Developing an eggshell marker based on a dominant female sterile mutation for the identification of complete follicle cell clones in *Drosophila melanogaster.*

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Abstract

Patterning of the body axes of the *Drosophila* embryo depends on maternally expressed genes, some of which function in the follicular epithelium of the developing egg chamber. Many such genes were identified in genetic screens for homozygous mutant females that produce abnormal embryos. However, mutations in zygotically required maternal effect genes are homozygous lethal, and therefore viable females cannot be recovered using this screening approach. This limitation can be overcome by generating homozygous mutant follicle cell clones in heterozygous females using a system that induces site-specific mitotic recombination events. However, to date, eggs produced from egg chambers with complete follicle cell clones cannot be directly identified. We have developed an eggshell marker for follicle cell clones using a dominant negative (DN) allele of the gene *defective chorion* (*dec*). Females with a single copy of this allele, dec^{DN}, lay collapsed eggs and are therefore sterile. Site-specific mitotic recombination events induced in females heterozygous for dec^{DN} and a mutation on the homologous chromosome arm result in homozygous mutant follicle cells that have lost dec^{DN}. Therefore, egg chambers with the entire follicular epithelium homozygous mutant generate intact eggs that can be unambiguously identified amongst otherwise collapsed eggs.

Résumé

L'établissement des axes du corps chez la drosophile est gouverné par l'expression de gènes maternels dont certains sont exprimés dans l'épithelium folliculaire de l'oeuf. Plusieurs de ces gènes ont été identifés au cours de dépistages génétiques afin d'isoler des mutations recessives qui affectent le développement normal des oeufs. Par contre, cet approche ne peut pas identifier les mutations recessives létales puisqu'aucune femelle mutante viable ne peut être obtenue. Les systèmes de recombinaisons mitotiques homologues sont utilisés afin de produire des clones de cellules folliculaires homozygotes mutantes dans une femelle hétérozygote permettant ainsi l'étude des mutations létales recessives. Cependant, les oeufs provenant d'épithelium folliculaires complètement mutants ne peuvent être identifié lorsque pondu, car les cellules folliculaires qui sécrètent la coquille dégénèrent avant la ponte de l'oeuf. Nous avons développé un marqueur phénotypique, une allèle dominante négative (DN) du gène *defective chorion* (*dec*), qui affecte la structure de la coquille de l'oeuf servant ainsi à identifier les oeufs provenant de clones folliculaires complets. Les femelles qui portent une seule copie de l'allele dec^{DN} sont stériles car elles pondent des oeufs affaissés infertilisable. La production de clones par recombinaisons mitotiques homologues dans les ovaires de femelles hétérozygotes pour dec^{DN} et une mutation d'intérêt permet d'obtenir des cellules folliculaires mutantes qui ne contiennent pas dec^{DN}. Ainsi, il est possible d'identifier des oeufs intacts provenant de clones folliculaires complet pour la mutation d'intérêt parmis une population d'oeufs affaissés.

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Chapter I. Introduction

I.1. The use of *Drosophila* as a model organism.

For over a century, *Drosophila melanogaster* has been studied as a model organism. Many molecular and genetic tools have been developed that make it a popular choice for the study of a variety of biological processes (Beckingham et al., 2005). How genetically similar the fruit fly is to human has been recently greatly appreciated: with both the human and *Drosophila* genome projects completed, many genes in humans were found to have homologs in *Drosophila* (Bier, 2005). Therefore, it remains an attractive model organism to study a wide range of genetic pathways and signaling cascades to deepen our understanding of the details of different biological events in higher organisms where they are largely conserved.

Development is the process by which organisms progress from embryonic stages to stages of maturity, following specific series of events to finally yield the complex unique patterns of the adult stage. These patterning events need to be tightly controlled to generate properly formed and functional adults. *Drosophila* has been appreciated as a valuable tool to study genes involved in developmental events such as axis specification and cell type specification, since a number of them have been found to be conserved across several species.

I.2. Overview of *Drosophila* oogenesis.

