly control *hh* expression.

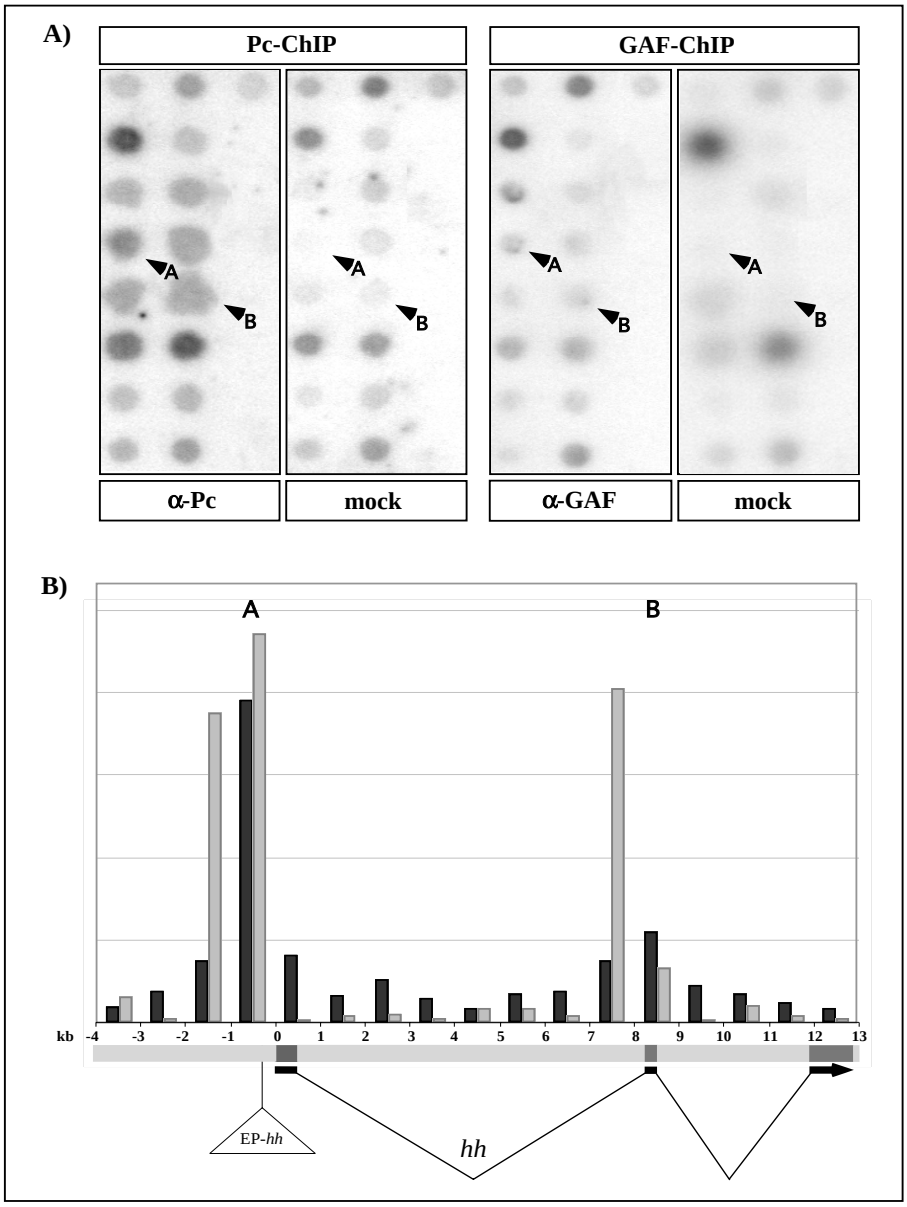



**Figure 6: Binding of PC and GAF factor to the *hh* genomic region in embryos**. A) *Slot blot hybridization;* chromatin from *Drosophila* wild-type embryos was either mock immunoprecipitated or immunoprecipitated with anti-PC or anti-GAGA antibodies. 1kb PCR-fragments from the *hh* genomic region were blotted on a nylon membrane and the immunopurified DNA was radiolabelled and used as a probe for hybridization (arrows A and B show the signals corresponding to the strongest enrichment compared to mock). B) The graph depicts the relative enrichments of immunopurified DNA compared to mock (PC enrichment is shown in black, GAF enrichment in gray). The protein distribution shows two main peaks of PC and GAF binding sites. One peak is situated upstream of the transcription start site (peak A) whereas the second one spans the second exon and spreads in the neighboring introns (peak B). The transposon EP-*hh* is inserted 364 bp upstream of the *hh* transcription start site.

To investigate this at the functional level, the accessibility of the *hh* promoter region to a trans-activating factor was assessed. It is known that a PRE placed in the vicinity of an Upstream Activating Sequence (UAS) is able to counteract GAL4 binding preventing expression of the reporter gene (Zink and Paro, 1995; Fitzgerald and Bender, 2001). I took advantage of the availability of an EP line possessing a UAS site close to the endogenous *hh* transcription start site (Rørth et al., 1998) to test whether the *hh-PRE*s could inhibit the activation of transcription induced by GAL4. The EP3521 line (termed here EP-*hh*) possesses an EP transposon containing several UAS sites, and is inserted in the *hh* promoter region (-0,36 kb, see Fig. 6B). The endogenous *hh* gene is not transcribed in salivary glands. By using a hs-*GAL4* line, which is known to be leaky at 25°C, weak expression of GAL4 in larval salivary glands is observed. When hs-*GAL4* is crossed to a line containing UAS-*hh* integrated randomly in the genome, *in situ* stainings reveal that at 25°C, by the action of GAL4, the *hh* mRNA is present in high amount in all the salivary gland cells (Fig. 7A). However, when hs-*GAL4* is crossed to the EP-*hh* line, in which the UAS sites are juxtaposed to the presumptive PRE, *hh* transcription was observed in only a very few cells situated mainly at the base of the glands (Fig. 7B). Since in most cells transcription is inhibited, it was reasonable to think that the PcG proteins binding the PREs in the vicinity of the *hh* promoter blocks the accessibility of GAL4 to the UAS sites. Accordingly, reducing the amount of some of the PcG proteins in the cells by repeating the experiment with flies heterozygote for the $Pc^3$ allele (Fig. 7C) or with males hemizygote for the $ph^{409}$ alleles (Fig. 7D), induces partial derepression of transcription of the endogenous *hh* gene in a substantial number of gland cells. These results indicate that the repression observed in most of the salivary gland cells in the EP line is due to the action of the PcG proteins through the binding to the identified PREs. These experiments together demonstrate that PcG proteins directly repress the transcription of *hh*.

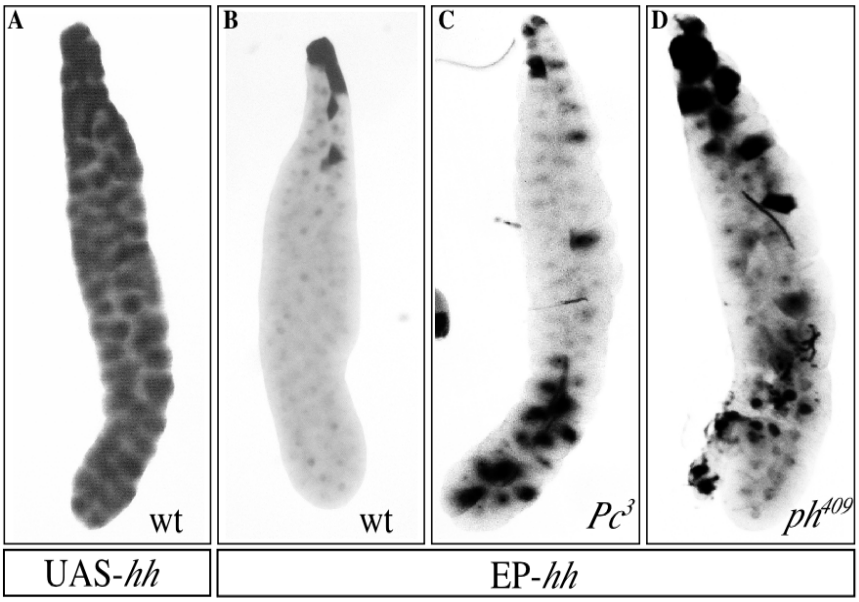



**Figure 7: The PcG proteins repress transcription of the *hh* gene in salivary glands**. At 25°C, the hs-*GAL4* driver is leaky in salivary glands. It can activate transcription of a UAS-*hh* reporter construct (A). However, when using the EP-*hh* line (in which an EP element is inserted near the endogenous hh promoter) in the same conditions, *hh* transcription is observed in a very few cells only (B). Repeating the same experiment in flies heterozygous mutant for $Pc^3$ (C) or $ph^{409}$ (D) shows that *hh* transcription becomes derepressed in more cells in the salivary glands.

*2.1.2.2 A fragment of the upstream regulatory region of hedgehog exhibits a CMM activity.*

Having shown that the *hh* gene is controlled by the PcG proteins, I was interested to see whether the mapped PC/GAF binding sites could function as PREs and CMMs *in vivo*. Transgenic flies were produced using the vector that allows to test for the maintenance of the reporter gene expression through cell divisions (Cavalli and Paro, 1998). A 3,4 kb fragment, starting from position -3760 to -402 bp upstream of the *hh* transcription start site (according to transcript CG4637 from Flybase), and containing the PRE identified in the *hh* promoter region by chromatin immunoprecipitation (peak A, Fig. 6), was linked to a GAL4 / UAS-inducible lacZ gene (UAS-lacZ) and mini-*white* as a reporter and transformation marker (Fig. 8A). Another construct was made containing a 3,6 kb fragment starting from position -3760 to -248 bp upstream of the *hh* transcription start site (according to transcript CG4637 from Flybase).

*a. Characterisation of the PRE-like activity*

Most of the lines obtained with the 3,4 kb fragment (15/22) exhibit pairing-sensitive silencing when homozygous for the construct, indicated by the variegated expression of mini-*white* in the eyes , a phenomenon often associated with PREs (Fig. 8D) (Fauvarque and Dura,

1993; Kassis, 1994; Zink and Paro, 1995). Similar results have been obtained with transgenics containing the 3,6 kb fragment (10/15).

In order to characterise more precisely the action of the 3.4 or 3.6kb fragment on the expression of the reporter genes, *lacZ* expression was assessed in embryos and imaginal discs of the transgenics. Some posterior cells of each parasegment seem to express *lacZ* (Fig. 8B). This reflects that the *hh* DNA fragment cloned in front of the reporter genes contains some regulatory elements able to drive *lacZ* in some of the posterior cells but is not sufficient to drive the correct expression pattern of the endogenous *hh* gene. In imaginal discs, *lacZ* is expressed in ectopic patches, more frequently in the posterior compartment (Fig. 8C). Interestingly the expression pattern of *lacZ* in imaginal discs is not reproducible and different for each disc. *LacZ* expression can be activated at any time of development since clones are of different sizes and activation seems to be maintained through cell divisions. This suggests an epigenetic kind of regulation of *lacZ* expression

In addition, the silencing of the mini-*white* reporter gene was tested in different mutant backgrounds. For the 5 tested lines (Table 3), derepression of mini-*white* was observed in flies heterozygous for the transgenic construct and for a $ph^{401}$ hypomorph mutation, showing a ph-dependent repression of the reporter gene (Fig. 8E). Surprisingly, for the 4 lines, mini-*white* was more strongly repressed in flies heterozygous for the transgene and for a $Pc^{3}$ or $Pc^{XT109}$ mutations (respectively strong antimorph and null allele). Mini-*white* expression was slightly repressed when flies were crossed in a double heterozygous mutant for the trx-G genes *trx* and *brm* ($trx^{E2}/brm^{2}$), consistently with a trx-dependent activation of the transgene (Fig. 8E).

Moreover, these lines exhibit a temperature-dependent silencing with lines grown at 25°C being more strongly repressed than lines grown at 18°C for several generations (Fig. 8F). This is consistent with the observed fact that Pc-mediated silencing is more efficient at higher temperature (Fauvarque and Dura, 1993; Cavalli and Paro, 1998).

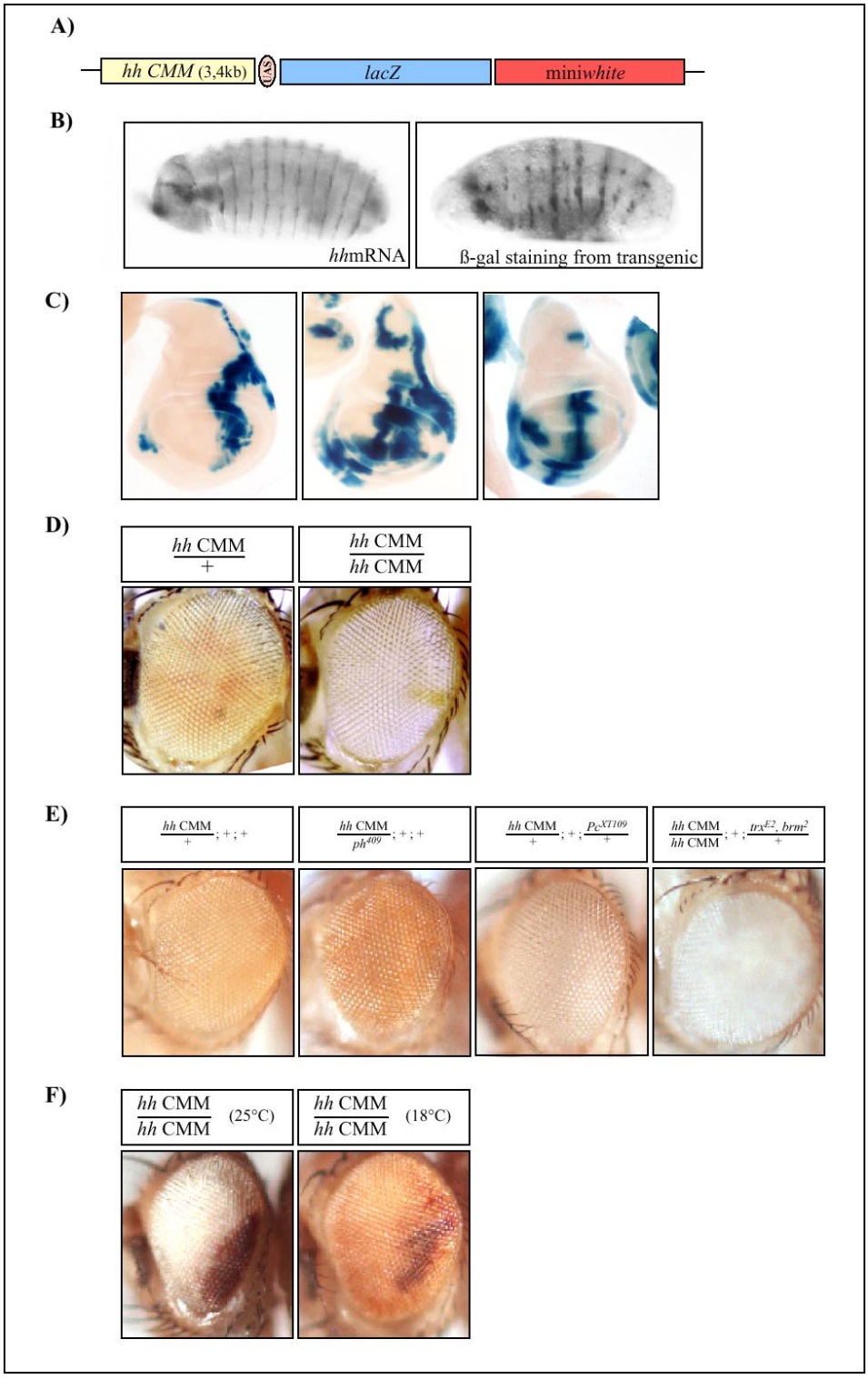



**Figure 8: Transgenic study of the influence of a 3.4 kb element of the hh promoter region on reporter gene expression.** A 3.4 kb fragment, termed *hh*CMM, containing the PRE identified in the *h h* promoter region was cloned into the pUZ transformation vector (A) and transgenic flies were generated. All pictures are taken from the line 7.2s. The 5 other transgenic lines tested show similar results. The 3.4kb DNA fragment contains regulatory regions which are able to drive *lacZ* expression in a pattern ressembling the endogenous hh pattern (B). However, in imaginal discs (C), ectopic randomly distributed patches of *lacZ* expression are seen. Flies heterozygous for the transgene show reduced mini-*white* expression (D). This is even more pronounced in flies homozygous for the transgene depicting pairing-dependent silencing of mini-*white* (E). Mini-*white* expression is derepressed in *ph* heterozygous mutants, whereas it is unexpectedly super-repressed in *Pc* heterozygous mutants. Consistently with the PRE-like activity of the 3.4 kb fragment, mini-*white* is more strongly repressed in doubly heterozygous mutants for some trxG genes, *trx* and *brm*. One week old flies raised for several generations at 18°C show a partial derepression of mini-*white* in the eyes compared to flies continuously raised at 25°C.

The insertion site of 3 of the collected lines, 7.2s, 11.6s, 8,1b, (Table 3) showing a strong pairing-dependent silencing was determined by *in situ* hybridisation on polytene chromosomes. Since they were not inserted into any known Pc-binding site, I tried to check whether they would create some new PC-binding site. Immunostainings of polytene chromosomes against PC and PH were performed, but no additional binding site could be detected at the position of insertion of the transgenes (Fig.9).

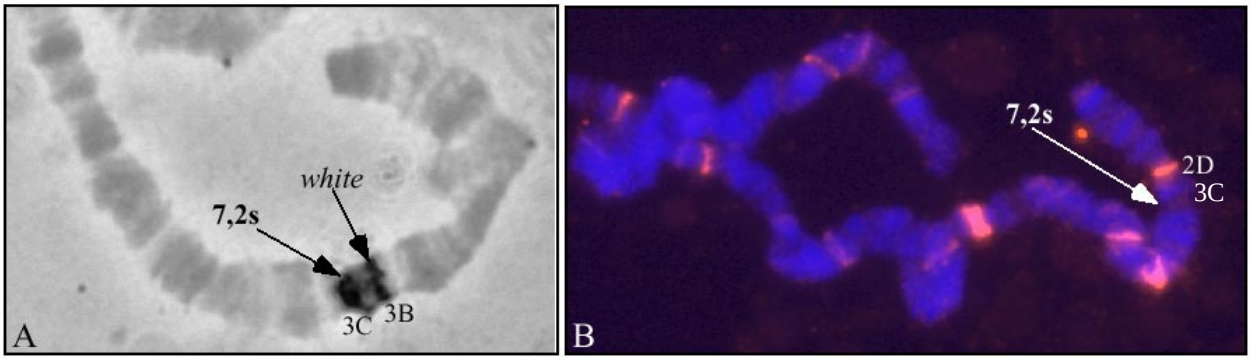



**Figure 9: No additional PC-binding site is detected at the insertion site of the transgene on polytene chromosomes.** In A is depicted the insertion site of the transgene in the line 7,2s at position 3C, close to the *white* gene (3B6-C1). Immunostainings with α-PC (B) or α-PH antibodies do not reveal additional binding sites for these two proteins. The DNA has been stained with DAPI (blue), in red are shown the PC-binding sites. Similar results have been observed for lines 11.6s and 8,1b.

Despite the absence of additional PC-binding sites on chromosomes, the 3,4 and 3,6 kb DNA fragment from the *hh* upstream regulatory region show some characteristics of known PREs, like pairing dependant silencing of the reporter genes, and silencing dependant on some trxG and PcG genes. Therefore, it is reasonable to define this region as a PRE-like element.

*b. Characterisation of the CMM activity*

A hs-*GAL4* driver was crossed into the lines containing the transgenes. This enables the transient production of GAL4 protein upon heat shocks, resulting in *lacZ* expression through the binding of GAL4 to the UAS elements in the construct. By this way, it is possible to test whether the *hh* PRE identified can function as a CMM and convey inheritance of the reporter gene expression throughout development.