An adult *Drosophila* female has a pair of ovaries, each consisting of about 16 ovarioles bunched together. An ovariole can be thought of as an assembly line for the production of eggs (Figure 1A)(Spradling, 1993). Egg production starts at the

very tip of the ovariole in a region called the germarium, where germline stem cells (GSCs) are found in a niche (Spradling et al., 2001). When a GSC divides asymmetrically one of the daughter cells maintains the stem cell identity and the other one proceeds to divide four successive times with incomplete cytokinesis to form a cyst consisting of 16 germline cells interconnected by structures called ring canals. Of the two cells that share 4 ring canals with neighboring cells, one becomes fated as the oocyte. The other 15 cells remain attached as nurse cells, providing the growing oocyte with cytoplasmic components. Soon after its formation, the 16-cell cyst becomes enveloped into a layer of somatic follicular epithelium, and the resultant package is called an egg chamber (Huynh and St Johnston, 2004) (Figure 1B).

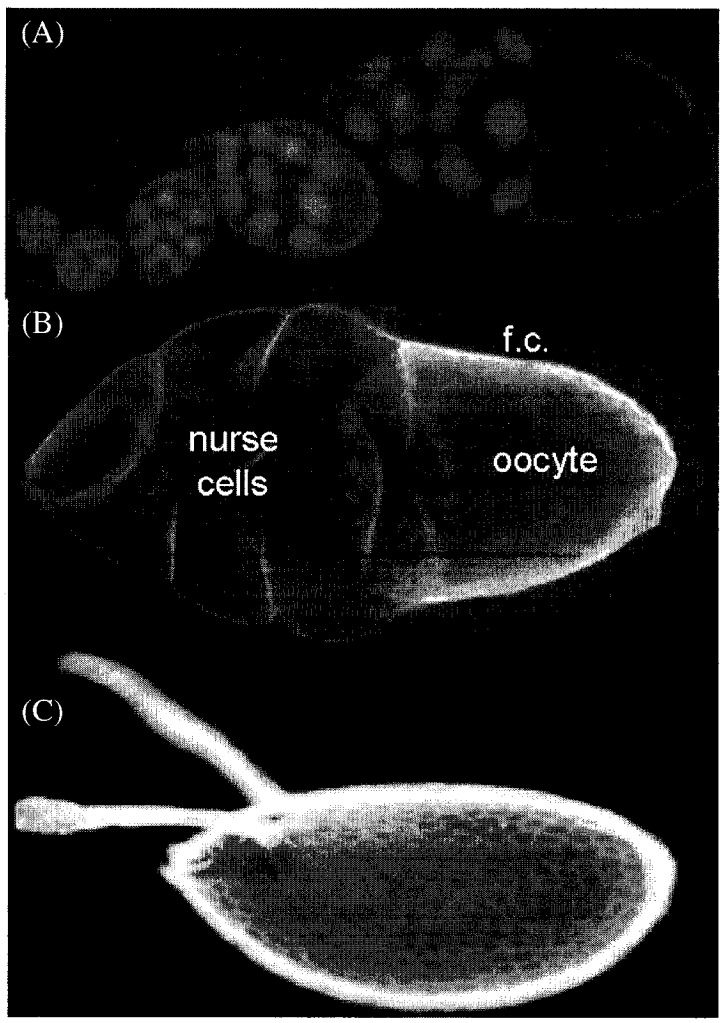
Each egg chamber matures as it travels down the ovariole towards the oviduct, passing through successive stages of oogenesis (Figure 1A). Towards the end of the process the nurse cells, which have been shrinking, finally degenerate. The follicle cells also degenerate before the egg is laid; but before they do, they secrete the eggshell material on top of the mature oocyte in the final stages of oogenesis, leaving their imprints on the surface of the eggshell (Spradling, 1993) (Figure 1C).

I.3. Maternal effect genes affect the A-P and D-V axes of the developing embryo.

An egg laid by a wild-type *Drosophila* female has a characteristic distinctive pattern exhibiting both dorsal-ventral (D-V) and anterior-posterior (A-P) axes of asymmetry. The ventral domain is slightly more rounded than the flatter dorsal

Figure 1. Examples of an ovariole, an egg chamber and a mature egg (not to scale).

(A) An ovariole, nuclei in blue due to DAPI staining. Egg chambers are assembled at the germarium (not shown) and they pass through successive stages of oogenesis as they travel down the ovariole from anterior (left) to posterior (right). **(B)** An egg chamber in mid-oogenesis. The nurse cells are at the anterior (left) and the growing oocyte is at the posterior (right). Most of the somatic follicle cells (f.c.) have migrated by this stage to the posterior on top of the oocyte. **(C)** A wild type egg laid by a wild-type female has a distinctive pattern. The dorsal side (top) is flatter than the more rounded ventral side (bottom). A pair of appendages is found on the dorsal side at the anterior domain (left). The imprints of the degenerated follicle cells are visible on the surface of the eggshell material, which they secrete in late stages of oogenesis.


(A)
(B)
f.c.
nurse
cells
oocyte
(C)

side, and a distinctive pair of appendages is found dorsally on the anterior end (see Figure 1C). The proper D-V and A-P patterning of the egg is crucial since many of the patterning mechanisms that determine the shape of the eggshell are also involved in embryo patterning. It has been long known that the eggs of many studied organisms contain localized products that affect the proper patterning of the developing embryo after fertilization (Wilson, 1928). Genes coding for such maternal signals are referred to as maternal effect genes. In the case of *Drosophila*, a number of such genes have been identified, expressed in different stages of oogenesis either by the germline or the follicle cells. They have been grouped into four classes according to the patterning feature of the embryo they affect. Genes in only one of these classes, the dorsal group genes, affect the D-V axis of the embryo, while genes in the other three classes, the anterior, posterior, and terminal genes, influence the patterning events along its A-P axis (St Johnston and Nusslein-Volhard, 1992).

Products of the maternal effect genes that are expressed in the germline are transported from the nurse cells into the growing oocyte where their intracellular localization is tightly controlled, which is crucial to the relay of patterning signals to the developing embryo at a later stage. A classical example is the gene *bicoid*, one of the anterior class genes. Transcribed in the nurse cells, the *bicoid* RNA is then delivered to the oocyte where it is localized exclusively to the anterior. Following fertilization, it gets translated resulting in a gradient of Bicoid protein concentration emanating from the anterior and absent at the posterior. This gradient controls a number of zygotic genes, such as *hunchback*, which gets turned on in the anterior-most region where Bicoid levels are highest, driving the

embryonic development of the thorax and parts of the head (Ephrussi and St Johnston, 2004; McGregor, 2005; St Johnston and Nusslein-Volhard, 1992).

However, some maternal patterning signals are not localized within the oocyte. They are expressed in specific subpopulations of the overlying layer of the somatic follicular epithelium (St Johnston and Nusslein-Volhard, 1992). But since follicle cells are known to degenerate in late stages of oogenesis, it is not yet clear how the signals are communicated to the embryo, which begins its development after the egg has been laid. One possibility is that the expressed products are secreted and deposited in restricted positions within the vitelline membrane or the perivitelline space beyond the egg membrane. After fertilization, these products function as ligands or activators of ligands that in turn relay the localized exterior signals to the developing embryo by binding to specific receptors in the egg membrane, which then trigger pathways that result in the localized control of zygotic gene expression (Gonzalez-Reyes and St Johnston, 1998; St Johnston and Nusslein-Volhard, 1992).

The terminal class, one of the three gene classes involved in the patterning of the A-P axis, specifically in the development of the non-segmented ends of the embryo, as well as the dorsal class that controls patterning events along the D-V axis, both contain maternal effect genes expressed in the follicle cells. In the case of the terminal system, follicle cells covering the termini of the developing oocyte express *torso-like*, most likely secreting the product into the perivitelline space that surrounds the developing egg (Stevens et al., 2003). After fertilization, Torso-like, along with products of other genes expressed in the germline and presented uniformly on the oocyte surface, is involved in the modification and subsequent

positional activation of the germline-secreted ligand Trunk. Although the details of the nature of this modification are still unclear, the activated ligand becomes available exclusively at the poles of the developing embryo, resulting in localized activation of the tyrosine kinase transmembrane receptor Torso, and thus limiting the consequential signaling cascades to the extremities (reviewed in LeMosy, 2003; Li, 2005).