A short GAL4 pulse produced in these flies, during embryogenesis, by activation of the hs-*GAL4* driver, leads to a homogeneous expression of the *lacZ* gene in the entire embryo (Fig. 10B). When these embryos are transferred back to 21°C and are allowed to develop to adulthood, more than 90% of the offspring of the two lines tested displayed partial or homogeneous mini-*white* derepression in the eyes (Fig. 10A,B). These results show that the

upstream 3,4 kb fragment, as well as the 3,6 kb fragment, are able to maintain the initial state of transcription of the reporter gene through the development and therefore exhibits CMM properties. In the same way as it was reported for the *Fab7* CMM (Cavalli and Paro, 1998), the *hh* CMM can not be switched to an activated state when the GAL4 pulse is produced during larval stages (Fig. 10C)

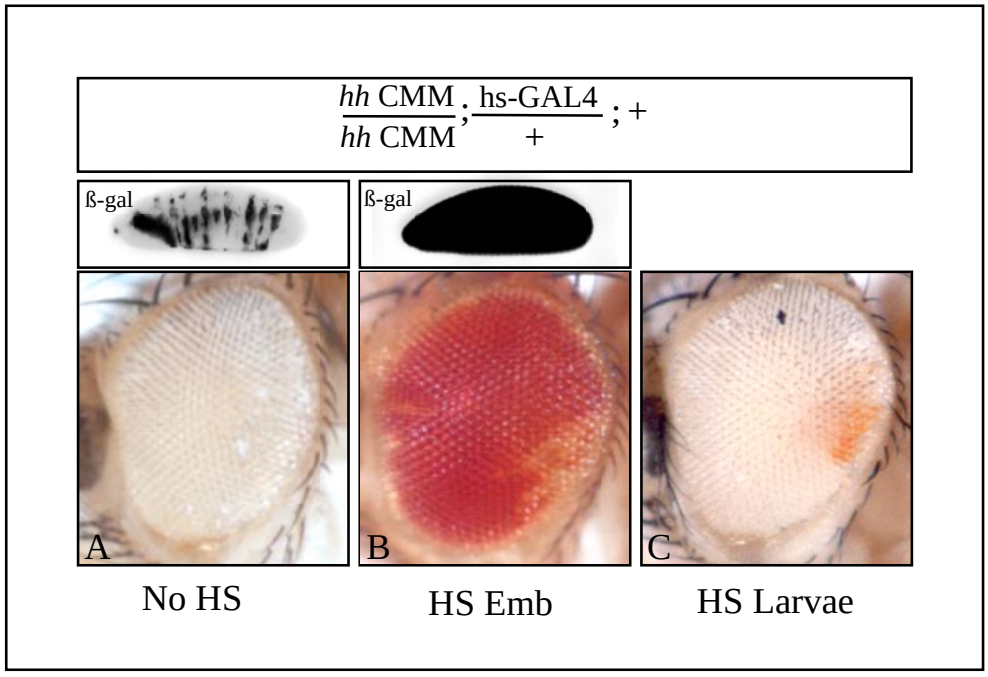



**Figure 10: A fragment of the upstream regulatory region of hedgehog exhibits a CMM activity.** A 3.4 kb fragment, termed *hh* CMM, containing the PRE identified in the *hh* promoter region was cloned into the pUZ transformation vector and transgenic flies were generated. Transgenic flies, homozygous for the *hh* CMM construct and containing the hs-*GAL4* driver raised at 21°C have repressed mini-*white* expression (A). When submitted to an embryonic GAL4 pulse. *LacZ* is homogeneously expressed in the embryo (B). When raised afterwards at 21°C until adulthood, the activation of the reporter genes is maintained from embryonic to adult stages and flies exhibit red eye color (B). When a GAL4 pulse is given during larval stages, the activation of the reporter genes is not maintained through the development and mini-*white* stays repressed in the eyes (C). Pictures are here taken from line 7,2s.

| **line** | **Insertion site** | **Pairing-dependent silencing** | **Epigenetic inheritance** |
|---|---|---|---|
| 7,2s | 3C (on X) | ++++ | Yes (can only be followed in females) |
| 11,6s | 2A (on X) | +++ | Yes (can only be followed in females) |
| 8,1b | 88E (on 3rd) | ++ | yes |
| 6,2b | on 3rd chromosome | ++ | yes |
| 10s | on 3rd chromosome | ++++ | yes |

**Table 3:** Transgenic lines containing either the 3,4 kb fragment (s) or the 3,6 kb fragment (b), and showing pairing-dependent silencing exhibit epigenetic inheritance of the transcriptional state.

*2.1.2.3 During imaginal disc development, hedgehog expression can be inherited through cell divisions independently of the initial trans-activator*

Having shown that the *hh* gene is controlled by PcG proteins and that a DNA-fragment upstream of the *hh* transcription start site can function as a CMM in a transgenic assay, I wanted to test whether the *hh* gene itself, in its original chromatin environment, is regulated by a CMM activity during imaginal disc development when cells undergo a high number of divisions. It is known that all wing pouch cells are progenies of the cells determined at the dorso-ventral (D-V) boundary at early larval stages (Klein, 2001). I hypothesized that if the transcription of a gene possessing a CMM is activated in cells during early larval development at the D-V boundary, then transcription should be inherited to daughter cells after mitosis, resulting in expression of the gene in all wing pouch cells.

During embryonic and larval development, En induces transcription of *hh* in the posterior compartment of leg and wing imaginal discs where the two factors substantially colocalize (Fig. 11A, B, C) (Tabata et al., 1992; Zecca et al., 1995; Guillen et al., 1995). Even though it is not presently clear whether En directly activates *hh* expression, this regulatory feature gives us a tool to test for CMM activity at the *hh* gene during imaginal disc development. UAS-*en* was overexpressed, at the D-V boundary using a vestigial-*GAL4* driver (vg-*GAL4*) (Simmonds at al., 1995). This transgene combination allows expression of GAL4 in a thin stripe (1 or 2 cells thick) along the D-V boundary during wing disc development (Fig. 11D). Double stainings of such late third instar wing discs reveal that surprisingly En does not only induce a thin stripe of *hh-lacZ* expression (reflecting the *hh* expression pattern in the P30 enhancer trap line) in cells along the D-V boundary as expected, but also in all the posterior and anterior wing pouch cells (except in a stripe along the A-P boundary (Fig. 11F). Strong UAS-*en* expression is detected in cells at the D-V boundary and lower levels of En in some regions of the anterior wing pouch (Fig. 11G). The repression of the endogenous *en* observed in some parts of the posterior compartment is explained by the fact that high levels of En could cause repression of the endogenous *en* in the P compartment (Guillen et al., 1995). Strikingly, the overlay of *hh*-LacZ and En stainings clearly reveals large domains, in both anterior and posterior wing pouch, with strong *hh* expression in the absence of En, suggesting that the transcription of *hh* in these cells becomes independent of En (Fig. 11H).

Furthermore, it is known that En represses *cubitus interruptus* (*ci*) expression (Eaton and Kornberg, 1990; Schwartz et al., 1995) and it has been shown that clones of A cells lacking Ci express low levels of Hh protein (Methot and Basler, 1999). In order to check whether the activation of *hh* in the wing pouch cells is not due to the repression of *ci*

expression by En, *ci* expression was examined in vg-*GAL4;* UAS-*en* wing imaginal disc. The stainings revealed that *ci* repression by En is restricted to the cells at the D-V boundary only (Fig. 11E), indicating that *hh* expression in the wing pouch cells of the A compartment is not due to a downregulation of *ci*. These observations suggest that *hh* expression is activated by En at the D-V boundary in early larval development, and is inherited, even in the absence of the initial trans-activator (En), through mitosis in the cells forming, in later stages, the wing pouch.

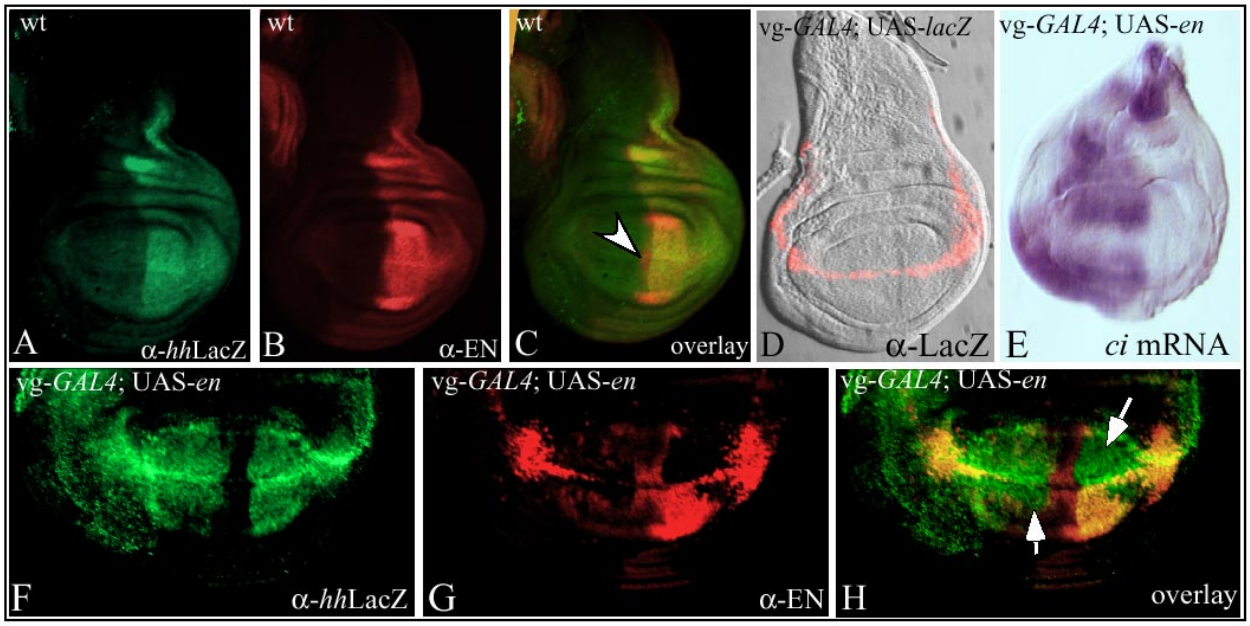



**Figure 11: UAS-*en* expressed at the D-V boundary induces expression of *hh* in most of the wing pouch cells.** All discs are shown dorsal side up, and anterior to the left. In wild type third instar wing imaginal disc, *hh-lacZ* (A) and *en* (B) are expressed in the posterior compartment. However in late discs, Hh induces an extension of *en* expression into the anterior compartment (C; arrowhead). The vg-*GAL4* driver induces expression of the UAS-*lacZ* reporter gene at the D-V boundary in wing imaginal discs (D). When UAS-*en* is misexpressed in a stripe along the D-V boundary using the vg-*GAL4* driver, *ci* is only repressed at the D-V boundary by En (E). However, En is able to activate *hh-lacZ* expression in most of the wing pouch cells (anterior and posterior) at a constant high level (F), whereas strong UAS-*en* expression is detected at the D-V boundary and lower levels of EN in some regions of the wing pouch (G). The overlay (H) of *hh-lacZ* and *en* expression domains shows large regions in the wing pouch where *hh-lacZ* is expressed in the complete absence of En (arrows), indicating that at this stage *hh* expression is maintained independently of En.

### *2.1.2.4 hedgehog inheritance of expression in the wing imaginal disc is not due to a positive feedback loop*

*hh* inheritance of transcription to daughter cells could be explained alternatively by the existence of a positive feedback loop allowing continuous maintenance of *hh* expression. This positive feedback loop would be activated once *hh* is expressed, either by auto-activation or cross-activation with another factor, like En, for instance. To investigate this possibility, *hh* was misexpressed along the D-V boundary, using the vg-*GAL4* driver and a UAS-*hh* transgene. Although UAS-*hh* is continuously strongly expressed at the D-V boundary from the second instar larval stage, *in situ* stainings do not reveal any inheritance of *hh* transcription to daughter cells, since the presence of *hh* mRNA is always restricted to a thin row of cells at the D-V boundary, even in late third instar wing discs (Fig. 12A). This result demonstrates that the previously observed inheritance of *hh* expression in wing pouch cells of vg-*GAL4*; UAS-*en* flies is not due to auto-activation by Hh itself nor to any positive feedback loop.

Furthermore, antibody stainings in such discs displays a progressive activation of *en* expression along the D-V boundary during development. In late third instar larvae, a strong En signal is observed, testifying the functional activity of the protein produced by UAS-*hh*. Higher magnification shows that in these discs, Hh is able to induce non cell-autonomously *en* expression in a stripe of about 7 rows of cells (Fig. 12B). However, the fact that at this stage, *hh* expression is only limited to a stripe of 2 rows of cells indicates that En is not able anymore to induce transcription of the endogenous *hh* gene, in contrast with early larval stages. It implies that the low levels of En protein observed in some of the anterior wing pouch cells of vg-*GAL4*; UAS-*en* third instar larvae (Fig. 11G), is most probably due to a late activation of *en* transcription by Hh. In addition, *hh* expression in these cells cannot be due to activation by low or undetectable levels of En protein, since we have now shown that even strong doses of En do not activate *hh* transcription in this region at this stage of development.

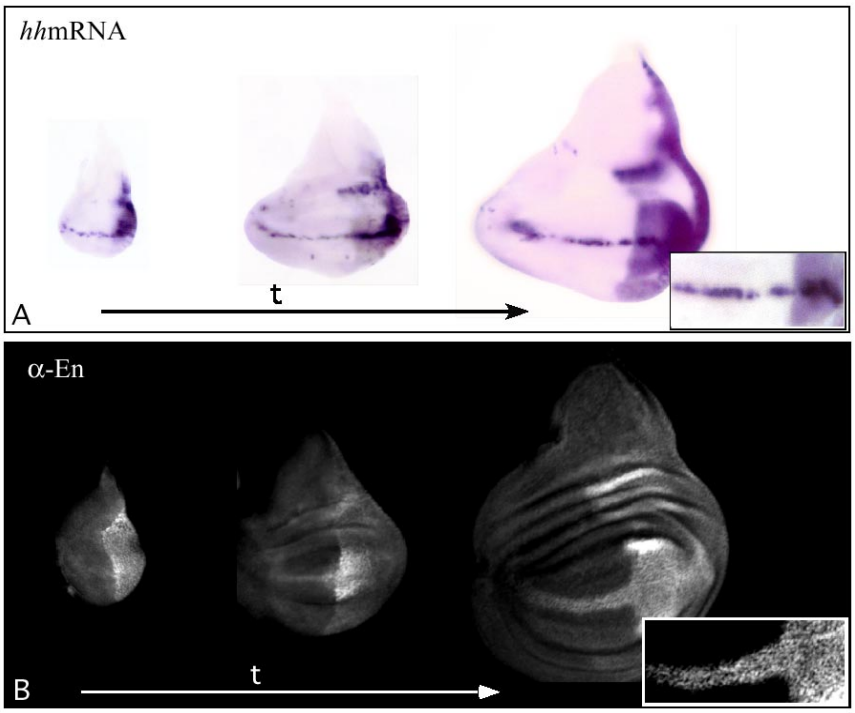



**Figure 12: Misexpression of UAS-*hh* at the D-V boundary induces *en* expression but does not activate transcription of the endogenous *hh* gene.** The figure shows wing imaginal discs from second instar larvae to late third instar larvae. UAS-*hh* is strongly misexpressed at the D-V boundary by the vg-*GAL4* driver, starting when the D-V boundary is established (A), but is not maintained in the progenitor cells in the wing pouch. *en* expression gets progressively activated at the D-V boundary in late larval development (B). The magnifications of the D-V boundary (inserts) shows that in late third instar wing imaginal disc, Hh induces *en* expression non cell-autonomously. The *en* expression domain is broader (7 cells thick) than that of *hh* (2 cells thick) indicating that in late larval wing pouch cells, En is not able to activate *hh* expression.

*2.1.2.5 The maintenance of the transcriptional state of hedgehog through cell division depends on PcG and trxG proteins*

When UAS-*en* is misexpressed at the D-V boundary in a wild type genetic background using vg-*GAL4* (Fig. 13A), it induces *hh* expression in most of the cells of the wing pouch except in a stripe along the A-P boundary where *hh* seems to be repressed. While UAS-*en* is strongly misexpressed at the D-V boundary, the endogenous *en* gene is weakly misactivated in some cells of the anterior wing pouch (Fig. 13B).

Repeating the same experiment in a genetic background hemizygous mutant for an hypomorphic allele of *polyhomeotic* ($ph^{409}$) leads to a broader domain of expression of *hh* (Fig. 13C). Remarkably, the region along the A-P boundary seems to be less refractory to activation of *hh* transcription since the territory of the repressed domain is reduced. Endogenous *en* is itself overexpressed in the anterior compartment (Fig. 13D). This is consistent with the previous findings demonstrating that its expression can be derepressed in PcG gene mutant background (Busturia and Morata, 1988; Moazed and O'Farrell, 1992; Randsholt et al., 2000). In this case in the anterior wing pouch cells, the activation of *en* transcription by Hh is probably more efficient than in a wild type background since *en* can not be correctly silenced by PH.

The same experiment repeated in a genetic background now doubly heterozygote for the trxG genes *trithorax* ($trx^{E2}$) and *brahma* ($brm^{2}$) consistently shows that *hh* expression is

activated at the D-V boundary, but can hardly be maintained through cell divisions in the anterior compartment, since in *in situ* staining the hh signal progressively fades away from the D-V boundary (Fig. 13E). As expected, in such a case, *en* expression, in the anterior compartment, is restricted to the D-V boundary since Hh might not be present in a sufficient amount to activate transcription of the endogenous *en* gene in the subsequent wing pouch cells (Fig. 13F).

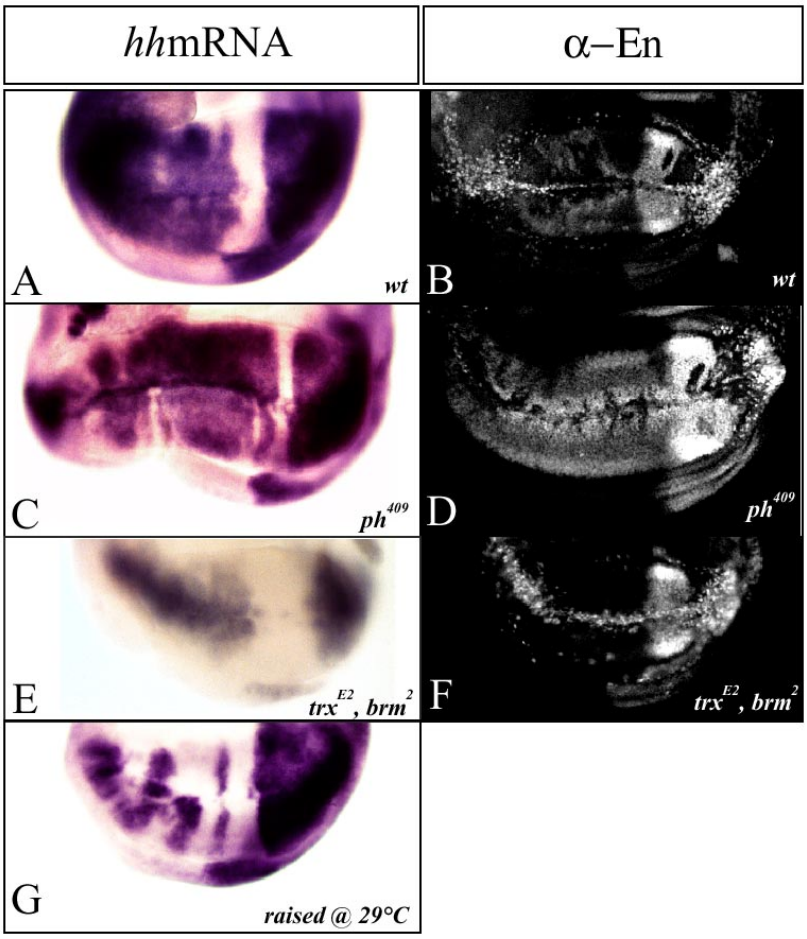



**Figure 13: The PcG and trxG proteins control the inheritance of *h h* expression in the wing pouch cells.** UAS-*en* is misexpressed using the vg-*GAL4* driver in all wing discs shown. In wild type background, a high level of *hh* mRNA is detected in most of the wing pouch cells except in a stripe at the A-P boundary (A), *en* is expressed strongly at the D-V boundary and more weakly in some region of the wing pouch (B). In $ph^{409}$ mutant background, *hh* (C) and *en* (D) are more strongly derepressed than in wild type flies. The stripe where *hh* was not expressed in a wild type background is reduced, indicating a dependence on PH-regulation. In double heterozygous mutants for $trx^{E2}$ and $brm^{2}$, *hh* expression is activated at the D-V but is not maintained through cell divisions and progressively fades away (E). *en* is strongly expressed at the D-V boundary but not in the other wing pouch cells (F). For embryos raised at 29°C until the start of the second instar larval stage, *hh* transcription is ectopically activated only in few clones in the wing pouch (G) indicating that, at this temperature, the Pc-repression of the *hh* gene is stronger and transcription is more difficult to be switched on. However, once switched, it is inherited through cell divisions, in contrast to the *trx*-G mutants.

Furthermore, it is known that PcG-mediated silencing is enhanced at higher temperature (Fauvarque and Dura, 1993) and this hyperrepressed state can be inherited through cell divisions (Cavalli and Paro, 1998). Based on these observations, it could be reasoned that raising embryos at 28°C instead of 18°C would make the Pc-mediated silencing more difficult to derepress, and influence the activation of *hh* transcription by En. Vg-*GAL4*, UAS-*en* embryos were allowed to develop at 28°C until the beginning of second instar larvae when the D-V boundary is established in wing discs and UAS-*en* expressed there. As

expected, stainings on third instar imaginal discs reveal ectopic clones of wing pouch cells expressing *hh* (Fig. 13G). However, the frequency of cells expressing *hh* is lower than in discs of larvae grown at 18°C indicating that the Pc-mediated silencing was harder to erase at 28°C. Nevertheless, in contrast with *trx*G mutant flies, once the transcription has initially been activated in this case, it is maintained in the subsequent daughter cells as suggested by the presence of clones spreading from the D-V midline to the limits of the wing pouch.

These experiments demonstrate that once initiated by En, the maintenance of the transcriptional state of *hh* to the daughter cells can be attributed to the action of the PcG and trxG proteins. It can be concluded that the CMM activity of the *hh* upstream region we have described in the transgenic assay is also efficient when considered in its natural chromatin environment and is responsible for the inheritance of the initial transcriptional state of *hh* from the initiation to the completion of the wing pouch development.