A similar situation is found in the D-V system. Genes expressed in the follicle cells are involved in signaling cascades that ultimately result in controlling embryonic ventral fates. Extensive research has shown that follicle cells take on the default ventral fate in the absence of EGF receptor activation due to the lack of the Gurken signal, which is normally localized exclusively to the dorsal side (Morisato and Anderson, 1995; Nilson and Schupbach, 1999; Riechmann and Ephrussi, 2001). The product of *windbeutel* is expressed in all follicle cells, where it was shown to be involved in ER trafficking (Konsolaki and Schupbach, 1998). One of the substrates of Windbeutel has been shown to be Pipe, which is expressed exclusively in the ventral follicle cells and is localized in the Golgi, where it was shown to be sufficient for zygotic D-V axis formation (Sen et al., 1998). In the absence of Windbeutel, Pipe is retained in the ER (Sen et al., 2000).

There is evidence that the function of Pipe in the Golgi is to modify another product of the follicle cells that turns on the processing steps for the cleavage and activation of the product of *spätzle*. The existence of a missing link is assumed, since Pipe localized to the Golgi cannot directly activate Spätzle, which is expressed in the germline. Therefore, Pipe is expected to be involved in the modification of another product or more that can in turn recognize Spätzle as a

substrate and be involved in its activation steps. Pipe being expressed only in the Golgi of the ventral follicle cells consequently spatially restricts activated Spätzle, which in turn recognizes and binds to the Toll receptors that are themselves found throughout the egg membrane. The subsequent localized signaling cascades ultimately result in the localization of the product of *dorsal*, a transcription factor, into the nuclei of ventral cells exclusively, triggering embryonic ventralizing genetic pathways (for review see Anderson, 1998; Moussian and Roth, 2005).

Some studies suggest the notion that the substrate of Pipe is the follicle cell product Nudel (Hong and Hashimoto, 1995; LeMosy et al., 1998), although experiments showing that activated Nudel is found in follicle cells lacking Pipe argue against this suggestion (Nilson and Schupbach, 1998). Therefore, to date, the missing link between the follicle cells and the cleavage cascades of Spätzle is still unclear.

I.4. The identification and characterization of maternal effect genes.

The late 1980s witnessed a landmark period in the field of *Drosophila* developmental genetics when mutagenesis screens systematically identified and characterized various maternal effect genes (Gans et al., 1975; Nusslein-Volhard et al., 1987; Schupbach and Wieschaus, 1986; Schupbach and Wieschaus, 1989). Random mutations were induced in the genome, followed by recovery of homozygous mutant females, whose eggs and embryos were studied for patterning defects. When such defects were recovered, the affected genes were subsequently identified and studied for their roles in various patterning events.

However, females homozygous for mutations in maternal effect genes that are also required zygotically, and are therefore essential for viability, could not be recovered using this approach. With these genes unidentified, our understanding of the early patterning genetic pathways remained incomplete. One solution has been to generate mosaic females containing homozygous germline or somatic cell clones. This is typically achieved by inducing mitotic recombination events in heterozygous females that would result in populations of cells being homozygous for the mutation, mirroring the genotype of cells of homozygous mutants. Initially, such recombination events were induced in heterozygous cells using irradiation treatments, which have been reported to cause breaks in homologous chromosomes that result in exchange events (Ashburner, 1989) (Figure 2A). However, the efficiency of generating mosaicism using irradiation treatments is low (Perrimon, 1984), which makes the subsequent screening process quite laborious.

In the late 1980s, the site-specific recombination system of the yeast 2μm plasmid, based on the production of the Flipase enzyme (Flp) that recognizes and induces recombination at specific target sites (*FRT*s), was successfully introduced into the *Drosophila* genome (Golic and Lindquist, 1989). Shortly after, the system was used to generate mitotic recombination events with high efficiency between *FRT* sites located on identical locations of homologous chromosomes (Golic, 1991). However, to be able to use the system to generate homozygous cells, the locations of the studied genes need to be distal to the *FRT* sites. Therefore, chromosome arms bearing *FRT* sites near the centromere were generated to

accommodate the great majority of such studied genes (Chou and Perrimon, 1996; Xu and Rubin, 1993) (Figure 2B).

After the generation of mitotic recombination, the cell clones need to be identified to be subsequently studied. This is typically done using the expression patterns of cell markers such as green fluorescent protein (GFP). When mother cells heterozygous for *GFP* distal to the *FRT* sites undergo mitotic recombination, one of the daughter cells acquires two copies of the *GFP* transgene, while the other daughter cell gets none. Cell populations derived from either of these two cases can therefore be easily identified due to expressing GFP at double the heterozygous intensity versus the lack of GFP expression, respectively (Figure 3).

I.5. Technique based on a dominant female sterile mutation for the identification of eggs from complete germline cell clones.

To study whether zygotically required genes were also required in the germline of adult females for the proper development of their eggs and embryos, a technique relying on dominant female sterile (DFS) mutations was developed. The DFS mutations used in this case affect genes that are also themselves expressed exclusively in the germline cells (Perrimon, 1984; Perrimon and Gans, 1983; Wieschaus, 1980). A widely and successfully used germline DFS mutation is ovo^{D1}, whose phenotype is the lack of egg laying by the mutant females (Perrimon and Gans, 1983). Females heterozygous for the mutation of interest as well as an ovo^{D1} mutation on the homologous chromosome were generated. Mitotic recombination was then induced to produce clones of homozygous mutant cells,

Figure 2. Inducing mitotic recombination events by irradiation versus the Flp-FRT system.

(A) If a cell is subjected to irradiation (red arrows), random mitotic recombination events due to double strand breaks could be induced (dashed red crosses). If such an event takes place proximal to a particular mutation (✱) in a heterozygous cell, then one of the generated daughter cells will be homozygous mutant and so will the population of cells derived from it. When using irradiation treatments, such events occur at a low frequency. **(B)** If a cell has corresponding *FRT* sites (▶) on homologous chromosomes, as well as a Flipase source (not shown here), then site-specific recombination events could take place specifically at the *FRT* sites during mitosis. If *FRT*-mediated recombination takes place in a cell heterozygous for a particular mutation (✱) then one of the generated daughter cells will be homozygous mutant and so will the population of cells derived from it. The Flp-FRT system induces mitotic recombination at a significantly high frequency.