### *2.1.2.6 The switching of a CMM during larval stages may require specific trans-activating factors*

It has been previously reported that in the GAL4/UAS system, a GAL4 pulse, when provided in larval stages, was only able to transiently activate transcription of the reporter gene, but no heritable switching of the *Fab7* CMM was observed since transcription was lost as soon as the trans-activator (GAL4) was down-regulated (Cavalli and Paro, 1998). These observations led to the hypothesis that Pc-mediated silencing might be more stable in larval stages than in embryonic stages and CMMs can not be switched to mitotically heritable activity at these later stages. Consistent with these data, the upstream 3,4 kb fragment showing a CMM activity could not be switched to an active state through a GAL4 pulse produced during larval stages as demonstrated by the lack of mini-*white* derepression in the eyes of the adult flies (Fig. 10C).

However, in contrast to these experiments, I have now shown that the endogenous *hh* CMM can be switched to an active state in larval wing pouch cells upon an En pulse. The switch occurs in second instar larval stages, when the D-V boundary is established through the action of the Notch pathway (Kim et al., 1996, Klein, 2001) and GAL4 expressed by the vg driver. At this moment, *en* misexpression induces a switch of the endogenous *hh* CMM at the D-V to an active state, leading to maintenance of *hh* transcription in all wing pouch cells. I wanted to test whether GAL4 is also able to directly switch the endogenous *hh* CMM, in its natural

chromatin environment, in larval stages or whether this feature is restricted to specific trans-activators like En. To perform this experiment, the previously described line containing an EP-element inserted into the *hh* promoter region (EP-*hh*) was used. By inducing GAL4 in the cells it is possible to activate expression of the endogenous *hh* gene. I postulated that, by promoting transcription of the endogenous *hh* gene, the *hh* CMM may be switched to an active state in wing pouch cells. As observed on *in situ* preparations of late third instar discs, endogenous *hh* transcription is activated by GAL4 at the D-V boundary, but not maintained through cell division in wing pouch cells (Fig. 14A). In comparison, also the well characterized *Fab7* CMM is itself not switched to the active state after GAL4 induction at the D-V boundary since expression of the reporter gene is not maintained in daughter wing pouch cells (Fig. 14B). Thus, it is possible to conclude that the GAL4 trans-activator is not able to switch a CMM in larval stages, though this can be carried out by the action of a gene specific trans-activator, alone or more likely in association with other factors.

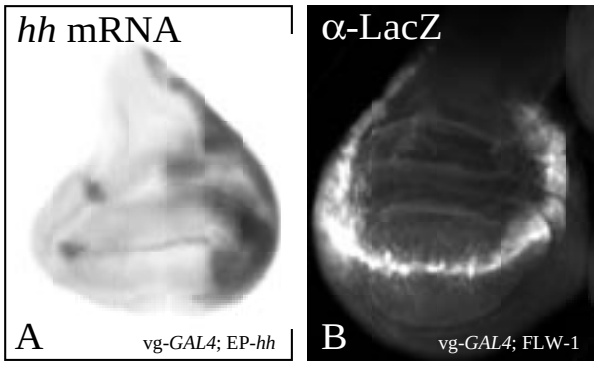



**Figure 14: The GAL4 trans-activator is not able to switch a CMM when expressed during larval stages.** *hh* is transcribed at the D-V boundary using the EP-*hh* line in combination with the vg-*GAL4* driver (A). However, transcription is not maintained in the daughter cells of the wing pouch. Similarly, expression of *lacZ* is not maintained when FLW-1 flies (Cavalli and Paro, 1998) are crossed with vg-*GAL4* (B). It indicates that at this stage the *Fab7* CMM can not be switched to the active state by GAL4.

### *2.1.2.7 The repression conferred by a CMM can not be overcome by inhibition of histone-deacetylases*

The fact that in imaginal discs, an epigenetic mark can apparently be erased, and a new one be established, upon specific circumstances addresses the question of the nature of this mark. A few years ago, the lab demonstrated that hyper-acetylation of histone H4 was an epigenetic mark for active CMMs (Cavalli and Paro, 1999). However, it is still unclear what is the role of histone-acetylation in this context. In general histone-acetylation has been shown to activate transcription although this is not a universal feature. It has been suggested that histone-deacetylases present in the Pc-complex could maintain the repressed state of the chromatin by continuously deacetylating the histones. Conversely, histone-acetylases in the

trx-complex would maintain the transcription in an active state by continuously acetylating the histones (Pirrotta, 1998). To investigate this hypothesis, I have decided to test whether inhibition of histone deacetylases alone could lead to an erasure of the epigenetic mark and induce derepression of a CMM. Treatment of cells by Trichostatin A (TSA), a histone-deacetylase inhibitor, has been shown to activate transcription of genes as a consequence of hyperacetylation of the promoter region due to the unbalanced action of histone-acetylases (Almouzni et al., 1994).

Because embryos are not permeable to chemicals, primary embryonic cell cultures deriving from a transgenic fly line transformed with the GAL4 inducible FLW-1 construct were established. This construct contains the *Fab-7* CMM upstream of some UAS enhancers controlling expression of the *lacZ* and m*W* reporter genes. As control, embryonic cell cultures with the LW-1 construct lacking the *Fab-7* CMM were established. These primary cultures were treated overnight with TSA (Fig. 15A).

This overnight treatment induces expression of the *lacZ* gene from the LW-1 construct. However, *lacZ* is not expressed in cells containing the FLW-1 construct (Fig. 15B).

In these primary cultures, cells are very fragile and difficult to handle, and small patches of embryonic tissues can often been seen. Therefore, it is very difficult to estimate the number of cells by directly counting them. In order to check whether the TSA treatment do not block cell division, we indirectly measured the rate of cell divisions by measuring the amount of proteins in our sample after cells were collected. This gives a rough idea of how the primary cells were dividing. It appears that mitosis still occurs in these cultures after TSA treatment since the amount of proteins continuously increased over time (Fig. 15C).

After the overnight TSA treatment, cells were maintained in culture up to 72 hours, and cell division was observed. Twenty-four hours, after TSA have been removed, the expression of *lacZ* in the LW-1 line start to decrease indicating that the histone-deacetylases are not inhibited anymore. In the FLW-1 cells, *lacZ* is never expressed even after cell division has occured (Fig. 15B). These results show that inhibition of histone-deacetylases does not overcome the repression mediated by the *Fab7* CMM, although being able to activate transcription in a reporter construct lacking the CMM. Therefore overacetylating histones at the CMM does not have any direct effect on the activation of transcription.

The possibility that histone-acetylation is just the main marker for an active CMM state, that would be needed as an anchor to recruit a trancriptionally active trx-complex after DNA replication, is implausible since an overnight TSA treatment followed by some cell divisions still does not switch the CMM to an active state.

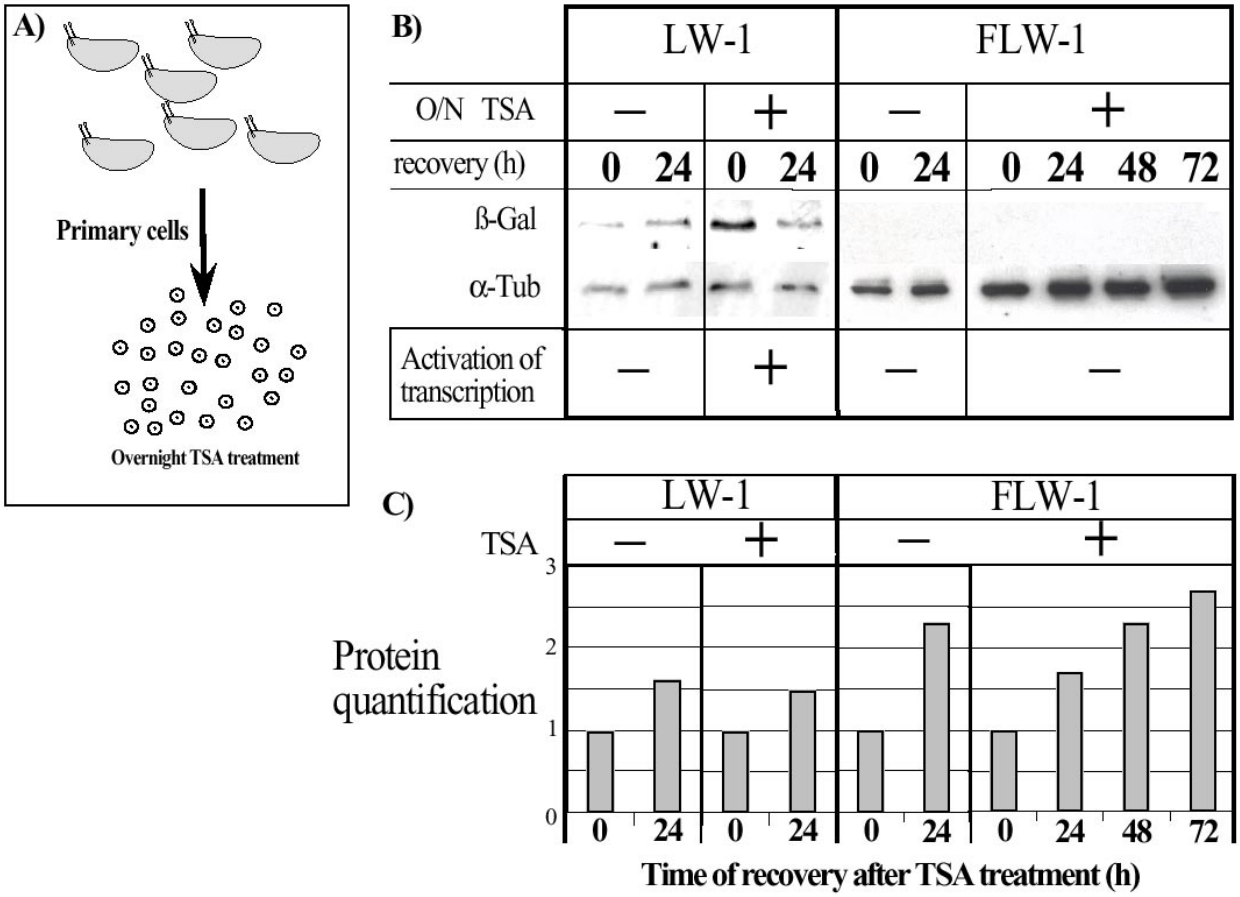



**Figure 15: TSA treatment of primary embryonic cells does not induce activation of the reporter genes in cells containing the FLW-1 construct.** A) Primary embryonic cell cultures from embryos of the LW-1 and FLW-1 fly lines have been generated and treated overnight with TSA. B) Inhibition of histone-deacetylases induces expression of the *lacZ* reporter gene in the LW-1 line lacking the *Fab7* CMM. However, TSA has no effect on the repression conferred by the *Fab7* CMM on the *lacZ* reporter gene in the FLW-1 line since no *lacZ* expression is induced, even after cell divisions. C) Since the direct counting of cells is difficult in these primary cultures, cell division was assayed indirectly by measuring the quantity of proteins after cells were collected following different times of recovery.

Therefore it can be concluded that the state of histone acetylation, although influencing the transcriptional activity of basal promoters, does not seem to be the main mechanism, as an epigenetic mark, responsible for the maintenance of epigenetic states.

### 2.1.3 Discussion

Initially, the first CMM was found to maintain the embryonically defined expression of *Abd-B* expression (Cavalli and Paro, 1998). By extension, it was admitted that CMMs maintain homeotic gene expression patterns until the completion of the development. However, it appears that other developmental genes, like *hedgehog*, are controlled by CMMs suggesting that this chromatin-based epigenetic inheritance of gene expression may be much more widely spread as expected.

Furthermore, CMMs may also be used to freeze developmental decisions taken at later stages upon signal from the cellular environment. The *hh* CMM could indeed be switched from a repressed to an active state during larval stages. However, in contrast with embryonic stages, specific trans-activators appear to be necessary to induce the switch in larval stages. These results highlight the dynamic of such CMMs during later stages of development and provide an additional layer of complexity for the control of morphogenic signaling used for tissue patterning.

#### *2.1.3.1 Developmental relevance of the presence of CMMs at the hedgehog gene and other segmentation genes*

Very little is known about how the gene expression pattern of cells building compartments in imaginal discs is inherited through cell divisions. Except for some homeotic genes, it is generally assumed that auto- and cross-regulations allow selector and segmentation gene expression to be maintained until the adult stage. However, here it was shown that at least in the case of *hh* a cellular memory system can take over to carry out the maintenance. It had already been proposed that trxG proteins might be needed to allow a proper inheritance of En expression in the cells of the posterior compartment (Breen, 1995). It was also suggested that a positive feedback loop between *en* and *hh* could achieve their own maintenance (de Celis and Ruiz-Gomez, 1995). My results indicate that this does not seem to be the case since the windows of time, when En can activate *hh* and Hh activate *en*, seem not to overlap over the entire wing development. During embryogenesis and early larval development (at least until the D-V boundary is established in wing disc), En is able to activate *hh*. However, experiments have proven that this competence disappears later, in particular in third instar larvae, when even high amounts of En can not activate *hh* transcription in at least the anterior compartment of the disc. On the other hand, Hh seems to

acquire the competence to activate *en* transcription in late larval stages. These results are consistent with the fact that in late larval stages, the Hh gradient is able to induce a stripe of *en* expression at the A-P boundary, whereas En does not in turn induce *hh* expression in this domain (Blair, 1992; Strigini and Cohen, 1997). Thus, since no feedback loop seems to exist, it suggests that the *hh* CMM has a role in maintaining *hh* expression in the posterior domain during late stages of development.

In an attempt to characterise the mechanisms by which the *hh* CMM mediates repression of the reporter genes in the transgenic lines, I tried to check whether additional PC and PH-binding sites would be visible at the insertion sites of the transgenes on polytene chromosomes. Although there is a visible PC-binding site at the cytological localisation of the endogenous *hh*, no obvious new sites could be detected in the three transgenics tested. On the chromatin-immunoprecipitation experiments, two mains PREs could be determined. The transgenes only contain the PRE located in the promoter region. Despite the fact that it is able to exhibit PRE-like and CMM activities on transgenic assays, this element on its own may not be able to recruit enough PC and PH proteins to be detectable by our antibodies on polytene chromosome immunostainings.

The endogenous *hh* gene seems to be directly repressed by PH and PC (Fig. 6 and 7) and consistently, the repression of the transgene seems to be impaired in *ph* mutations, whereas it is enforced by trxG gene mutations. However, *Pc* mutated alleles induce the opposite effect as expected. The transgene is more strongly repressed in such mutants. One explanation for this result is that the *Pc* mutation may misregulate a gene that could act on mini-*white* expression through the binding to the 3,4 kb region which, we know, possesses some regulatory elements in addition to the CMM. Subdivisions of the 3,4 kb fragment and their study in transgenic assays may help to isolate the minimum *hh* CMM which should be used to study more directly the real action of PcG and trxG proteins.

A domain along the A-P boundary seems to be refractory to a switch of the *hh CMM* to an active state (see Fig. 11 and 13). Interestingly, it appears that in this region Groucho and PH contribute to a strong repression system preventing *hh* expression to be activated in the anterior compartment in wild type flies (de Celis and Ruiz-Gomez, 1995; Maschat et al., 1998; Apidianakis et al., 2001). Thus, these proteins may counteract a stable switch of the CMM to an active state. Consistent with this result is the reduction of the thickness of this refractory domain in flies mutant for *ph* (Fig. 13C).

It has been reported that large clones lacking *en*/*inv* expression in the posterior compartment of wing discs show reduced or no Hh protein although this was not a universal

feature of small clones (Tabata et al., 1995; Sanicola et al., 1995). Apparently, in this situation the loss of *en*/*inv* in the cells, especially when induced early in development, might cause a substantial reprogramming of the gene expression pattern leading to repression of *hh*, perhaps due to the appearance of new repressors. In this case, the initially activated CMM would not be able to overcome the repression.

Interestingly, in 3rd instar imaginal discs in which *en* have been overexpressed at the D-V boundary, it was noticed that the endogenous *en* is sometimes completely repressed from large domains of the posterior compartment of the wing pouch (Fig. 11G). This was explained by the fact that En is able to directly downregulate its own expression when it is overexpressed in a cell (Guillen et al., 1995). However, it is clear from the immunostainings that once silenced by the exogenous En, the endogenous *engrailed* gene is able to maintain its transcription repressed even when the exogenous En protein has disappeared. Since it is known that En is regulated by PcG and trxG genes, it is actually likely that *en* is also regulated by a CMM. In this case, we observe here a switch of the *en* CMM from an active to a repressed state. This observation brings us some more proofs that CMMs are switchable elements which can not only be switched from a repressed to an active state, but also from an active to a repressed state, and this at any time during development.

The experiments described in this chapter strongly suggest that CMMs have major direct roles in the inheritance of the expression of *hh*, and most probably *en*, in the development of wing imaginal discs. Furthermore, *hh* and its vertebrate homologues are expressed in many other tissues during development, in which its activation and/or maintenance are independent of En and not yet elucidated (i.e. eye, gut, lung) (Bitgood and McMahon, 1995; Strutt and Mlodzik, 1996; Hoch and Pankratz, 1996; Warburton et al., 2000). Further studies should help to understand how the *hh* CMM may be involved in regulating the gene in different tissues.

*2.1.3.2 Dynamic CMM states during development*

The finding that genes necessary to pattern imaginal discs can be regulated by CMMs is in disagreement with models in which the elaboration of pattern in multi-cellular fields is solely based on information conferred by the local concentration of secreted signaling molecules (morphogen model). In addition to this, it is now clear that, the establishment of a specific gene expression program in cells at various developmental stages depends on both

the information conferred by the morphogens surrounding the cell and its history. Thus, a cell fate will be specified by the transcriptional activation or repression of new genes, as a result from surrounding information, as well as by the maintenance of old transcriptional states established earlier and inherited by CMMs through the action of the PcG and trxG proteins. It has already been suggested that the gene *optomotor-blind* could be regulated by a cellular memory mechanism in imaginal discs (Lecuit et al., 1996), although it was not directly demonstrated which mechanism could allow inheritance of transcription.

It is important to note that the state of activation of a CMM does not have to be established, once and for all, during embryogenesis, but can be modified or stably switched later in development. This may be especially true for genes patterning imaginal discs for which the expression pattern is established during larval development in contrast to homeotic genes defining the A-P axis during embryogenesis. However, it seems that general trans-activating factors such as GAL4, which are able to establish the active state of a CMM during embryogenesis, are not able to modify or switch the CMM state later in development, suggesting that the chromatin state of a CMM is more difficult to reprogram at late developmental stages. During larval stages, many cell divisions have been accomplished and cells are getting more and more restricted in their determination state. The chromatin could then be in a "mature" conformation stable enough to transmit a previously established transcriptional state despite the potentially contradictory actions of other transcription factors found concomitantly in the nucleus. Nevertheless, other transcription factors such as En (in the case where En directly activates *hh*) seem to be able, alone or by recruiting cofactors, to stably switch a CMM from a repressed to an active state during larval stages. At these stages, the switching of CMMs could require specific factors to set epigenetic marks. It could be envisaged that the En complex is able to attract some kind of chromatin remodeling machinery which would have the potency to erase the memory imprint and leave the chromatin competent to be reprogrammed.

In this way, it seems that the cell memory system is a complex and dynamic process during development, in which the role of CMMs is to heritably maintain a previously established transcriptional state until new specific patterning events are able to redirect the epigenetic marks of the CMMs.

The nature of the epigenetic mark responsible for the maintenance of a specific state of activation of a CMM is still not clear. Since it has been shown that hyperacetylation of histone H4 is an epigenetic mark for active CMMs, we tried to assess more carefully what is the influence of histone-acetylation on the activity of CMMs. We could demonstrate that, at

least in embryonic cells, the state of histone-acetylation is not by itself able to switch a CMM from a repressed to an active state (however the possibility that TSA is not able to inhibit a specific histone-deacetylase responsible for deacetylation at CMMs can not be rejected). This argues that histone-acetylation may not be the main or the only mark defining the activity of a CMM. This would be consistent with the recent results suggesting that histone-methylation would have a preponderant role in marking the different epigenetic states of the chromatin (Beisel et al., 2002; Czermin et al., 2002; Müller et al., 2002; Cao et al., 2002; Kuzmichev et al., 2002). It is likely that the epigenetic states are actually defined by different combinations of histone modifications, in which methylation may be responsible for the maintenance of defined transcriptional states, and acetylation a consequence of it.

*2.1.3.3 Implications for diseases, cancer and cell reprogramming*

*hh* possesses three homologues in mammals whose functions are highly similar those found in *Drosophila* (Fietz, 1994). The mammalian trxG and PcG proteins control homeotic gene expression (Deschamps et al., 1999) as well as hematopoiesis (Takihara and Hara, 2000). It can be predicted that such a cellular memory system, as found in *Drosophila*, also exists in mammals. In this way, the *hh* genes and many other developmental genes in mammals could very well be regulated by chromosomal elements with CMM function.

The fact that the transcriptional state of essential developmental genes can potentially be stably switched at any time during *Drosophila* development, could have serious consequences in case of accidental switches. Misactivation of the *hh* pathways causes dramatic developmental malformations and diseases such as cancer in mammals (Villavicencio et al., 2000; Toftgard, 2000; Taipale and Beachy, 2001). It would not be surprising to find such syndromes associated with a misexpression of one of the mammalian *hh* genes due to some alterations in the epigenetic marks setting the corresponding CMM status.