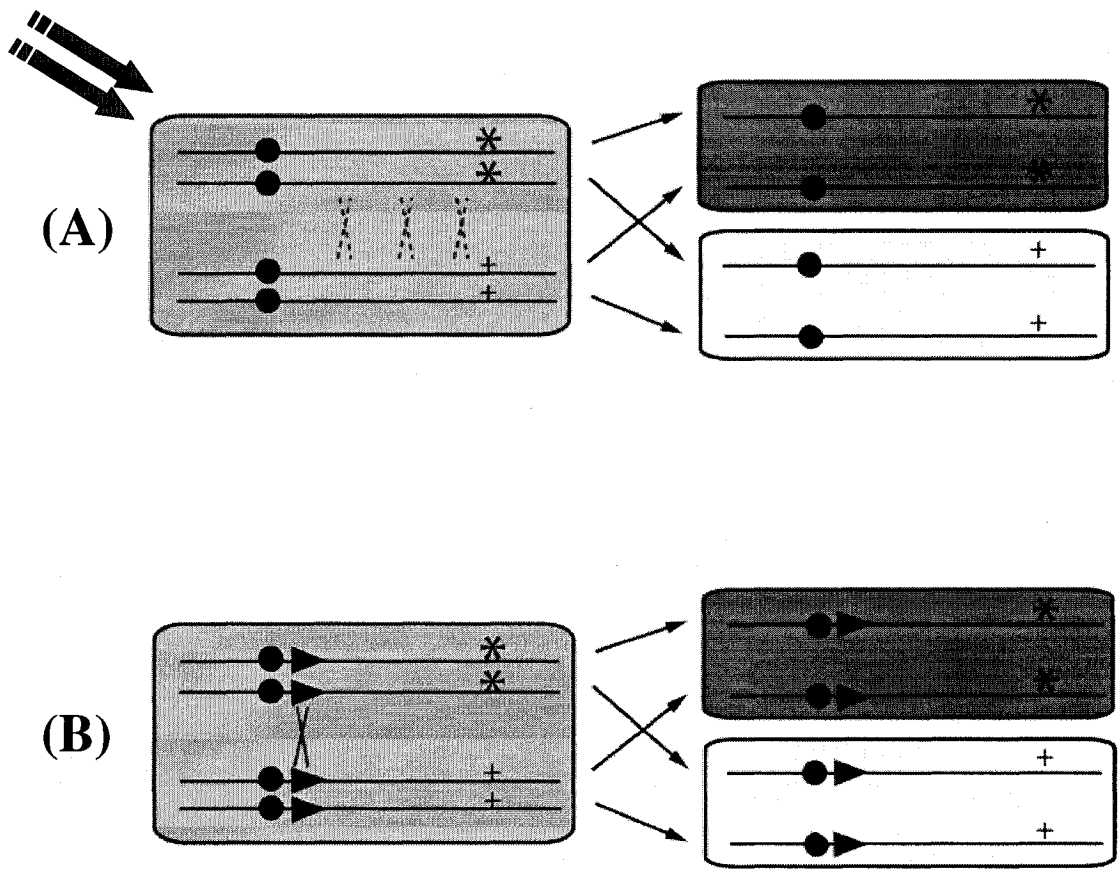
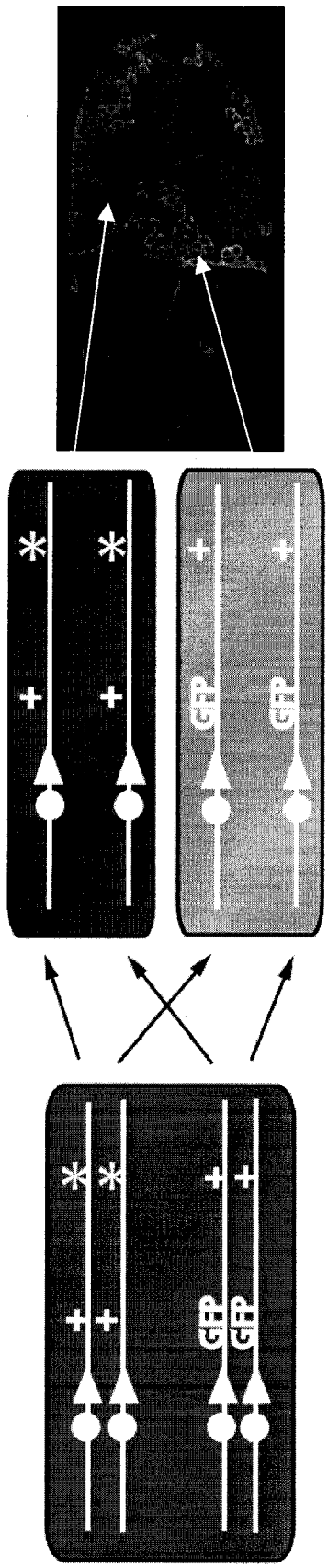

(A)
(B)

Figure 3. Identifying cell clones using the cell marker GFP.

Site-specific mitotic recombination (red cross) can be efficiently induced at the corresponding *FRT* sites (▶) using the Flp-FRT system in a cell heterozygous *in trans* for a mutation of interest (✱) and *GFP,* both distal to the *FRT* sites. This gives rise to a daughter cell homozygous for the mutation and therefore not expressing any GFP, and another daughter cell homozygous for *GFP* and therefore expressing the protein at twice the intensity as the expression in heterozygous cells; as shown on the right in the example of an egg chamber with visible cell clones in the follicular epithelium.