Furthermore, this study offers clues to how stem cells can be reprogrammed and can differentiate in different cell fates after the initial action of transcription factor bursts, and may maintain their new transcription pattern for the required round of cell divisions. In this direction, an accidental CMM switching of developmental genes during larval *Drosophila* development could explain transdetermination events (Maves and Schubinger, 1999). Under certain circumstances, some imaginal disc cells suddenly change their determination state and

are able to propagate their new cell fate over many divisions ending by creating new tissue structures.

# II. Results.

## *Part 2*

The role of Cellular Memory Modules in the transdetermination of *Drosophila* imaginal disc cells

### 2.2.1 Introduction

In the first part, it has been demonstrated that, besides homeotic genes, the expression of different kinds of genes involved in regulating developmental pathways, like *hh*, may be regulated by CMMs. The homeotic genes in the bithorax complex, which expression pattern defines the segment identity along the antero-posterior axe of the embryo, are regulated by several CMMs (Cavalli and Paro, 1998; Rank et al., 2002). Other genes, like *hh*, which are responsible to define the cell identities in imaginal discs are regulated by CMMs during development, however in a dynamic way. The CMM of these genes would be able to maintain an established state of transcription through cell divisions, but under certain circumstances it seems that the epigenetic mark responsible for the maintenance of the previously established transcription state could be erased and the transcription state switched to a new one, which would in turn be inherited through cell divisions.

Transdetermination is a switch of disc identity that occurs in some cells, under specific conditions, when the cell is already determined. It was shown to appear in *Drosophila melanogaster* when fragmented discs were cultivated for a period of time allowing several cell divisions. Cells at the wound start an aggressive regeneration program involving cell proliferation and fate respecification. One feature of the fragmented prothoracic leg (L1) compared to the mesothoracic (L2) and (L3) legs is that L1 anterior 1/4 (A1/4) fragments almost always regenerate, while complementing posterior 3/4 (P3/4) fragments almost always duplicate (Schubiger, 1971). In both cases, some anterior (A) cells convert directly to posterior (P) identity (Abbott et al., 1981), in direct violation of lineage restrictions imposed during normal development (Garcia-Bellido et al., 1973; Garcia-Bellido, 1975). Only a few founder cells are engaged in this proliferation program.

Recently, new molecular tools have allowed a better understanding of the transdetermination phenomenon. The reason why among the leg discs, only L1 legs are able to start this regeneration program may be to be due to the presence of peripodial cells expressing *hedgehog* in L1. Indeed, Gibson and Schubiger (1999) demonstrated that the secretion of Hh from the peripodial cells is responsible for the start of this regeneration and respecification program. A distinct population of squamous *en/hh*-expressing peripodial cells (specific to L1) fuses to the cut disc edge and acts as a transient ectopic source of Hh in cultured disc fragments. Hh signaling induces A/P conversion in both fragments by activating *engrailed* (*en*) in anterior cells. Furthermore, this transient *hh* expression is apparently responsible for the induction of an ectopic wg domain at the wound site in P3/4 fragments, overlapping with the endogenous dpp gradient. The new interaction between wg and dpp signaling generates

the activation of *vg* expression, a marker for cell identity (Kim et al.m 1996), inducing a leg-to-wing transdetermination event (Maves and Schubiger, 1995; 1998; Johnston and Schubiger, 1996). This new cell identity is clonally inherited since sub-cultures could be established containing only a population of transdetermined cell (Gehring, 1967).

Whereas it is thought that the new combination of signaling molecules, resulting from the juxtaposition of the two edges of the fragmented disc, has a major role in establishing a new gene expression pattern in the cells at the wound, it is not clear to what extents can PcG and trxG genes influence the switching to the new expression program. Furthermore, it is still not known which mechanisms allow these transdetermined cells to maintain their new identity through mitosis, in a clonally inheritable manner.

In the light of the results described in the first part of the thesis, I will demonstrate that PcG and trxG genes also have a role in the switching of cell identity during transdetermination events. Their influence may be concentrated at CMMs. I will show experiments suggesting that transdetermination consists of the switching of the CMM of some genes to a new state of activation. This new state of activation may be maintained through cell divisions by the interplay of PcG and trxG proteins at CMMs.

### 2.2.2 Results

*2.2.2.1 In imaginal disc cells, Polycomb restricts activation of specific developmental pathways*

*Pc* is expressed in all imaginal disc cells suggesting a general role in the maintenance of imaginal disc cell identity during development. To assess this question more carefully, clones of cells lacking any functional PC protein were produced during imaginal disc development. The FLP/FRT recombination technique was developed to induce "loss of function" clones (Dang and Perrimon, 1992). This technique allows mitotic recombination between homologous genetically engineered chromosomes through FRT (FLP-Recombinase-Target) sites after a transient expression of the FLP-recombinase. If recombination is induced between two homologous chromosomes, one containing a mutated allele of your gene of interest, the segregation of the chromosomes to the daughter cells after the mitosis will produce progenies exhibiting new genotypes: either homozygous for the mutated allele or completely wild type in an heterozygotic environment. By this way, it is possible to study the behaviour of clones of cells homozygous mutant for a specific gene in an heterozygotic environment. In the mean time, cells are genetically marked with the Green Fluorescent Protein (GFP). It is possible to visualise the clones because they do not express *GFP*, whereas the cells wild type for the gene have two copies of GFP and the cells heterozygous for the mutated allele have one copy of *GFP* (Fig. 16).

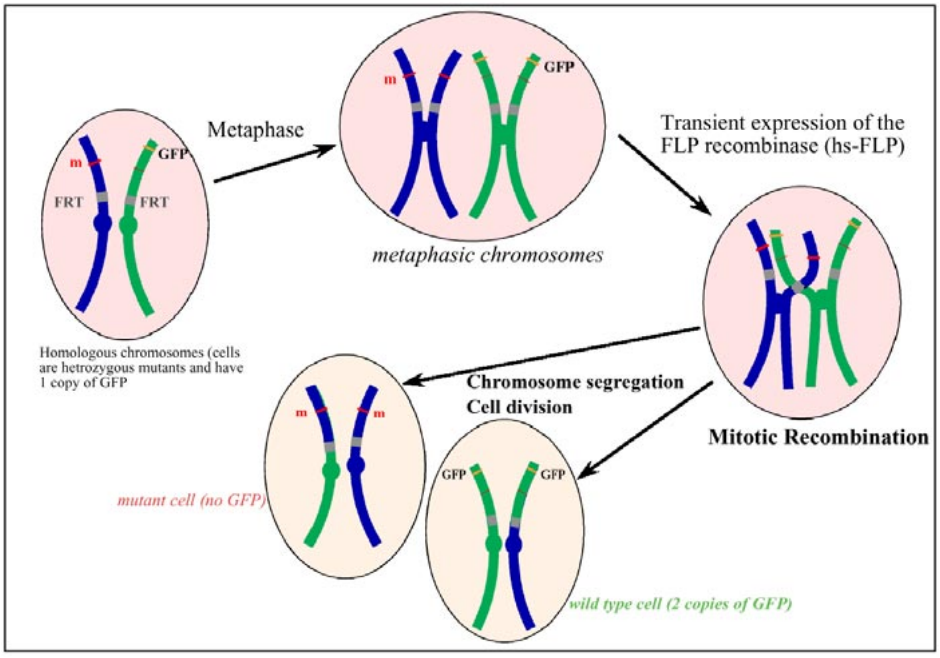



**Figure 16: The production of loss of function clones.** The scheme shows how mitotic recombination induced during development can be used to produce clones of cells homozygous for a mutated allele of a specific gene. This allow studying the behavior of muated cells in a wild type environment.

For this purpose, the strong antimorphic $Pc^3$ allele, on the third chromosome, was recombined with an FRT containing chromosome. Clones homozygous for the mutant *Pc* allele were induced during early larval stages, and 3rd instar imaginal discs were stained with different antibodies to observe how the lack of PC during development affects expression of genes involved in defining cell identity in the discs. All the clones shown here were produced with the $Pc^3$ mutation. However, the experiments have been reproduced with the $Pc^{(XT)}$ null mutant, and results observed were identical.

A striking feature that appears is that most of the clones observed do not mix with the normal population of cells. Cells lacking PC appear to minimize the contacts with the surrounding cells and form small islands of tissue in the disc. This fact may reflect the inability of the cells in the clones to remember their initial determination state. This may give rise to altered properties, affinities and identities by opening new developmental pathways. Immunostainings revealed that, as expected, homeotic genes like *Ubx* which is normally not expressed in wings, are derepressed in most of the clones lacking PC (Fig. 17A). More interestingly, expression of segmentation genes, like *en*, *wg and vg* (*vg* expression is represented by the expression of the *lacZ* gene which is under the control of the *vg boundary-enhancer*), is directly or indirectly affected by the loss of PC (Fig. 17B,C,D). It appears indeed that, depending on the region were the clones are situated, the expression of these genes can be repressed or activated. For instance, *en*, normally expressed in the P compartment, is repressed in clones made in the P wing pouch cells. Furthermore, *en* expression is not derepressed in the clones located in the A wing pouch cells. However, *en* expression is activated in clones formed in the putative dorsal hinge compartment.

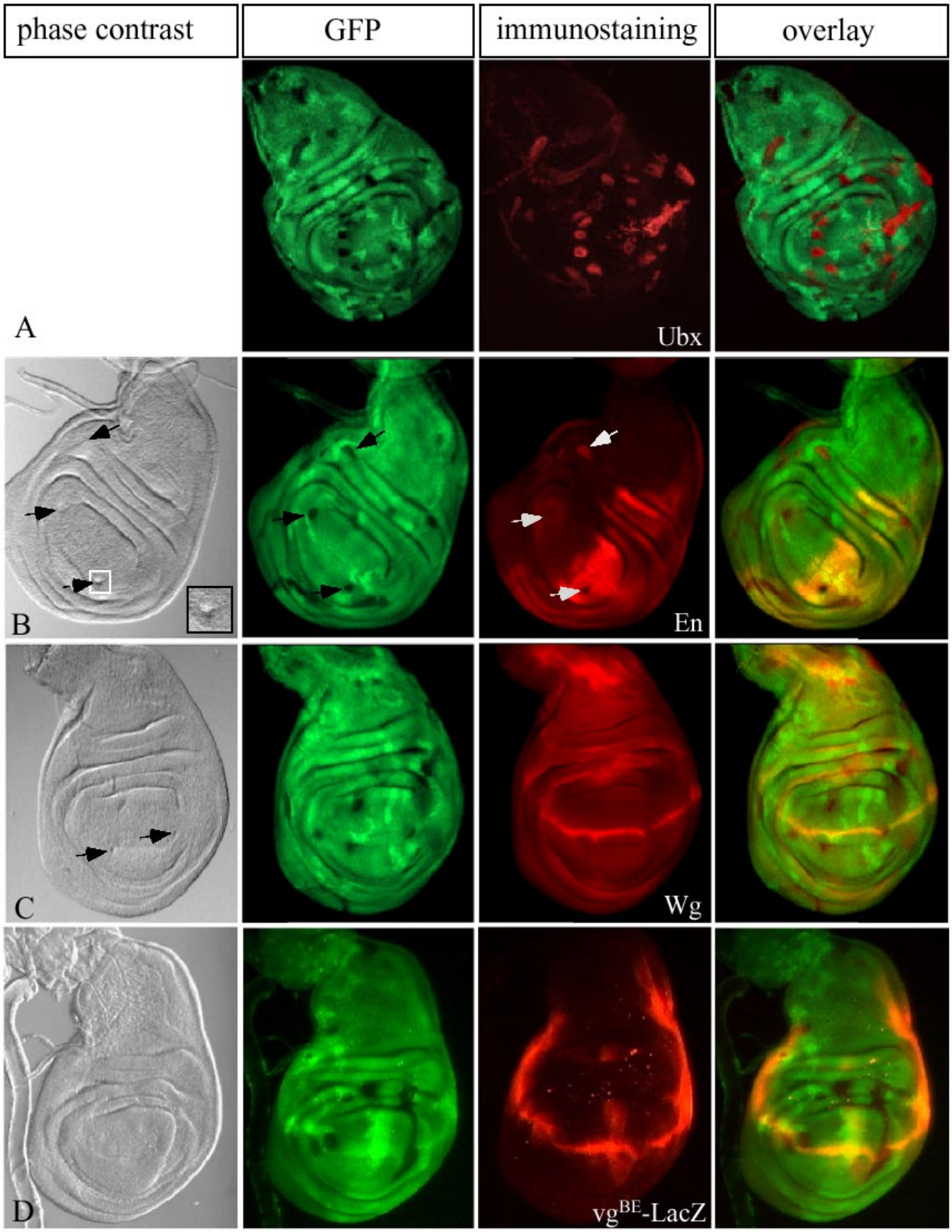



**Figure 17: Cells lacking Polycomb lose their identities**. Discs are all wing imaginal discs of late 3rd instar larvae. They are presented dorsal side up and posterior to the right. One copy of GFP is expressed in cells heteroyzygous mutant for *Pc*, 2 copies of *GFP* are expressed in cells being wild type for *Pc* and there is no GFP present in cells homozygous mutant for *Pc*. In the A row, *Ubx* is derepressed in most of the clones lacking PC. In B, it is possible to see that *en* is either repressed or activated in the clones lacking PC depending on the region of the disc where the clone is made. An enlarged view of one clones is seen in insert, in the phase contrast picture, showing how clones sort out from the wild type tissue. Similar results are observed in C for *wingless* or in D for *vestigia*l. In D, the pattern of expression of *lacZ* is dependent on the boundary enhancer of *vestigial*.

In adult cuticles several kinds of defects caused by misregulation of gene expression are observed. For example, most of the wings show additional or loss of wing veins (Fig. 18). The wing margin is sometimes notched and the corresponding rows of bristles are absent (Fig. 18D). This is a typical phenotype caused by an inactivation of the Notch pathway at the D-V boundary. *Vestigial* expression at the D-V boundary, which is directly activated by Notch and Suppressor of Hairless (Kim et al., 1996), is indeed down-regulated in clones lacking PC (Fig. 17D). Therefore, the Notch pathway is affected by the loss of PC at the D-V boundary. Moreover, bubbles are often observed between the dorsal and the ventral cell layers of the wing surface, indicating that cells of the two layers do not attach to each other perhaps because their cell identities are changed (Fig. 18B). Another striking defect lies in the duplication of a wing in the notum, which could be due to the derepression of *vg* and/or *scalloped* (Simmonds, 1998) in some clones (Fig. 18B).

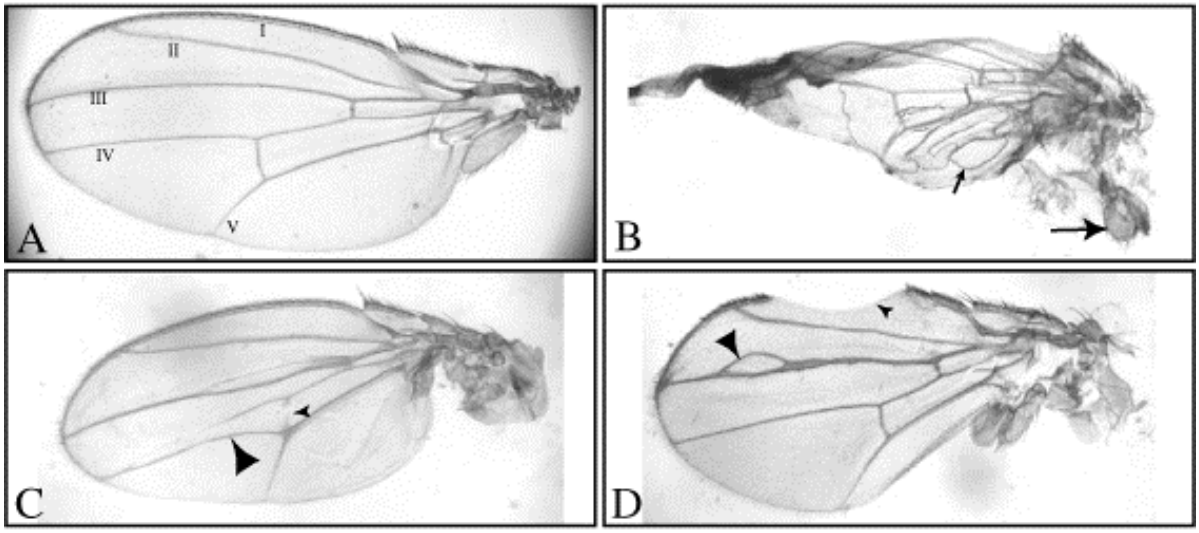



**Figure 18: Adult cuticle phenotypes of Pc- clones in wings.** In A is a wild type adult wing. Some typical defects in B show wing duplication (big arrow) or bubles between the dorsal and ventral cell layers in the wing (small arrow). In C, the arrowhead points out the appearance of a new vein connecting vein IV and vein V. On the other hand, the small arrowhead marks a gap in the intervein normaly linking vein 4 to vein 5. In D, the big arrowhead points out a duplication of vein III, whereas the small one shows a notch at the wing margin in the anterior compartment.

These results show that cells lacking PC do not follow the gene expression program they were initially determined for. They rather change their gene expression pattern in ways depending on the region where they are formed, and open new developmental pathways. It can be concluded that during development in the cells, the Pc-mediated repression pattern is established to restrict the number of developmental pathways that could potentially be activated, and indirectly promote the activation of specific ones.

*2.2.2.2 Cells lacking Pc can potentially show transdetermination-like events*

*Vestigial* can be used as a marker for leg-to-wing transdetermination, since it is normally expressed in the wing discs only (and halteres which have wing origins). Furthermore, when overexpressed, it is sufficient to induce outgrowths of wing tissue from eyes, legs and antenna (Kim et al., 1996). It was previously used as a marker for leg-to-wing transdetermination (Maves and Schubiger, 1995; 1998). Therefore, experiments were designed to ask whether cells lacking PC could transdetermine by searching for *vg* expression in discs different from wing imaginal discs. An enhancer trap line driving expression of *lacZ* in a pattern governed by the *vg boundary enhancer* was used as a marker for *vg* expression. Immunostainings showed that *lacZ* is expressed in some clones situated in the eye disc, posterior to the morphogenetic furrow, in a region which will form the head capsule in the adult. In this case, head imaginal disc cells derepress a gene marker for wing identity (Fig. 19A,B,C). In this respect, we can conclude that these cells show head-to-wing transdetermination-like events. The fact that only clones located in a specific region of the disc can transdetermine to a wing identity suggests that, in this region, the combination of signaling molecules are such that they can potentially open new developmental pathways in cells lacking PC, leading to a wing identity. The *boundary enhancer* normally induces expression of *vg* at the dorso-ventral boundary of wing imaginal disc (Fig. 17D). *Vestigial* expression under the *boundary enhancer* is unlikely to be regulated by a CMM, otherwise *lacZ* expression in the enhancer trap line would be maintained in the wing pouch cells through cell divisions (see Part I). Therefore, in this case, ectopic expression of *lacZ* driven by the *vg boundary enhancer* reflects the expression of *vg* and most probably results from an indirect misregulation of *vg* consecutive to the removal of PC from the cells.

Cuticle preparations of the adult fly head did not allow the nature of the tissue outgrowths to be precisely determined(Fig. 19D). It is likely that other genes are derepressed in these clones preventing the wing determination pathway from acting correctly and leading to an undefined tissue.

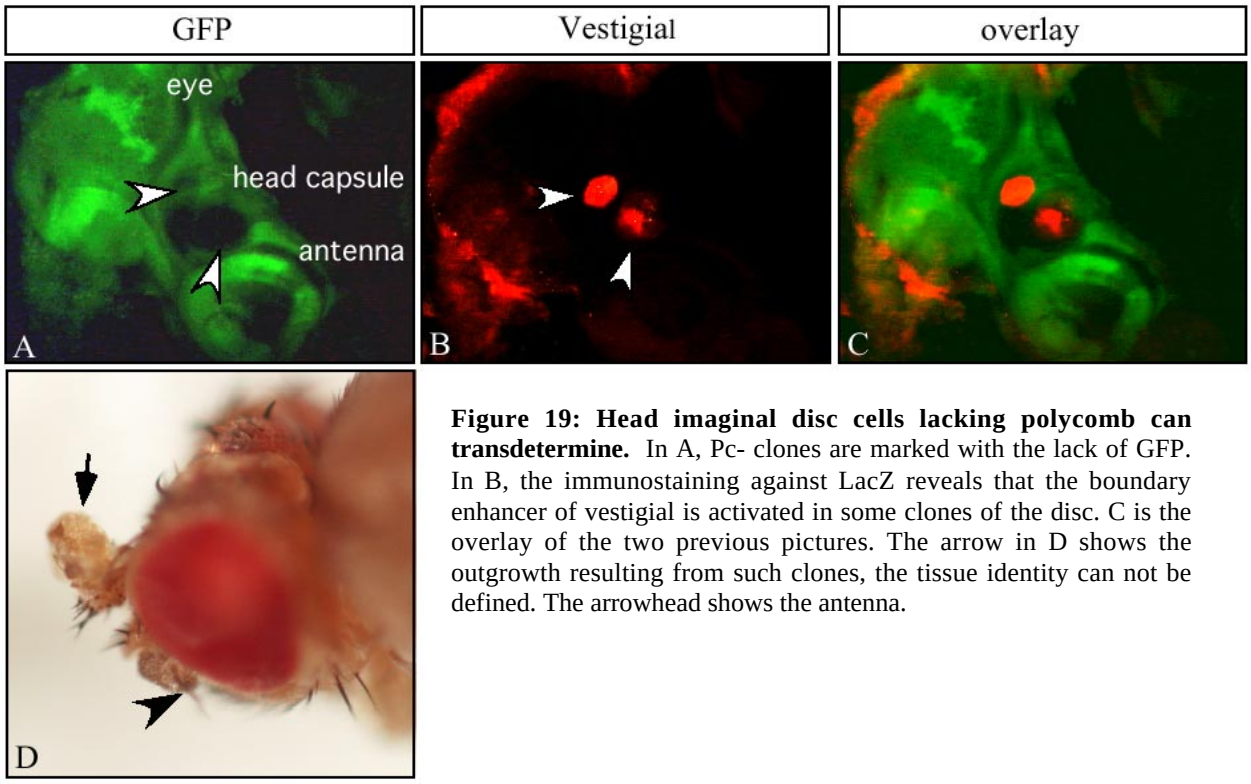



**Figure 19: Head imaginal disc cells lacking polycomb can transdetermine.** In A, Pc- clones are marked with the lack of GFP. In B, the immunostaining against LacZ reveals that the boundary enhancer of vestigial is activated in some clones of the disc. C is the overlay of the two previous pictures. The arrow in D shows the outgrowth resulting from such clones, the tissue identity can not be defined. The arrowhead shows the antenna.

*2.2.2.3 The frequency of transdetermination is affected by the dosage of PC protein in the cell*

Maves and Schubiger (1995) demonstrated that the production, in the leg discs, of ectopic "gain of function" clones expressing *wg*, induces leg-to-wing transdetermination. Interestingly, as in fragmentation experiments, this event always occurs in the same predicted proximal-dorsal region of the leg discs which has been called a "weak point". Thus, it has been postulated that the ectopic *wg* expression experiment may mimic the fragmentation experiments by juxtaposing "ventral" *wg*-expressing cells with dorsal leg disc cells creating new combinations of signaling molecules. In this assay, the cells will transdetermine if the right amount of Wg signaling molecule is produced at the right location, in order to induce a change in the gene expression program leading to the expression of *vestigial*. In the previous paragraphs, experiments show that removing PC from cells allows new gene expression

programs to be opened. It can be assumed that reducing the amount of PC protein in the cells should increase their flexibility in changing their initial gene expression program upon external influences. Therefore, if PC is required for the transcriptional regulation of genes involved in transdetermination, the frequency of transdetermination should vary in cells lacking half of the normal amount of PC protein.

To validate this hypothesis, I investigated the influence of PC in Wg-induced transdetermination by inducing *wg* expressing clones in flies heterozygous for the $Pc^3$ antimorphic mutation. A variant of the FRT/FLP recombination technique allows to produce ectopic clones of cells expressing your gene of interest. By this mean, *wg* was ectopically expressed in imaginal discs during larval stages. The leg-to-wing transdetermination was detected by the examination of *vg* expression in the discs (using the enhancer trap line previously described in which *lacZ* expression is activated by the *vg boundary enhancer*) (Fig. 20). In my hands, in flies wild type for the *Pc* gene, a frequency of transdetermination of 26% (69/270) of the leg discs possessing *wg*-expressing clones was observed. In comparison, this frequency was increased up to 50% (97/194) in discs heterozygous for the $Pc^3$ allele. This result indicates that cells having a smaller amount of PC are more amenable to switch their fate. It can also be concluded that some genes involved in the process of leg-to-wing transdetermination are controlled by PC.

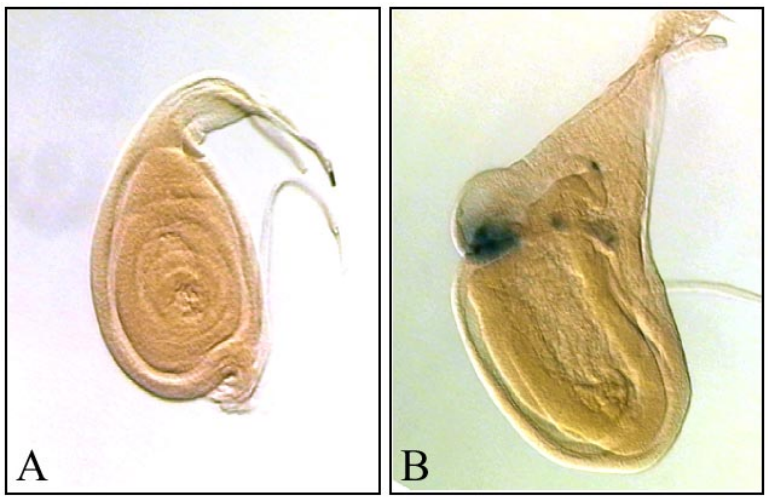



**Figure 20: Induction of ectopic *wg*-expressing clones induces leg-to-wing transdetermination.** In A, a wild type leg imaginal disc is shown. In B, ectopic clones expressing *wingless* have been produced during larval development resulting in cell proliferation and leg-to-wing transdetermination as visualized by the ß-gal staining which represents *vg* expression. Repeating the experiment in disc cells lacking one copy of *Pc* increased the frequency of transdetermination by 2 fold.

*2.2.2.4 PcG proteins may be needed for the maintenance of the transdetermined state through mitosis*

Production of ectopic *wg*-expressing clones in PcG mutant discs allows assessing the role of PcG proteins in the establishment of the new cell identity. However, even if the ectopic expression of *wg* induces some extra-cell divisions, inducing *wg*-expressing clones in the disc during larval stages may not be the right way to study the role of PcG and trxG genes in the maintenance of the transdetermined state through mitosis since the number of cell divisions is restricted in time by the entry in metamorphosis.

With the fragmentation/transplantation experiment, it is possible to cultivate discs for a long period *in vivo* in the abdomen of adult flies where a high number of cell divisions can be accomplished. Therefore, it could be an ideal mean to study how the new transdetermined state is inherited through a high number of cell divisions.

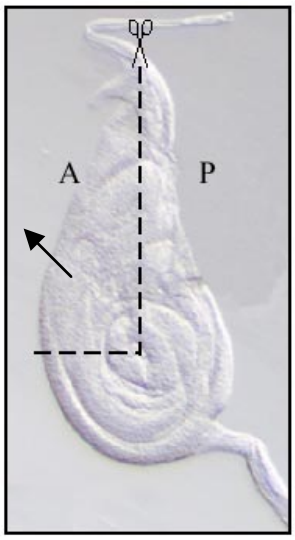
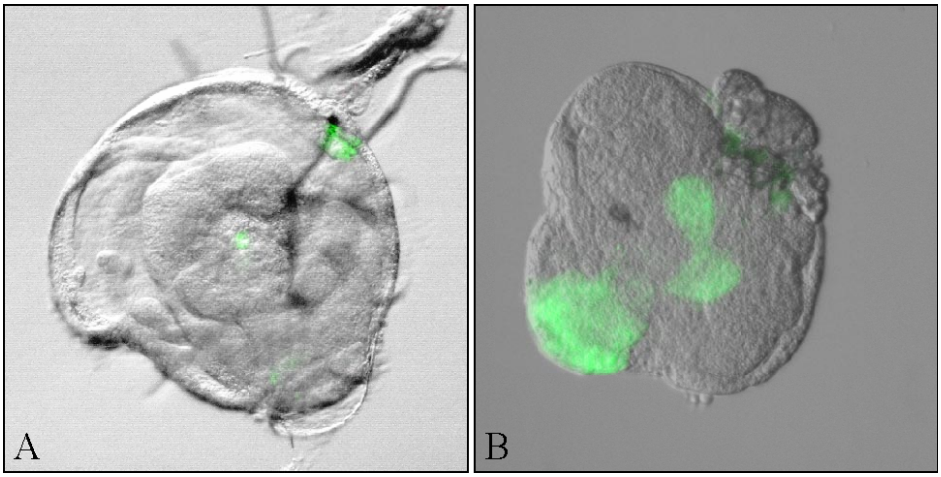



**Figure 21: Fragmentation of first leg imaginal disc.** The picture shows a first leg imaginal disc, anterior to the left and posterior to the right. To induce transdetermination during *in vivo* culture, the upper part of the anterior compartment (A1/4) must be cut out. Then the complementing 3/4 posterior fragment (P3/4) can be transplanted into the abdomen of an adult female fly. The P3/4 fragment of wild type male leg discs was transplanted as well as male the P3/4 fragment of male leg discs hemyzigote for the $ph^{409}$ allele.

For this purpose, I performed *in vivo* cultures of fragmented leg imaginal discs as described by Hadorn (1963) to test whether PcG proteins were involved in the accurate transmission of the transdetermined state through cell divisions. The P3/4 fragment of fragmented male wild type foreleg discs (Fig. 21) were injected into one day old adult females. The same was proceed for discs hemizygous mutant for the $ph^{409}$ hypomorphic allele. After an incubation of 13 days at 25°C, leg-to-wing transdetermination in male foreleg discs was checked by looking at UAS-*GFP* expression under the control of a *vg boundary enhancer*-GAL4 driver. Three different phenotypes were screened: no *GFP* expressed in the disc, discs possessing small domains expressing *GFP* (Fig. 22A), and discs having large domains of *GFP* expression (Fig.

22B). 43 wild type discs and 39 *ph* mutant discs were recovered after *in vivo* culture. Surprisingly, results shows that the frequency of transdetermination slightly decreases in mutant flies compared to wild type. The frequency of discs having large transdetermined domains seem to be also reduced in mutant discs (Fig. 22C). 54% of the wild type discs have a large domain of *vg* expression whereas this number is reduced to 38% is discs mutant for the *ph* gene.

This could mean that a fewer number of founder cells transdetermine in *ph* mutants, or that the new transdetermined cells can not maintain their new fate through mitosis as efficiently than in wild type discs arguing that PcG genes are needed for the accurate transmission of the new transdetermined state to the cell progenies.




C)

| **genotype of the fragmented discs** | **days of incubation** | **Number of discs recovered after *in vivo* culture** | **number of discs with or without *vg* expression** | | |
|---|---|---|---|---|---|
| | | | **no *vg*** | **small domain** | **large domain** |
| **WT** | 13 | 43 | 4 (9%) | 16 (37%) | 23 (54%) |
| **$ph^{409}$** | 13 | 39 | 7 (18%) | 17 (44%) | 15 (38%) |

**Figure 22: Transdetermination in leg discs after fragmentation and *in vivo* culture**. Transdetermination is visualized by the GFP signal which reflects expression of *vestigial.* In A, only small domains show *GFP* expression whereas in B, *GFP* is expressed in larger domains. The table (C) shows the percentages of transdetermination events in wild type discs and discs heterozygous for the $ph^{409}$ mutant allele.

### *2.2.2.5 Expression of some PcG and trxG genes may be downregulated at the wound of fragmented discs*

The last experiments highlight the fact that downregulation of some members of the PcG gene expression favours the opening of new developmental pathways and affects the frequency of transdetermination. Therefore if such an event would exist in a period of time when cell proliferation is required and when cells are exposed to new signaling cascades, one could envisage that cells would be more amenable to change their determination state and possibly transdetermine.

After leg imaginal disc fragmentation, when the 3/4 fragment is cultured *in vivo*, the dorsal and ventral cut edges undergo wound healing. In the adult *Drosophila melanogaster*, the Jun N-terminal kinase (JNK) signal transduction pathway is activated at the edge of wounds in epithelial cells (Ramet et al., 2002). Interestingly, a recent study of the genome-wide transcriptional response to activation of the JNK pathway in the *Drosophila* embryo showed that a number of genes encoding proteins involved in PcG and trxG-mediated mecanisms, like *Rpd3, brahma, polyhomeotic,* and *ASH2,* are down-regulated in cells in which the JNK pathway is activated (Jasper et al., 2001).

I investigated whether the JNK pathway was activated at the wound of fragmented imaginal discs. Activation of the JNK pathway was revealed by assaying the expression of the *puckered* (*puc*) gene, which is known a target of the JUN N-terminal kinase signaling pathway (Martin-Blanco et al., 1998). For this purpose, an enhancer trap line containing the *lacZ* gene inserted into the *puc* locus was used to reflect *puc* expression. After 48h of *in vivo* culture, fragmented disc clearly show expression of the *puc-lacZ* gene at the wound meaning that the JNK pathway is activated in cells at the wound (Fig. 23).

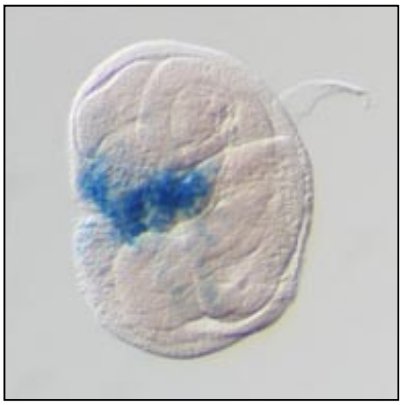


**Figure 23: The JNK pathway is activated at the wound of fragmented imaginal disc during regeneration.** The expression of *puc* (*puckered*) was investigated in fragmented leg imaginal discs cultivated 48 hours in the abdomen of adult Drosophilas. Cells in blue express *puc.*

These cells will later start to proliferate to form a blastema potentially containing transdetermined cells. If some of the PcG and trxG genes are indeed transiently downregulated in these cells due to the activation of the JNK pathway, it would certainly make them more susceptible to the variations of their new environment and therefore these cells could be preferential potential targets for transdetermination events.

### 2.2.3 Discussion

*2.2.3.1 Pc-mediated repression ensures that the cell is competent to follow specific developmental programs*

The lack of functional PC alters repression mechanisms, leading to aberrant gene expression programs being activated and change in determination. One striking observation resulting from the lack of PC in clones of cells is that the activation of developmental genes is not random at all and depends on the region were the clone is produced even for the genes known to be directly regulated by PcG proteins like *en*. For example, *en* was not systematically activated in the clones produced in the wing. Likewise, for the homeotic gene *Ubx*, regions in the wing were present where it was not systematically derepressed. However there seems to be a global change in the gene expression program of each clones since they all show a rounded morphology, very different from their twin clone (having two copies of GFP), perhaps reflecting their need to minimize contact with the surrounding wild type cells. The progeny of cells in which a functional *PC* protein has been eliminated do not remember their initial fate and change their developmental gene expression program. In fact, in the different regions of the discs, the clones seem to activate different transcription programs which is very likely due to the specificity of the environment. Lacking the repression mecanism induced by PC, the cells may start to react more freely to the instructions provided by the cellular environment and new developmental programs may thus be activated depending on the combination of the surrounding signaling molecules. This situation may then lead to aberrant gene expression patterns, such as *en* being expressed in anterior compartments or *vg* being expressed in head imaginal disc cells.

In a new bid to define cell determination in molecular terms, it has been linked to the distribution of superrepressed genes (Zuckerkandl, 1997; 1999). In this sense, it is possible to say that the Polycomb-mediated repression pattern amounts to the limitation of the cell to a certain subset of fates that remain potentially accessible to it or to its progeny. In cells advancing toward terminal differentiation, the Pc-mediated repression pattern would be responsible for the progressive narrowing of the range of developmental pathways that could potentially still be activated for later development. In this way, the repression pattern conferred by PC and the PcG protein distribution on chromatin renders the cells competent to be determined in specific directions by allowing them to switch on the requested developmental program only.

On the other hand, the trxG protein distribution pattern on chromatin defines for a cell which developmental genes must be transcribed (or competent for transcription) in the cell progenies even in the absence of the transactivator.

*2.2.3.2 Some genes involved in leg-to-wing transdetermination events are regulated by PcG genes*

As a molecular marker for leg-to-wing transdetermination, *vg* expression was assessed by the help of a *lacZ* gene inserted into the *vg boundary enhancer*. This *boundary enhancer* normally drives *vg* expression at the dorso-ventral boundary of the wing disc. Therefore in this line, *lacZ* expression is restricted to the dorso-ventral boundary. This makes *vg* unlikely to be regulated by a CMM because otherwise *lacZ* expression would be maintained in the wing pouch cells through cell divisions (see Part 1). Therefore, *vg* expression seems not to be directly influenced by PcG genes. In this respect, it represents a good general marker to follow how PcG/trxG genes can influence the state of determination of a cell.

I could demonstrate that at least some PcG genes are involved in the process of transdetermination since the frequency of transdetermination is affected by the dosage of PC and PH in the cells. One assay used molecular tools to skip the "fragmentation/transplantation" procedure and induce ectopic *wg*-expressing domains in the leg disc that may be juxtaposed with high concentration of the Dpp morphogen possibly resulting in a leg-to-wing transdetermination event. This experiment allows studying the process of identity switching. The frequency of transdetermination is doubled in our hands in leg discs from flies having only half on the normal amount of PC. This result suggests that a lower concentration of PC in the cells render them more susceptible to switch their developmental pathway and transdetermine.

Another strategy consisted in comparing the transdetermination frequencies in wild type leg discs versus *ph* mutant discs by using the "fragmentation/transplantation" experiment. In this case, variations in the frequency of transdetermination were not very striking and rather decreased in the mutant discs.

Unfortunately, for technical reasons, the same mutated PcG gene was not used for the two experiments and therefore this makes a comparison difficult to interpret. PC and PH may for example have different target genes. It is clear that ideally the fragmentation/transplantation experiment should be repeated with discs mutant for the same *Pc* allele that was used in the experiment that uses ectopic *wg*-expressing clones.

However, it may be difficult to compare the effect of such PcG mutants between the two experiments. In one strategy, the fragmentation and wounding steps were skipped and replaced by directly promoting ectopic *wg* expression which is normally a consequence of the fragmentation itself (Gibson and Schubiger, 1999). The establishment of the new *wg*-expressing domain after fragmentation and *in vivo* culture represents a change in the gene expression program of the cells and could itself be influenced by PcG genes. In the fragmentation/transplantation experiment, PcG proteins could have an influence in the two steps leading to transdetermination (Fig. 24). Therefore, if a mutation in a PcG group interferes with the establishment of the *wg*-expressing domain, *vg* may not be expressed and the frequency of transdetermination will decrease.

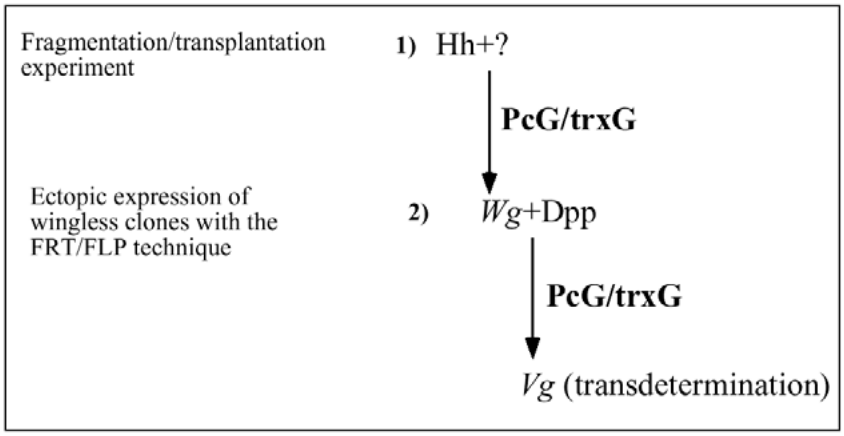



**Figure 24: PcG/trxG proteins may influence transdetermination events in a two step process.** In the fragmentation/transplantation experiment, Hh secretion emmanating from peripodial cells induces a *wg*-expressing domain in cells at the wound. The establishment of this new expression program may be influenced by PcG/trxG proteins. In a second step, the new Wg signaling from cells at the wound is combined with Dpp signalling from cells at the A-P boundary, inducing *vg* expression which is also influenced by PcG/trxG proteins as we could show. Therefore, the fragmentation/transplantation experiment establishes transdetermination in a two step process both probably influenced by PcG/trxG genes. However, ectopic expression of *wg*-expressing clones using the FRT/FLP technique skips the first step.

One gene known to be regulated by PcG/trxG genes via a PRE/TRE (Gindhart and Kaufman, 1995) and that may be downregulated during leg-to-wing transdetermination is the homeotic gene *Sex comb reduced* (*Scr*). *Scr* is expressed in larvae in legs, antennas and thorax. It is required for tarsus determination in leg and antenna discs (Percival-Smith et al., 1997). To allow the leg-to-wing transdetermination event, one can predict that *Scr* should be turned off in the first leg. In addition, the leg imaginal disc 3/4 fragment that have undergone regeneration after *in vivo* culture show expression of *wg* in a region at the wound after a new posterior compartment has been duplicated resulting from the secretion of Hh from the

peripodial cells (Gibson and Schubiger, 1999). This new posterior compartment expresses *en* and most probably *hh*. Both these genes are under the control of PcG genes. Therefore these genes represent targets that may affect the frequency of transdetermination in the mutant discs lacking one functional copy of *ph* or *Pc*.

#### *2.2.3.3 The fragmentation/transplantation technique can be used to study the role of CMMs in the mitotic inheritance of the transdetermined state*

One advantage of the fragmentation/transplantation experiment is that it is possible to cultivate discs *in vivo* as long as desired (the disc can also be retransplanted to a new host later on if necessary). By this way, a high number of cell divisions can be induced. This could be very useful to study the mechanisms responsible for the maintenance of the transdetermined state through cell divisions.