GFP
GFP
GFP
GFP

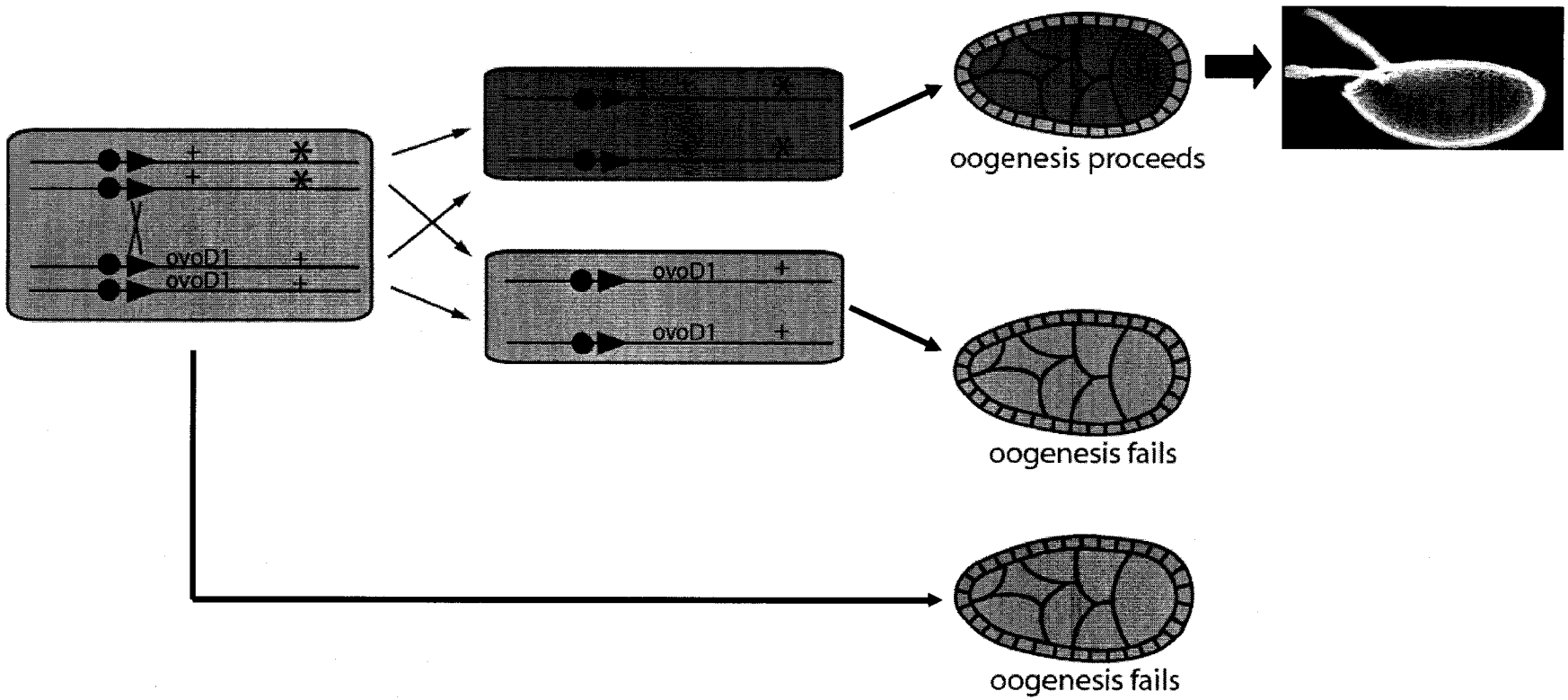
which lack the ovo^{D1} mutation. Using this approach, all the eggs that are recovered from mosaic females are products of egg chambers with complete germline clones: all the 16 germline cells have to lack the expression of ovo^{D1} for oogenesis to proceed, which therefore means that they have to be homozygous for the mutation of interest (Perrimon et al., 1989).

Initially, mosaicism was generated using irradiation treatments. However, after the development of the Flp-FRT system in *Drosophila*, it was used in conjunction with the ovo^{D1} DFS mutation system to generate females mosaic for mutations on the X-chromosome; where the ovo^{D1} locus is found in the genome (Chou and Perrimon, 1992). Later, to use the system to study genes on the autosomal chromosomes as well, first the ovo^{D1} mutation was cloned and copies were recovered on each of the four autosomal arms (Chou et al., 1993), and then proximal *FRT* sites were recombined onto each of these ovo^{D1} arms (Chou and Perrimon, 1996). Therefore, the technique currently provides an invaluable method for studying zygotically required maternal effect genes expressed in the germline: it allows the recovery and identification of eggs from complete germline clones; which are identical to those from homozygous mutant females, had they been viable (for review see Perrimon, 1998) (Figure 4).