Interestingly, when the "fragmentation/transplantation" experiment was performed, I could observed that wild type discs tend to have larger domains of transdetermined cells than mutant discs. Although the *ph* mutation may reduce the rate of cell proliferation, which is unlikely because *ph* mutant discs in larvae do not have proliferation defects, this result could suggest that mutations in the *ph* gene may impair the stable inheritance of the new transdetermined state through cell divisions. This could indicate that a stable PcG-mediated repression pattern has to be established and transmitted to the progenies in order to allow a proper inheritance of the transdetermined state. In other words, PcG proteins would be necessary to maintain the new cell identity by providing a stable repression of other developmental pathways that could interfere.

Obviously more experiments must be performed to confirm the role PcG and trxG genes in the inheritance of the transdetermined state. It can be assumed that trxG proteins are responsible for the maintenance of active transcriptional states at CMMs through mitosis allowing the newly open developmental pathways to be inherited, whereas PcG proteins ensure that other developmental pathways are kept repressed. One way to investigate if the leg-to-wing switch of identity is clonally inheritable in imaginal disc cells, and if this inheritance depends on trxG proteins, would be to produce, at the moment of fragmentation, clones of cells genetically marked with GFP, using the FRT/FLP technique. After transplantation and *in vivo* culture of the fragmented imaginal leg disc, large *GFP*-expressing clones should be observed at the blastema. An immunostaining against Vg should tell whether

cells of these clones have transdetermined, and how the new identity is clonally inherited, in a wild type background, and in a background containing mutations in the trxG or PcG genes.

*2.2.3.4 Defining conditions needed to induce a change in the determination state*

Hadorn (1966; 1978) proposed the Dilution-Equilibrium theory as an explanation of how transdetermination might arise. This theory is based on the role of proliferation in transdetermination although proliferation by itself is not sufficient to induce transdetermination (Shearn et al., 1984). The theory posits that factors controlling determination are present in different equilibria in different cell types and that such factors normally become diluted and resynthesized with each cell cycle. Changes in proliferation rate could disrupt an equilibrium of determination factors such that a new equilibrium, which would represent a new determined state might be reached. I indeed could show that reducing the amount of PC or PH in the cells modifies the frequency of transdetermination. It is possible that in highly proliferating cells forming the blastema at the wound, epigenetic factors like the PcG and trxG proteins might be transiently diluted. Rather than defining new specific determined state, this dilution may render the CMMs instable and therefore more "easily" switchable upon strong transactivator pulses following the creation of new morphogen gradiant combinations (*wg* expressed at the wound in a region where Dpp is in high concentration). Furthermore, cells at the wound, which will later undergo an extensive proliferation period, may have been subject to a consistent transient downregulation of some chromatin proteins (trxG and PcG proteins as well as the histone-deacetylase Rpd3) consequently to the activation of the JNK pathway. It is certainly important that downregulation of chromatin factors is only transient in order to facilitate CMM switching, but later on normal concentrations should be restored for proper inheritance of the transdetermined state.

A high rate of cell proliferation may help in switching the epigenetic mark on the DNA. It appeared in the last months that the state of histone methylation, conferred by E(z) for PcG-mediated repression (Czermin et al., 2002; Müller et al., 2002; Cao et al., 2002; Kuzmichev et al., 2002) and ASH1 for trx-mediated activation (Beisel et al., 2002), may provide the epigenetic mark needed to maintain the initial CMM activation state through mitosis. Since no histone-demethylase could be found so far despite extensive efforts (Jenuwein and Allis, 2001), it is assumed that there is no active histone demethylation process in the cell which actually make histone methylation a good candidate for being a stable epigenetic imprint. The

histone methylation state at CMMs is certainly maintained through DNA methylation by the binding of the methylase-complex to the previously methylated nucleosomes, semi-conservatively distributed after DNA replication, allowing newly deposed nucleosomes to be in turn methylated. However, if the Pc/Trx-complex containing a methylase is destabilized by downregulation of some of its components, and prevented for several rounds of cell divisions from binding to the methylated histones at the CMMs because of the presence at the promoter of new counteracting transcription factors, one could envisage that the epigenetic mark could be progressively lost after a few rounds of cell divisions, and a new one may be established. In this way, CMMs could be progressively switched to a new stable state of activation. Interestingly, the switch in the *hh* CMM activation state we observed in the wing pouch cells (see Part I of the thesis) after a transient expression of *engrailed* takes place at a moment in which these cells undergo an extensive proliferation period ($2^{nd}$ and $3^{rd}$ instar larvae).

Therefore, changes of the signaling molecule combination in the environment, a high rate of cell proliferation, and a transient downregulation of proteins involved in PcG/trxG-mediated mecanisms may be conditions necessary to induce switches of CMMs potentially leading to inheritable transdetermination events in organisms.

# III. Overall discussion and perspectives.

In this thesis I have shown that the state of determination of a cell, defined by a specific gene expression program, although being clonally inheritable is not fixed once and for all. Under certain conditions, cells can actually switch to a new identity, to a new gene expression program, and maintain it again through mitosis. I could demonstrate, that the *hh* gene is regulated by epigenetic mechanisms allowing inheritance of the initial transcriptional state through cell divisions. However, this transcriptional state can apparently, by a still mostly unknown mechanism, be switched during development to a new mitotically inheritable state leading to a stable change in the identity of the cell. PcG and trxG proteins are involved in this phenomenon through their interplay at CMMs.

Removing PC from the cells during imaginal disc development can induce transdetermination-like events, and diluting the concentration of PC in the cells facilitates transdetermination. Therefore, the change of cell identity observed in transdetermination events may be explained by a switch in the CMMs of some genes, leading to a new, mitotically inheritable, gene expression program in some cells. The maintenance of the new cell identity would be processed by trxG and PcG genes. Several conditions favour or are necessary for transdetermination: i) A new environment providing new signaling molecules establishes a new gene expression pattern (Maves and Schubiger, 1995; 1998; Johnston and Schubiger, 1996; Gibson and Schubiger, 1999). ii) Extensive cell proliferation appears to be necessary (Hadorn, 1966), and it could contribute to a transient dilution of the concentration of PcG and trxG-proteins in the cells, which favours the switch of developmental pathways by destabilizing CMMs. Maybe even more importantly, DNA replication may help in changing the epigenetic mark by a progressive dilution of modified histones. iii) Activation of the JNK pathway at the wound of fragmented imaginal discs or vertebrate tissue may help CMMs switching by transiently downregulating expression of proteins involved in PcG and trxG-mediated mechanisms. However, it is still necessary to confirm by Western blot or RT-PCR the fact that genes like the histone-deacetylase *Rpd3*, or the trxG gene *brahma* and the PcG gene *polyhomeotic* are indeed downregulated as reported by the recent study on the genome-wide transcriptional response to activation of the JNK pathway in the *Drosophila* embryo (Jasper *et al.*, 2001).

It remains to be determined whether possible switches in cell identity have any relevance for development in normal physiological conditions. It must necessarily be under a strict control, however it is becoming increasingly clear that the programming of determined state may not be totally hard-wired. How often can a cell change its gene expression program during development in order to switch to a new identity? When could it be used? Further experiments

must be conducted to decipher more precisely this the mechanism by which CMMs are switched, and for instance look more carefully at the role of histone-methylation.
In addition, it remains to determine more clearly how PcG and trxG are able, through their interplays at CMMs, to maintain the new transdetermined state through many cell divisions. This will give insights of how a specific state of determination is maintained or eventually switched to a new one during development.

One exciting consequence of controlling cell determination and transdetermination may lie in possible medical applications deriving from tissue remodelling studies.
In mammals, stem cell function is to maintain the cellular homeostasis of tissues and regenerate cells after injury. These stem cells are found in a variety of tissues like in the developing and adult brain, liver, intestine and hematopoietic system. They were initially believed to be restricted in their potential and limited to generate the types of cells present in the tissue. However, in recent years, it has became increasingly clear that in mammal adult tissues, a variety of stem cells are, under specific signals, able to divide and change their fate into a remarkably wide range of identities. For example, some striking results have shown that hematopoiesis in irradiated mice could be reconstituted after intraveneous injection of neural stem cells. Likewise, generation of liver cells, myocytes and neurons could be observed deriving from mouse bone marrow cells (Ferrari et al., 1998; Bjornson et al., 1999; Lagasse et al., 2000; Brazelton et al., 2000; Mezey et al., 2000; Krause et al., 2001). Interestingly, studies realised in humans having received a transplant demonstrated that some stem cells deriving from the blood and bone marrow grafted cells could generate hepatocytes, suggesting that stem cells in humans also show some plasticity (Alison et al., 2000; Theise et al., 2000).
The production of heterokaryons, resulting of the fusion of the nuclei of two cells from different types, may also induce a change of identity of the cells. For example, after the fusion of mouse muscle cells containing the factor MyoD with human fibroblasts, human muscle proteins were produced in the heterokaryon and its progeny (Pavlath and Blau, 1986). It suggests that a new expression program is established and influenced by the epigenetic signals in the cytoplasm of the new partner. Even more strikingly, recent studies reported that in cocultures of embryonic stem cells with brain or bone marrow cells, pluripotent hybrid cells emerged spontaneously, suggesting that stem cell plasticity could result from cell fusion (Terada et al., 2002; Ying et al. 2002). Even if it is hard to believe that tetraploid heterokaryons could be at the origin of regenerated tissues, these experiments suggest however that a change in the intracellular concentration of epigenetic factors could influence

the capacity of a determined cell to switch to another fate. Therefore, the ability of mammalian stem cells or progenitor cells to dramatically change their fate and produce completely new types of cells may reflect their capacity to answer to a new environment and open new developmental pathways. In addition their plasticity may be favoured by conditions influencing the intracellular concentration of epigenetic factors.

One could envisage in the future to be able to control the conditions that would allow some cells to easily switch their fate to a new defined one. By using specific signaling molecules, inducing cell proliferation, and transiently downregulating or inhibiting a number of proteins involved in PcG/trxG-mediated mechanisms, one may be able to switch the determination of some cells to a new desired direction. This could be extremely useful for therapeutic applications.

# IV. Materials and Methods.

### 4.1 Strains and handling

The following stocks were obtained from Bloomington, Indiana, USA, unless otherwise noted. Stocks were raised on standard fly food (10 L water, 80 g agar, 180 g dry yeast, 100 g soya-flour, 220 g honey, 800 g cornmeal, 24 g nipagin (methyl-4-hydroxybensoate; Merck), 62.5 ml propionic acid (Sigma)). Flies were maintained on standard culture medium at 18°C, except when stated otherwise with 60-70% relative humidity. Embryos of the strain $w^{1118}$ were used as a host for generating the transgenic lines.

In a modified version of the GAL47-1 (Brand et al., 1994), the hsGAL4 construct is inserted into the CyO chromosomes and the mini-*white* marker gene was mutated with EMS. This allows the hs-*GAL4* driver to be followed during crossings (gift from M. Prestel and R. Paro).

$ph^{409}$ is an hypomorphic viable mutation, $Pc^{3}$ is considered to be a strong antimorph mutant. $trx^{E2}$ and $brm^{2}$ are two amorphic mutations, recombined on the same chromosome. The vg-*GAL4* line expresses GAL4 in a thin stripe at the dorso-ventral boundary of wing imaginal discs (Simmonds et al., 1995). The hs-*GAL4* line is able to produce high amount of GAL4 protein upon heatshock. However, at 25°C it is known to be leaky in salivary glands, as low amount of GAL4 are produced. The EP3521 line, termed here EP-*hh*, (Rørth et al., 1998) possesses an EP element inserted upstream of the *hh* gene. Upon GAL4 induction a functional Hh protein is expressed (Rørth et al., 1998). The FLW-1 line possesses the *Fab7*-CMM controlling expression of the reporter genes *lacZ* and mini-*white* (Cavalli and Paro, 1998). UAS-*en* (Guillen et al., 1995; Tabata et al., 1995), UAS-*hh* (Fietz et al 1995), and UAS-*GFP* are lines able to express functional En, Hh and GFP proteins respectively upon a GAL4 pulse. The *hh-lacZ* line P30 (Lee et al., 1992) in which *lacZ* expression reflects expression of the endogenous *hh* gene was used for immunostainings. An enhancer-trap line with *lacZ* reflecting *puc* expression pattern (Martin-Blanco et al., 1998), and enhancer-trap line with *lacZ* reflecting *vg* expression at the dorso-ventral boundary were used.

For the heat-shock experiments, in order to produce a short pulse of GAL4 protein in the embryos, flies were allowed to lay overnight on apple juice agar plates at 21°C and embryos (4 to 16 hours old) were heat shocked at 37°C in a water bath for 55 minutes. 2nd instar larvae were heat shocked in small vials incubated in a water bath at 37°C for 1 hour.

| **Fly stocks** | **Obtained from** |
|---|---|
| Oregon R | R. Paro |
| $w^{1118}$ | R. Paro |
| *w* hs-*FLP*; Dr/TM3 | |

| | |
|---|---|
| *w Pc*$^{3}$ / TM3 | R. Paro |
| *w Pc*$^{XT109}$ / TM3 | R. Paro |
| *w ph*$^{401}$ | N. Randsholt |
| *w ph*$^{409}$ | N. Randsholt |
| *w; trx*$^{E2}$, *brm*$^{2}$ / TM6 | R. Paro |
| *w*, UAS-*en ftz.lacZ* | P. Lawrence |
| *w* hs-*FLP*; UAS-*hh* (III) | |
| *ry; hh[P30]* (*hh-lacZ*) (enhancer trap) | |
| vg boundary enhancer-*lacZ* (II) (enhancer trap) | S. Cohen |
| pucE69-*lacZ* (enhancer trap) | D. Bohman |
| *w*; Ubi-*GFP* FRT*w*$^{+}$ / TM3 (Insertion sites: 61E-F; 79D-F) | |
| *w*; FRT*w*$^{+}$ (Insertion site 79D-F) | |
| UAS-*GFP* (II) | R. Paro |
| FLW-I | R. Paro |
| EP3521 / TM3 (EP*hh*) | |
| hs-*GAL4* (II) | Brandt |
| *w*; hs-*GAL4* Cyo / Sp | M. Prestle and R. Paro |
| w; Act>*y*$^{+}$>*wingless* | K. Basler |

## 4.2 Antibodies

| Antigen | Source | Obtained from | Dilution |
|---|---|---|---|
| engrailed | mouse | P. Lawrence | 1/300 (immunostaining) |
| Ultrabithorax | mouse | R. White | 1/20 (immunostaining) |
| wingless | mouse | S. Cohen | 1/10 (immunostaining) |
| ß-Galactosidase | mouse | Roche | (immunostaining) |
| ß-Galactosidase | rabbit | Abcam | 1/1000 (immunostaining) |
| ß-Galactosidase | mouse | Sigma | 1/1000 (Western) |
| Polycomb | rabbit | B. Koch (Paro) | 1/100 (polytenes) |
| polyhomeotic | rabbit | H. Strutt (Paro) | 1/500 (polytenes) |
| DIG-AP | | Roche | 1/2000 (in situ) |
| α-tubulin | mouse | Sigma | 1/1200 (Western) |
| α-rabbit Cy3 | goat | Dianova | 1/800 (immunostaining) |
| α-mouse Cy3 | goat | Dianova | 1/800 (immunostaining) |
| α-rabbit Alexa 488 | donkey | Molecular Probes | 1/100 (immunostaining) |
| α-mouse HRP | goat | Amersham Life Sciences | 1/2000 (Western) |

## 4.3 Primers

| Name | Description | Sequence |
|---|---|---|
| Pair1LP1036 | hedgehog genomic region. Was used to amplify DNA fragments for the dot blot. | GGATCACCTCCATCTCC ATCTCCACATC |
| Pair1UP28 | ion. Was used to amplify DNA fragments for the | ATCCCAATCCCTGGTA GCCGTAAATGTC |
| Pair2LP2028 | hedgehog genomic region. Was used to amplify DNA fragments for the dot blot. | TCGAGTGTGTGTGCGA GAGTCTATGTGG |

| | | |
|---|---|---|
| Pair2UP1036 | hedgehog genomic region. Was used to amplify DNA fragments for the dot blot. | GATGTGGAGATGGAGA TGGAGGTGATCC |
| Pair3LP2973 | hedgehog genomic region. Was used to amplify DNA fragments for the dot blot. | TACATATGGGTTCAAT GCTGCTTCCGTT |
| Pair3UP2028 | hedgehog genomic region. Was used to amplify DNA fragments for the dot blot. | CCACATAGACTCTCGC ACACACACTCGA |
| Pair4LP3943 | hedgehog genomic region. Was used to amplify DNA fragments for the dot blot. | ACTTCACTTTTGGCACA CAGACACGCTT |
| Pair4UP2973 | hedgehog genomic region. Was used to amplify DNA fragments for the dot blot. | AACGGAAGCAGCATTG AACCCATATGTA |
| Pair5LP5075 | hedgehog genomic region. Was used to amplify DNA fragments for the dot blot. | GAGCGATAAGCGATCG CTAATTTGACAA |
| Pair5UP3943 | hedgehog genomic region. Was used to amplify DNA fragments for the dot blot. | AAGCGTGTCTGTGTGC CAAAAGTGAAGT |
| Pair6LP5988 | hedgehog genomic region. Was used to amplify DNA fragments for the dot blot. | AGCACACATTTTGTTCT GGCACCGATAT |
| Pair6UP5075 | hedgehog genomic region. Was used to amplify DNA fragments for the dot blot. | TTGTCAAATTAGCGAT CGCTTATCGCTC |
| Pair7LP7017 | hedgehog genomic region. Was used to amplify DNA fragments for the dot blot. | GCAGGAATGGCAAAAG ATTTCAATGTCA |
| Pair7UP5988 | hedgehog genomic region. Was used to amplify DNA fragments for the dot blot. | ATATCGGTGCCAGAAC AAAATGTGTGCT |
| Pair8LP7989 | hedgehog genomic region. Was used to amplify DNA fragments for the dot blot. | TAAGTTTGATTACTTTG TTCGCCGCAGG |
| Pair8UP7017 | hedgehog genomic region. Was used to amplify DNA fragments for the dot blot. | TGACATTGAAATCTTTT GCCATTCCTGC |
| Pair9LP9024 | hedgehog genomic region. Was used to amplify DNA fragments for the dot blot. | GATTAAGGGTGCTGTA TCGCGGCTAATT |
| Pair9UP7989 | hedgehog genomic region. Was used to amplify DNA fragments for the dot blot. | CCTGCGGCGAACAAAG TAATCAAACTTA |
| Pair10LP10017 | hedgehog genomic region. Was used to amplify DNA fragments for the dot blot. | ATGTACCATCTCCCATC TGTGGGCTTTT |
| Pair10UP9024 | hedgehog genomic region. Was used to amplify DNA fragments for the dot blot. | AATTAGCCGCGATACA GCACCCTTAATC |
| Pair11LP11004 | hedgehog genomic region. Was used to amplify DNA fragments for the dot blot. | AGAGGATCGCTGCCAA CGAGTGTGTATA |
| Pair11UP10017 | hedgehog genomic region. Was used to amplify DNA fragments for the dot blot. | AAAAGCCCACAGATGG GAGATGGTACAT |
| Pair12LP11929 | hedgehog genomic region. Was used to amplify DNA fragments for the dot blot. | GAGTGCTTCTACCTCTT TGCGCTCTGTG |
| Pair12UP11004 | hedgehog genomic region. Was used to amplify DNA fragments for the dot blot. | TATACACACTCGTTGG CAGCGATCCTCT |
| Pair13LP13024 | hedgehog genomic region. Was used to amplify DNA fragments for the dot blot. | GTCGTGTCTTTTAACTG GCACTGGCACT |
| Pair13UP11929 | hedgehog genomic region. Was used to amplify DNA fragments for the dot blot. | CACAGAGCGCAAAGAG GTAGAAGCACTC |
| Pair14LP14016 | hedgehog genomic region. Was used to amplify DNA fragments for the dot blot. | GATATTGCCCAAGGAG ACCAAGTTGGAG |
| Pair14UP13024 | hedgehog genomic region. Was used to amplify DNA fragments for the dot blot. | AGTGCCAGTGCCAGTT AAAAGACACGAC |
| Pair15LP15014 | hedgehog genomic region. Was used to amplify DNA fragments for the dot blot. | TAAAACCCATAATCGA TGTGGATGGACG |
| Pair15UP14016 | hedgehog genomic region. Was used to amplify DNA fragments for the dot blot. | CTCCAACTTGGTCTCCT TGGGCAATATC |
| Pair16LP15913 | hedgehog genomic region. Was used to amplify DNA fragments for the dot blot. | TCTAATTTGGCGTTAAG CATCCAATTGC |
| Pair16UP15014 | hedgehog genomic region. Was used to amplify DNA fragments for the dot blot. | CGTCCATCCACATCGA TTATGGGTTTTA |
| Pair17LP17543 | hedgehog genomic region. Was used to amplify | TGCATTTTAGTATTTCA |

| | | |
|---|---|---|
| | DNA fragments for the dot blot. | AGGCTGGCTGC |
| Pair17LP15913 | hedgehog genomic region. Was used to amplify DNA fragments for the dot blot. | GCAATTGGATGCTTAA CGCCAAATTAGA |
| UphhPRE.UP243.T3.Not1 | hedgehog upstream region + not1 site+ T3 promoter (can be used to make RNA probe). Was used to clone the hh CMM in pUZ | AATTAACCCTCACTAA AGGGAGAgcggccgcCGT TTTTAGTTTGCTGCCTG CATT |
| UphhPRELP1.3735.T7. Spe1: | hedgehog upstream region + Spe1 site+ T7 promoter (can be used to make RNA probe). Was used to clone the hh CMM in pUZ | TAATACGACTCACTAT AGGGAGActagtAATGAC ATTTCCGAGCGGAGTA TC |
| UphhPRELP2.3580.T7. Spe1: | hedgehog upstream region + Spe1 site+ T7 promoter (can be used to make RNA probe). Was used to clone the hh CMM in pUZ | TAATACGACTCACTAT AGGGAGactagtACACTA TCGCCTCGAGTTCATTC C |
| 5'CI | Sequence in the *Cubitus Interruptus* gene (can be used to make RNA probe) | ATGGAGTTCGAGCATC TGAC |
| 3'CIT7 | Sequence in the *Cubitus Interruptus* gene (can be used to make RNA probe). The T7 promoter sequence allows to make antisens RNA probe for in situs | taatacgactcactatagggAGAG CTGCTAACATCGGGA |

### 4.4 DNA Vectors and cloning strategy

*Preparation of the genomic stretch containing the hedgehog gene region*

A genomic stretch containing the hh gene region from Drosophila Melanogater could be obtained from BACPAC (BAC RPCI 98-3N12) (Genbank number: AC008365). It contains a genomic insert of about 160 kbp. To shorten this fragment to the region of interest, 8mg of the BAC DNA has been digested overnight with 6 U of Fse I (New England Biolab) at 37°C in 50 ml. After phenol extraction, the digested DNA was resuspended in 30 ml dd$H_2O$ and ligated in a total volume of 300 ml overnight at room temperature. Electro MAX DH10B competent cells (Gibco) were transformed. From this procedure, a BAC was recovered with an insert of only about 45 kb containing the hedgehog gene as well as 30 kb of upstream sequence. This 53kb long BAC was used as genomic DNA to which immunoprecipitated DNA could be hybridized for a southern blot.

*Construction of the transgenes*

The 3,4 kb fragment upstream of the transcription start site was amplified by PCR (Table 4) using the Expand High Fidelity PCR System from Roche which contains a thermostable Taq DNA polymerase and a proofreading polymerase.
aattaaccctcactaaagggagagcggccgcCGTTTTTAGTTTGCTGCCTGCATT was used as upper primer, and taatacgactcactatagggagactagtACACTATCGCCTCGAGTTCATTCC as lower primer (the capital letters denote the sequence homologous to the genomic hh upstream region). For the 3,6 kb fragment the same cloning strategy was followed but the following

primer was used instead as lower primer: taatacgactcactatagggagactagtAATGACATTTCCGAGCGGAGTATC.

| | |
|---|---|
| 1 x | denature template 2min at 94°C |
| 15 x | denaturation at 94°C for 20 s<br>annealing from 70 to 55°C (decrease from 1°C every new cycle) for 30 s<br>elongation at 72°C for 3.30 min |
| 20x | denaturation at 94°C for 20 s<br>annealing at 55°C<br>elongation at 72°C for 3.30 min + add 20 s each new cycle |
| 1 x | prolonged elongation 7 min at 72°C |

**Table 4: PCR cycle scheme for the amplification of the upstream region of *hh*.**

Thereby, new restriction sites were created at both ends. The PCR product was digested with the *Not1* and *Spe1* restriction enzymes and the resulting fragment was cloned via the *Not1* and *Spe1* sites into the pUZ vector (Lyko et al. 1997).

### 4.5 Transformation of $w^{1118}$ with pUAST constructs

The appropriate pUZ constructs (300 ng/ml, described in "DNA Constructs") were coinjected into $w^{1118}$ embryos with the helper DNA pUChspD2-3 (100 ng/ml). Embryos were recovered from 30 minutes egg lays at 25 ºC. The embryos were transferred to 18 ºC, were dechorionised with a 1:5 dilution of bleach (14%)-water solution, and then were washed extensively with water until bleach odor was no longer detectable. Around 70-100 embryos were lined on 16% agar plates and were transferred onto a double-sided sticky tape on a coverslip. The embryos were dehydrated in a closed chamber containing Silica gel for 7 minutes and then covered with Voltalef 10 S oil (Lehmann & Voss & Co.). Microinjection of the pUAST/helper DNA solution into embryos was performed using the Eppendorf FemtoJet microinjector. Larvae from injected embryos were collected into standard fly food and allowed to develop. Eclosed flies were crossed to $w^{1118}$ virgins or males; progenies were then scored for pigmented eyes. At least 5 different independent transformants were kept as stocks and the chromosome in which the construct was inserted was defined by established genetic methods.

### 4.6 In situ hybridisation of a DNA probe on polytene chromosomes from salivary glands

*Preparation of the polytene chromosomes*

Wandering 3rd instar larvae grown at 18°C on rich medium supplemented with yeast were dissected in PBS/ 0,1% TritonX-100 and salivary glands were transferred in 30ml 45% acetic acid on a 22x22 mm coverslip for 3-5 min for fixation. The coverslip was then picked up with a poly-L-Lysin coated slide and tapped a few times with the tip of a pencil. If the spreading was sufficient, as determined by inspection under the microscope, the slide was turned over onto blotting paper and pressed heavily with the thumb. After freezing in liquid nitrogen, the coverslip was flipped off with a razor blade. The slides were incubated immediately in ethanol:acetic acid (3:1) for 3-5 min, dehydrated 10 min in 100% ethanol and air-dried. The slides can be used immediately after air-drying, but aged preparations (at least one week at room temperature) give better results.

*Labelling the probe*

The whole pUZ vector plus the 3,4 kb insert were used as template DNA. The vector was digested with BamH1 in order to produce DNA fragments smaller than 5kb long. The labelling was performed following the DIG-High Prime labelling kit (Roche) protocol. The labelling reaction was performed overnight. After the reaction, the DIG-labelled DNA was precipitated and the pellet washed. The pellet is resuspended in 50ml hybridisation buffer.

*Hybridization*

10 ml of this solution was applied to one chromosome preparation, mounted with a coverslip and sealed with rubber cement. The probe and the chromosomal DNA were denatured simultaneously by incubating the slide 3 min in a metalbox warmed at 70°C in a waterbath. Hybridization was performed overnight at 37°C in a humid chamber.

*Detection of the signal*

After hybridisation, coverslips were removed, and the slides were washed twice for 30 min in 2x SSC at 53°C. For blocking, the slides were incubated for 45 min in TNM-A. Then the slides were incubated for 1 hour at room temperature with the anti-DIG-AP in PBS and afterwards washed 3 times for 5 min in PBS/0,1% Triton X-100. Then the slides were stained with the detection solution containing a mix of NBT/BCIP in AP-buffer. The reaction was followed under the microscope and stopped by washing the slides with PBS. Chromosomes were then mounted with a coverslip for microscopy.

*Solutions*

Hybridization-solution: 50% Formamide; 2x SSC; 10% Dextransulfate, 100mg/ml sheared salmon sperm.

TNM-A: 100 mM Tris-HCl pH 7,5; 100 mM NaCl; 2mM $MgCl_2$; 0,05% Triton X-100; 3% BSA

AP-buffer: 20mM Tris/HCl; 100mM NaCl; 50mM $MgCl_2$.

1x SSC: 0,15 M NaCl; 0,015 M Na-Citrat.

1x PBS: 130 mM NaCl; 2,7 mM KCl; 1,5 mM $KH_2PO_4$; 8mM $Na_2HPO_4$.

### 4.7 The FRT/FLP-recombination techniques

*To produce "loss of function clones"*

This technique takes benefit from engineered chromosomes containing a specific yeast sequence (FRT) which is a target for the yeast FLP recombinase (Dang and Perrimon, 1992). By low level induction of the FLP-recombinase in transgenic flies, recombination between two homologous chromosomes containing the FRT sequence can be induced in a small number of cells. It is used to produce clones of homozygous mutant cells in discs that are heterozygous for recessive alleles of interest (mosaics) (Fig. 25). This can be of special interest to study the role of a gene at a stage of development which can not be reached my homozygous mutants because of lethality. It is also used to study the behavior of mutant (homozygous) cells in a wild type environment (heterozygous).

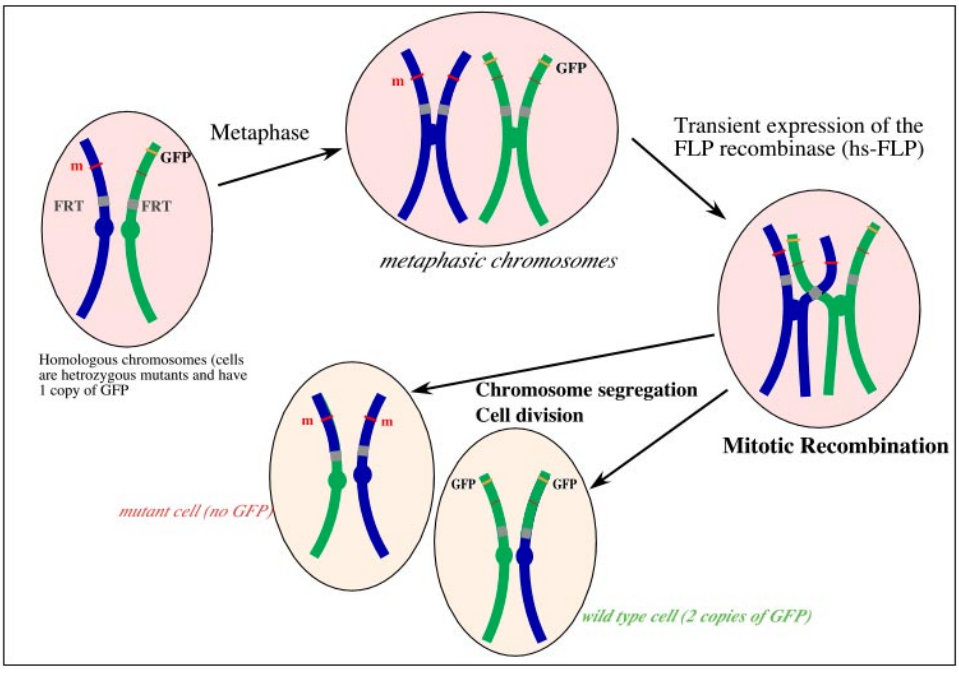



**Figure 25: The FRT/FLP recombination technique to produce "loss of function clones".**

Based on this scheme, imaginal discs of heterozygous flies for the $Pc^3$ allele should possess clones of cells homozygous for the *Polycomb* mutated allele and without any GFP, whereas "twin" clones will have two wild type copies of *Polycomb* and two copies of GFP.

**Genotype of Flies:** hsFLP ; + ; frt:GFP / frt:$Pc^3$

The FLP-recombinase is under the control of the heat-shock promoter (hsp). A small heat shock at a chosen stage of the development can induce a transient expression of the FLP-recombinase in the cells. 2nd instar larvae were heat-shocked 1 hour at 37°C in order to induce a transient expression of the FLP-recombinase which promotes mitotic recombination at the FRT sites.

*To produce "gain of function clones"*

Another use of the FLP/FRT-recombination technique allows the production of "gain of function" clones in a wild type environment (Struhl and Basler, 1993). In this case, the FRT-cassette is made in which two FRT sequences frames a marker gene which expression is controlled by a constitutive promoter downstream of this gene a termination sequence stops the transcription (Fig. 26). Downstream of the FRT-cassette is placed the gene of interest. Upon a transient expression of FLP in the cells during development, the recombination of the two FRT sequence can occur and the FRT-cassette containing the marker gene and the termination sequence is flipped out. This leads to an activation of the transcription of the gene of the interest thanks to the constitutive promoter. In this case, the cells which will have flipped out the cassette will produce clones overexpressing a new gene.

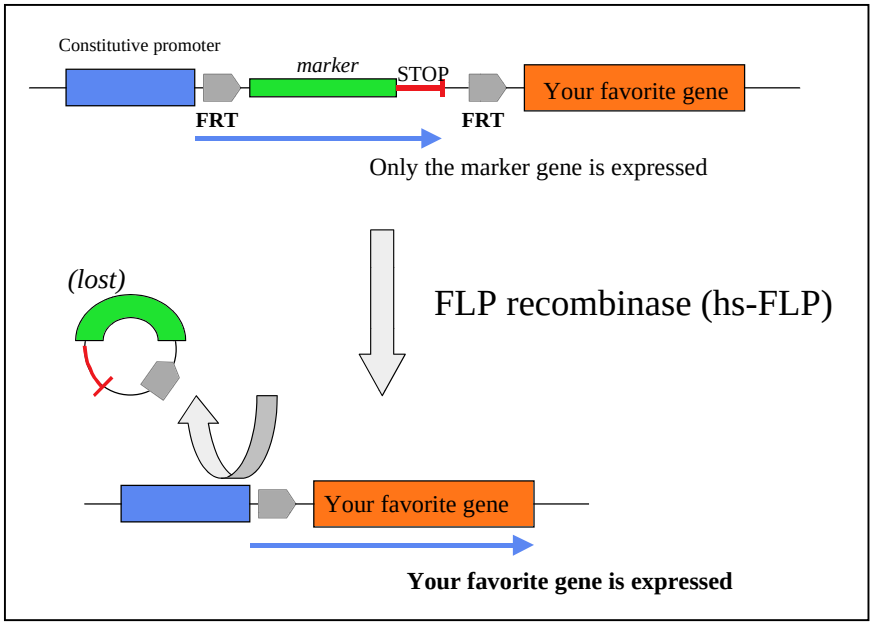



**Figure 26: Production of gain of function clones.** In cells, the constitutive promoter activate transcription of the marker gene which is blocked further by the termination sequence. When the FLP recombinase is transiently expressed, the FRT-cassette jumps out and the constitutive promoter can activate transcription of the favorite gene. The cells will produce gain of function clone.

*Production of the recombined FRT/Pc$^3$ chromosome.*

The *P c$^3$* mutation which is considered to be a strong antimorph was recombined with a stock containing the FRT sequence on the 3$^{rd}$ chromosome and marked with the mini-*white* gene. Flies heterozygotes for the *Pc$^3$* mutation exhibit visible homeotic phenotypes like additional sex combs on midllegs and posterior legs of males and crumpled wings. However this phenotype is not fully penetrant. In order to simplify the screening procedure, an additional step have been made in which flies are crossed to a stock heterozygous for the *Pcl$^{(XM3)}$* mutation, on the 2$^{nd}$ chromosomes (Fig. 27). Flies heterozygote for this mutation do not show any strong phenotype by themselves. However, flies transheterozygote for *Pcl$^{(XM3)}$* and *Pc$^3$* show a fully penetrant enhanced phenotype. Therefore, during the screening procedure, flies showing strong homeotic phenotypes and red eyes are considered to have recombined the FRT locus with the *Pc$^3$* allele. The *Pcl$^{(XM3)}$* allele is crossed out afterwards.

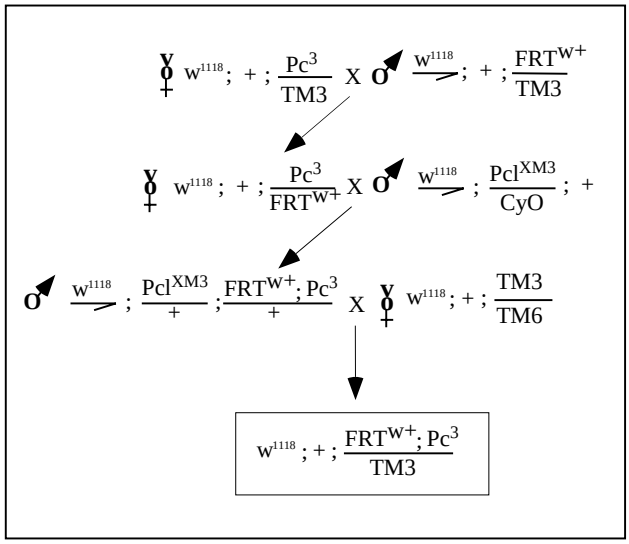



**Figure 27: Genetic scheme for the generation of the FRT/Pc3 chromosome**

## 4.8 Protocol for immunostaining of imaginal discs

*Dissection of imaginal discs*

Dissection was done in PBS. Using forceps, the larvae was catched by the head, and with other hand, cut the larvae in two parts by pulling out from the middle of the body. The cuticle of the larvae was everted “inside-out” along the forceps by keeping on holding the head (Fig. 28). Imaginal discs appear, stuck to the cuticle. Salivary glands and the fat tissues (in white) must be carefully removed (Can be stored in Eppendorf caps in PBS, on ice, up to 1 hour).

*Fixation*

The discs and the cuticle were fixed in PBS / 3%PFA for 30 min, and then rinsed twice and washed once for 5 min in PBS.

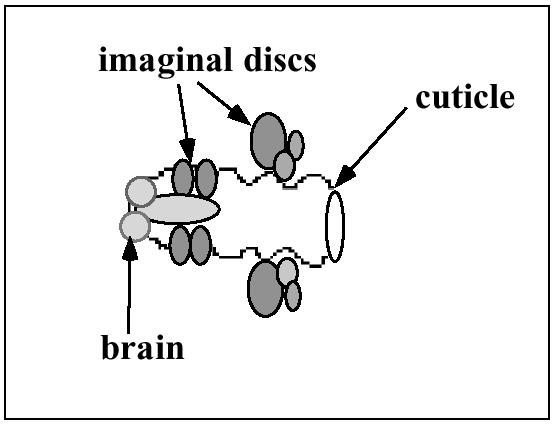



**Figure 28: The "inside-out" method for dissection of larvae.** The larva is first torn in two part with the forceps. One forceps then catch the mouth part while the other one everts the cuticle "inside-out". The imaginal discs are now sticking out into the dissection medium. The fat body and the gut should be removed. The scheme depicts how the preparation should look like after the dissection.

*Immunostaining*

After blocking in PBS / 0,3%TritonX100 / 5%BSA (3x10 min), the cuticles were incubated in PBS / 0,1%TritonX100 / 1%BSA + *primary antibodies* (dilution for antibodies must be checked). The preparation was incubated overnight at 4°C, on a rotating tray, in a humid chamber to prevent from evaporation.

On the next morning, the preparation was rinsed two times in PBS / 0,1% TritonX100 and incubated in PBS / 0,1%TritonX100 / 1%BSA (3x15 min). Afterwards, the preparation was incubated in PBS / 0,1%TritonX100 / 1%BSA / 2% goat serum + *secondary antibody* (check dilution of antibody*)* and again incubated 1 hour at room temperature on a rotating plate. It was rinsed once in PBS / 0,1%TritonX100, and washed in PBS / 0,1%TritonX100 (3x15 min) and finally rinsed in PBS.

*Final dissection*

A drop of 70% glycerol / PBS was put on a slide with two larval cuticles in the drop. Using needles, imaginal discs were detached from cuticles. When all imaginal discs (wing, leg, eye-antenna) were removed from the cuticles, cuticles were removed from the slide. A coverslip was carefully layed on the slide, and sealed with nailpolish. Results were observed under the fluorescence microscope.

### 4.9 Histochemical detection of ß-galactosidase activity in embryos and imaginal discs

An overnight collection of embryos was dechorionated for 2-3 min in 3% Na-Hypoclorid and washed in PBS. The embryos were fixed in small baskets for 10 min in the fix-solution. Finally the embryos were carefully washed in PBS, shortly dried and transferred with a brush to an Eppendorf tube filled with staining solution.

For imaginal discs, after the dissection of the larvae by the “inside-out” technique, the cuticles with the discs were fixed for 15 min in PBS/1% glutaraldehyde at room temperature. After fixation, they were washed twice with PBS and a staining solution was added.
One can stain from 5 min to overnight at 37°C (check staining under stereoview). After staining, it should be washed in PBS, and mounted in PBS/70% glycerol.

*Solution:*

Fix-solution for embryos: 5 ml Heptan; 0,9 ml 25% Glutaraldehyde; 0,1 ml 1M Phosphate buffer pH 7.

Mix vigorously, and take the upper phase for fixation.

Staining-solution: 10 mM Na-phosphate buffer pH 7,2; 150mM NaCl; 1 mM $MgCl_2$; 6 mM $K_4\{Fe^{II}(CN)_6\}$; 6 mM $K_3\{Fe^{III}(CN)_6\}$; 0,3% Triton X-100.

**4.10 In situ hybridization of imaginal discs**

*DIG-labeling of RNA Probes*

The work was done in Rnase-free conditions.

Two methods were used to produce RNA-probes. The DNA template was either cloned in pBlueScript and transcribed using the T3 or T7 promoter, or the DNA fragment to be transcribed was amplified with primers containing one of the two promoters. The labeling reaction was performed at 37°C for 2 hours. It contained the template DNA, some Rnase inhibitor (Promega), transcription buffer (Boeringer Mannheim), DIG RNA-labeling mix (Roche), T7 or T3 RNA Polymerase (Roche). The DNA was subsequently digested by addition of Dnase (Roche) to the reaction. The labeled RNA was afterwards purified using the QIAquick PCR purification Kit (Qiagen), and resuspended in 50ml Rnase free $ddH_2O$. The same volume of formamide was added and the RNA was stored at -80°C.

*Tissue preparation*

Imaginal discs were dissected in PBS according to the “inside-out” technique described in the protocol for immunostaining of the imaginal discs section. The cuticle containing the discs was immediately transferred to 4% formaldehyde/PBS on ice and fixed for 15-20 min followed by a fixation in 4% formalfehyde/PBS containing 0,6% Triton X-100 at room

temperature for an additional 15-20 min. Then, it was washed three times in PBS containing 0,3% Triton X-100 (PBS/TX) with rocking (5 min each wash).

At this stage, if it was needed to store discs for future use, they were rinsed in PBS, dehydrated through an ethanol series (25%, 50%, 75%, twice absolute, 10 min each) and stored in ethanol at –20°C. Storage in ethanol reduces nonspecific background staining.

After rehydration through an ethanol series, the preparation was rinsed in PBS containing 0,1% Tween20 (PBT). In the mean time, a tube with fixed discs or embryos was prepared, which was used for the preabsorbtion of antibodies. Fixation with Fix-solution/PBT (1:1) for 10 min was performed, followed by rinsing (three times) and washing once for 5 min with PBT. Disc were subjected to limited Proteinase K digestion for 4 min exactly in 500ml of 50mg/ml Proteinase K in PBT (a 50mg/ml Proteinase K stock solution must be made and 5ml of the stock solution is diluted in 5ml PBT). 500ml Glycin/PBT (4mg/ml) was added and the discs were incubated 3 min and rinsed twice with PBT. Be excessively gentle with the tissue from this point on, as it is very fragile and the discs tend to break away. When adding solutions to the tube, let them drip down the wall of the tube to minimize disruption of the tissue. The preparation was post-fixed with Fix-solution/PBT (1:1) for 20 min and rinse 3 times, washed once for 5 min with PBT (At this point the tube containing the discs or the embryos for the preabsorbtion of the antibodies were stored at 4°C). Washing with 500ml HybeB-solution/PBT (1:1) was done and equilibrium waited. This procedure was repeated with 250ml HybeB-solution, and with 250ml Hybe-solution.

*Prehybridization, hybridization and washing*

Discs were prehybridized with 250ml Hybe-solution at 65°C for 30-60 min. 12 min before the end of the prehybridization, 1-3ml of the Dig-labelled probe was added to 30ml of Hybe-solution and pre-heated at 65°C for 10 min. Hybe-solution was removed from the tubes containing the discs and 30ml of the pre-heated solution containing the probe was added. Incubation overnight in a waterbath at 65°C was performed.

The next morning, the anti-Dig-AP antibody was diluted (1:200 in PBT) and incubated with the prepared discs, or embryos for preabsorbtion. 500ml of prewarmed Hybe-solution was added to the tubes containing the discs and the probe and then replaced by 500ml of prewarmed HybeB-solution for 15 min at 65°C. This was repeated once. Then, 500ml PBT was added, and the preparation was rinsed twice with PBT and washed 5 min and twice 20 min in PBT.

*Detection*

Discs were incubated with preabsorbed Anti-Dig-AP were incubated for 2 hours (900ml PBT plus 100ml of preabsorbed antibodies), rinsed twice with PBT and washed twice 15 min. After equilibration with AP-buffer (twice), incubate in staining solution was performed (200ml of NBT/PCIP stock solution (Roche) in 10 ml AP-buffer) in a 24-well plate. The progression of the staining was observed under the microscope and stopped before background appeared. Then discs were rinsed 3 times in PBT, washed twice for 5 min, and mounted in 70% glycerol/PBS.

*Solutions*

HybeB-solution: 50% Formamide; 5x SSC; pH 5.

Hybe solution: 50% Formamide; 5x SSC; 5mg/ml Heparin (Fluka); 5mg/ml Torula Yeast (Sigma); 0,1% Tween20 (Sigma); pH 5.

Fix-solution: 10% paraformaldehyde (Sigma) in PBS; 50mM EGTA.

1x SSC: 0,15 M NaCl; 0,015 M Na-Citrat.

1x PBS: 130 mM NaCl; 2,7 mM KCl; 1,5 mM $KH_2PO_4$; 8mM $Na_2HPO_4$.

PBT: 1x PBS (pH 7,5); 0,1% Tween20.

AP-buffer:20mM Tris/HCl; 100mM NaCl; 50mM $MgCl_2$.

### 4.11 Chromatin immunoprecipitation and dot blot analysis

The chromatin immunoprecipitation was performed following a standard procedure described in Strutt and Paro (2000). For the dot-blot, 14 primer pairs were designed for the elaboration of 1 kb sized PCR fragments covering the hh genomic region. PCRs were performed using genomic DNA as template and the Taq Polymerase from Qiagen (Table 5).

| | |
|---|---|
| **1 x** | **denature template 2min at 94°C** |
| **15 x** | **denaturation at 94°C for10 s**<br>**annealing from 70 to 55°C (decrease from 1°C every new cycle) for 30 s**<br>**elongation at 72°C for 1 min** |
| **20x** | **denaturation at 94°C for10 s**<br>**annealing at 55°C**<br>**elongation at 72°C for 1 min + add 10 s each new cycle** |
| **1 x** | **prolonged elongation 7 min at 72°C** |

**Table 5: PCR scheme for production of 1kb fragment covering the hh genomic region.** Genomic DNA is used as template and the reaction volume used is 50ml.

After the first PCR, another PCR was performed using the same cycle scheme and the same primers, but using, for each DNA fragment, 0,5ml of the product of the first PCR as template DNA for the second PCR. This procedure allows minimizing the concentration of genomic DNA in the PCR product and should reduce background in the dot blot.

After blotting the PCR-products on nylon membranes, the immunoprecipitated and the mock DNA were radiolabelled using the *redi*prime™ kit II (Amersham Pharmacia Biotech) and the membranes were probed individually with their respective labeled DNA. Filters were exposed overnight to a Phosphorimager screen, and scanned. Signals were quantified by using NIH image software (version 1,62). For each dot, the intensity of the signal was quantified and the background subtracted to it. Then, relative enrichment of the immunoprecipitated material was calculated by dividing the intensity of the signal obtained for the PC and GAF chromatin-immunoprecipitation with the one obtained for their respective mock.

**4.12 Antibody staining of polytene chromosomes**

Two pairs of salivary glands were isolated in PBS and were transferred onto one poly-L-lysine (PLL) coated slide. The glands were incubated into a drop of 45% Acetic Acid / 5% fresh para-formaldehyde / dd$H_20$ fixative for 10 minutes. Salivary glands were then squashed and were spread by gently moving the cover slip. Slides were immersed in liquid nitrogen, and coverslips flicked off. Slides were washed in PBS twice for 5 minutes, permeabilised for 10 minutes in 1% Triton-X/PBS, blocked in a saturated solution of non-fat milk powder (blocking solution) for 30 minutes, and incubated with appropriate primary antibody dilution for 1 hour at room temperature, followed by overnight at 4ºC. After washing three times with saturated solution of milk powder, slides were incubated for 1 hour with secondary antibodies (a-rabbit Alexa-488, 1:200; a-mouse Cy3, 1:500) at room temperature. Slides were rinsed in PBS, and washed for 15 minutes in 0.2% NP-40, 0.2% Tween 20-80, 300 mM NaCl, and in 0.2% NP-40, 0.2% Tween 20-80, 400 mM NaCl solutions. Finally, the slides were DAPI (100 ng/ml) stained for 10 minutes and were mounted in 70% Glycerol/PBS.

**4.13 Embryonic primary cell culture protocol and TSA treatment**

An overnight laying of eggs was collected from apple-juice plates and poured through a 110 micron mesh sieve and washed with large amount of water. Embryos were dechorionated in 4% sodium hypochlorite solution and washed through the sieve with large amount of sterile dd$H_2O$ (all the yeast must be removed). At this point, the work was pursued under the hood. Embryos were washed again with sterile dd$H_2O$ and transfered into a 50ml

Falcon tube. Embryos were washed again with 40ml 90% ethanol, incubated twice in 40ml 70% ethanol for 5 minutes each and then washed 3 times in *Schneider Drosophila* medium. Embryos were transferred into a glass dounce tissue homogenizer. Embryos were disrupted and cells dissociated with a type A (loose) pestle by twisting five times under fair pressure. A slow withdrawing must be done, avoiding foaming. It was repeated if clumps of tissues were visible by eye. Pipetting helped disaggregation. Cell were resuspended from homogenisator into a steril 15-ml Falcon tube and spin for 1 min at 620g. The edium was then pored off and cells were resuspended in fresh medium. This step was repeated twice. and cells were finally resuspended in Schneider medium (Gibco) containing an antibiotics cocktail (penicillin/streptamycin/ 0,05mg/ml gentamycin), 15% fetal calf serum. Adding 4,5 U/ml insulin favors proliferation. Cells were resuspended in tissue culture flasks with a high density, to allow them to grow. If it is better that cells attach the flask it is better not to put the calf serum the first 1 or 2 hours. For TSA treatment, 120ng/ml of TSA was added in the culture medium the cells were incubated overnight. The next morning, the cells were rinsed once with medium, and incubated for the desired time.

After the cells were collected for Western blotting, a protein quantification was made using the Bradford method. By this way, it was possible to roughly checked whether cells were still dividing after TSA treatment.

**4.14 Western Immunoblotting**

Cells were collected and lysed in 10 mM Tris/0,3% Triton X-100. The amount of protein was measured by the Bradford method. Electrophoresis was performed under Laemmli conditions with the mini-gel system from Hoefer in a 12% SDS-Polyacrylamide gel and the same amount of proteins (15 to 20 mg) was loaded in each well in loading buffer after 10 min boiling. Migrating conditions were : 170 V, 60 mA per gel, 60 min.

The transfer of proteins was realized on nitrocellulose membrane with the transfer cell from Hoefer. Gels and membranes were immerged in the transfer-buffer. The transfer was performed at 4%C under the following conditions: 100V, 350 mA, 60 min.

The detection was realized with the chemiluminescence method using the ECL western blotting kit from Amersham Pharmacia Biotech. Signal was detected thanks to the HRP-substrate ECL when reacting with HRP-conjugated secondary antibody.

Solutions:

Loading-buffer: 20 mM Tris/HCl pH 6,8; 20% Glycerol; 2% SDS; 2% ß-Mercaptoethanol; 0,125% Bromophenol blue.

Separating-gel (12%): 12% acrylamide/Bisacrylamide; 1,5 M Tris pH 8,8; 10% SDS; 10% APS; TEMED

Stacking gel: 5% acrylamide/Bisacrylamide; 1,0 M Tris pH 6,8; 10% SDS; 10% APS; TEMED.

Running-buffer: 25 mM Tris/HCl pH 8,3; 192 mM Glycin; 0,1% SDS.

Transfer-buffer: 25 mM Tris-Base, 192 mM Glycin, 20% Methanol.

Blocking-solution: 5% milkpowder in PBS

Washing-solution: PBS-0,05 Tween20

### 4.14 *In vivo* culture of Drosophila imaginal discs

*Principle*

The transplantation of imaginal discs from the larvae to the abdomen of a young female adult fly allows the culture of discs in a natural incubation medium, the hemolymph. By this way, discs or fragmented discs can be kept for many days in this *in vivo* culture system allowing cells to divide. After a while, discs or fragmented discs can be recovered from the host abdomen and used for further manipulation. The technique was originally devised by Ephrussi and Beadle (1936).

*4.14.2 Dissection*

Wandering third instar larvae were used as imaginal discs donors. Care should be taken that larvae are not entering pupariation because discs may have started the prepupal morphogenesis process. Larvae were washed in water and in 100% ethanol and rinsed in sterile Ringer's solution. For the study of leg-to-wing transdetermination, first leg discs were dissected in sterile Ringer's medium using the "inside-out" technique (see paragraph: 4.8 Protocol for immunostaining of imaginal discs). They are transferred into a new clean drop of Ringer's medium. Up to 6 pairs of first leg imaginal discs can be dissected in a row and left in the drop of Ringer's until fragmentation and transplantation.

Thin tungsten needles were used to cut the discs into fragments. Tungsten needles could be sharpen by damping them in a solution of 1N NaOH in which an electric current (10 to 20V) was generated (Fig. 29).

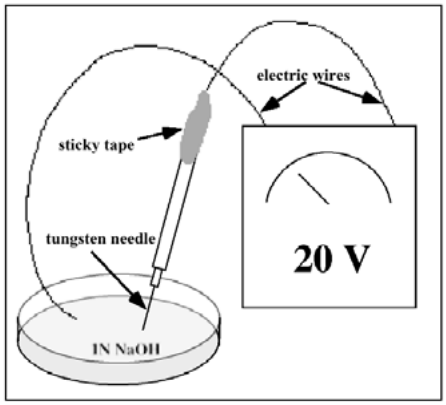



**Figure 29: Sharpening of the tungsten needles through a 20V electric current.** The tungsten wire is damped into the 1N NaOH solution. An electric wire is attached to the needle holder. Some sticky tape can be used to handle the needle when the current is applied in the solution. The sharpening should be checked under a microscope.

It was shown that fragmented leg discs in which the upper anterior quarter has been removed could transdetermine with a high efficiency. Therefore first leg discs has been cut this way and transplanted in the abdomen of adult flies.

For the transplantation, a special injection apparatus should be mounted. An all-glass 2ml syringe (Eterna-Matic) with a well-fitting glass plunger has been used. The syringe was mounted on a heavy metal base. A 80 cm length of tygon tubing, anywhere from 1 to 4 mm inside diameter was connected to a commercial needle holder. The tubing should be filled with distilled water, avoiding air bubbles (Fig. 30). Needles for injections were drawn from Pyrex capillaries (outside diameter 1 mm, inside diameter 0,8 mm) on a homemade microburner. Pipettes with a diameter of 130 µ suits well for leg imaginal discs. The tip must was given the shape of a hypodermic needle tip. This was done by pushing down a dissecting needle onto the pulled portion of the capillary (Fig. 31). Next, the injection pipette was equipped with a constriction et which the implant could come to halt when sucked up into the pipette. A microforge was used for this effect.

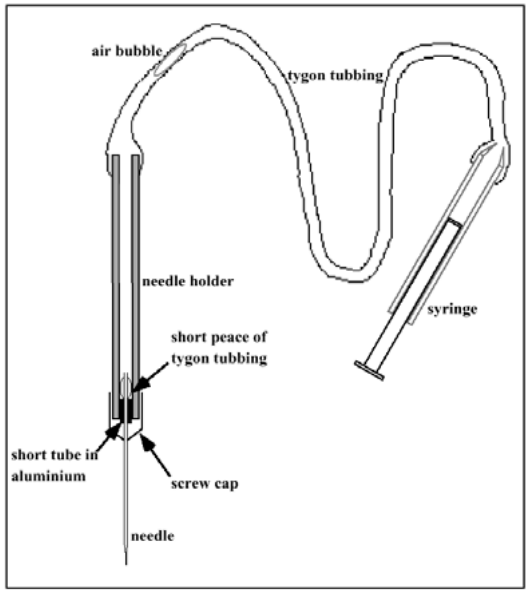



**Figure 30: Injection apparatus for transplantation.** The syringe should be mounted on a laboratory stand and operated with the left hand. the needle and the extremity of the tygon tubbing are filled with Ringer's solution. An air bubble must separate the water from the Ringer's solution.

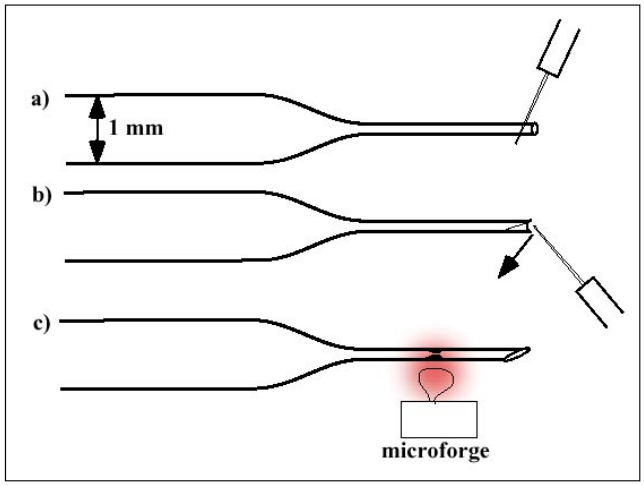



**Figure 31: Preparation of the needle for injection.** After pulling the pipette, pressure is applied from above with a dissection needle in order to crush the capillary near its tip (a). This often results in a U-shaped break. Then, one spurs is pried of gently with a needle in the direction of the arrow (b). If necessary this may be repeated but should end up with a tip with the shape shown. Finally, a constriction is produced close to the tip by heating the glass with a microforge (c).

*Injection*

A microscope slide was covered with a strip of double-faced sticky tape. Etherized one day old female flies were then affixed to the sticky tape, belly up, in a row, by gently tapping their wings against the tape.

The needle holder with the injection pipette was held in the right hand. The left hand was operating the plunger of the syringe, which was connected to the needle holder by the tygon tubing. In the tube the water phase must be separated from the *Ringer's* solution by an air bubble. Then the fragmented disc was gently sucked up into the needle. The discs must never be in direct contact with water. Then the needle was brought to the abdomen of the host flies while the left hand could help maintaining the abdomen stretched with a blunted needle. The pipette was gently inserted into the abdomen about in the middle. The left hand then had to leave the needle and operate the plunger again, pushing or rotating it gently so that the disc is injected. The pipette was then withdrawn quickly. Because quite frequently, the fragmented disc was rejected by the host, it is important to carefully check whether there is no rejection after the injection. Ideally, there must not be any hemolymph rejected from the abdomen after the transplantation. Flies were then carefully unstuck from the tape with forceps and transferred is fresh food.

To get a high frequency of transdetermination it was important to leave the discs incubated for several days in the abdomen. I left them 13 days at 25°C.

After the incubation period, the adult flies were dissected in PBS in order to recover the discs from the abdomen. After the transplantation, needles are conserved in 10N NaOH.

*Solutions*

Ringer's solution: 182 mM KCl; 46mM NaCl; 3mM $CaCl_2$; 10 mM Tris-HCl. Adjust to pH 7,2 with 1 N HCl and autoclave.

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# Abbreviations

| | |
|---|---|
| A | Anterior |
| ANT-C | Antennapedia complex |
| *antp* | *antennapedia* |
| *ash1/ ash2* | *absent, small and homeotic discs 1/ 2* |
| b-gal | b-galactosidase |
| BX-C | Bithorax complex |
| bp | base pair |
| *brm* | *brahma* |
| BSA | Bovine Serum Albumine |
| cDNA | complementary DNA |
| ChIP | Chromatin ImunoPrecipitation |
| CMM | Cellular Memory Module |
| D | dorsal |
| $ddH_20$ | double-distilled water |
| DNA | Desoxyribonucleic Acid |
| DNase | Desoxyribonuclease |
| *dpp* | *decapentaplegic* |
| *E.coli* | *Escherichia coli* |
| EDTA | ethylenediaminetetraacetic acid |
| *en* | *engrailed* |
| *esc* | *extra sex combs* |
| ETP | Enhancer of Polycomb and trithorax |
| *E(z)* | *Enhancer of Zeste* |
| FLP | Flip-Recombinase |
| FRT | Flip-Recombinase Target |
| g | gram/ gravity |
| GFP | Green Fluorescent Protein |
| HAT | Histone acetyl-transferase |
| HCl | hydrochloric acid |
| HDAC | histone deacetylase complex |
| HMT | histone methyl-transferase |
| HOX | homeobox gene |
| *hh* | *hedgehog* |
| HP1 | heterochromatin protein 1 |
| hs | heat shock |
| Hsp70 | heat-shock protein 70 |
| kb | kilobase |
| KCl | potassium chloride |
| *lacZ* | *ß-galactosidase gene* |
| LB | Luria-Bertani bacterial medium |
| M | molar |
| mg | microgram |
| ml | microliter |
| mM | micromolar |
| mg | milligram |

| | |
|---|---|
| ml | milliliter |
| mM | millimolar |
| min | minute |
| mRNA | messenger ribonucleic acid |
| NaCl | sodium chloride |
| nmol | nanomol |
| NP-40 | Nonidet P-40 |
| P | Posterior |
| PBS | Phosphate Buffered Saline |
| *Pc* | *Polycomb* |
| PCR | Polymerase Chain Reaction |
| PcG | Polycomb Group |
| *Pcl* | *Polycomb-like* |
| PFA | para-formaldehyde |
| *ph* | *polyhomeotic* |
| PHD | plant homology domain |
| *pho* | *pleiohomeotic* |
| PRC1 | Polycomb Repressive Complex 1 |
| *psc* | *posterior sex combs* |
| *puc* | *puckered* |
| RNA | Ribonucleic Acid |
| RNase | Ribonuclease |
| PRE | Polycomb Response Element |
| *Sce* | *Sex comb extra* |
| *scm* | *sex comb midleg* |
| SDS | sodium lauryl sulfate |
| SET | Su(var)3-9, E(z), Trx |
| *Su(var)3-9* | *Suppression of variegation 3-9* |
| *Su(z)2* | *Suppressor of zeste 2* |
| SWI/SNF | switch/ sucrose non-fermenting gene (yeast) |
| TRE | Trithorax Response Element |
| Tris | tris(hydroxymethyl)aminomethane |
| *trx* | *trithorax* |
| trxG | trithorax Group |
| TSA | Trichostatin A |
| Tween-20 | polyoxyethylene sorbitan-20 |
| V | Ventral |
| UAS | Upstream Activating Sequence (GAL4 binding site) |
| *vg* | *vestigial* |
| *w* | *white* |
| *wg* | *wingless* |
| **Z** | *Zeste* |
| $^{o}$C | degree Celsius |

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