It is important to note that since the DFS mutations such as ovo^{D1} affect the germline exclusively, the technique described above can only be used to study maternal effect genes required in the germline. However, as addressed above, a number of maternal effect genes are expressed in the follicular epithelium. Products of these genes are directly involved in signaling cascades leading to embryonic patterning events. Further studies are needed to uncover more of them,

Figure 4. The use of *ovo*^{D1} in the Flp-DFS system to identify eggs from complete germline clones.

Females heterozygous for *ovo*^{D1}, which is expressed in the germline cells, do not lay eggs due to failed oogenesis. In the Flp-DFS system, the Flp-FRT technique is used to generate site-specific mitotic recombination (red cross) at corresponding *FRT* sites (▶) in females heterozygous for *ovo*^{D1} as well as a mutation of interest (✱). The resulting homozygous mutant daughter cells, and subsequently the cell population derived from them, will lack the expression of *ovo*^{D1}. When such generated homozygous mutant cells make up the entire germline cell population of an egg chamber, oogenesis can proceed to give rise to a mature egg. Therefore the only eggs laid by the heterozygous mosaic females are products of egg chambers with homozygous mutant germline.


ovoD1
ovoD1
ovoD1
ovoD1
oogenesis proceeds
oogenesis fails
oogenesis fails

which would deepen our understanding of a large number of early developmental processes. For example, in the case of embryonic dorsoventral axis specification discussed above, Pipe in the Golgi modifies the product of an unknown maternal effect gene that in turn is involved in the activation of the ligand Spätzle. A Flp-DFS system for the recovery and identification of eggs from complete follicle cell clones would be of great value to uncover such maternal effect genes expressed in the follicle cells.

I.6. Lack of technique based on a dominant female sterile mutation for the identification of eggs from complete follicle cell clones.

As described above, when studying the maternal effect of zygotically lethal mutations the common approach is to generate mosaic females and study their eggs and embryos for patterning defects. When studying maternal effect genes expressed in the follicle cells, a most interesting class of eggs laid by such mosaic females contains those that are products of complete follicle cell clones, where the mutant clone comprises the entire follicular epithelium. With respect to the follicle cells, such eggs would be identical to ones recovered from homozygous mutant females (Figure 5). However, as explained above, when the egg is laid the follicle cells are no longer present. They secrete the eggshell material on top of the mature oocyte in the late stages of oogenesis before they themselves degenerate (Spradling, 1993). Therefore, once the egg is deposited and embryonic development begins, there are no cells available to be examined for the expression of conventional cell markers like GFP as described above (see Figure 3).

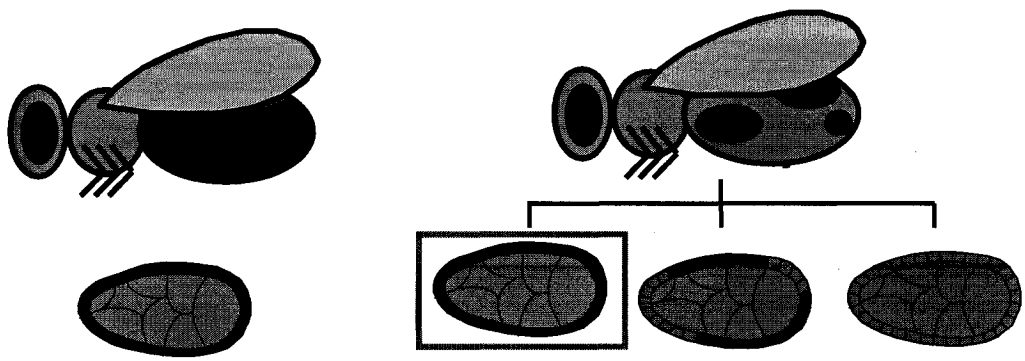


Figure 5. The follicular epithelia of egg chambers with complete follicle cell clones are identical those of homozygous mutant egg chambers.

When a female is homozygous for a mutation of interest, the follicular epithelium in all of her egg chambers are also homozygous mutant (left). To study homozygous lethal mutations, homozygous cells are routinely generated in viable heterozygous females (right) by inducing mitotic recombination events. The follicular epithelia of egg chambers with complete follicle cell clones in such mosaic females are identical to the follicular epithelia of egg chambers found in homozygous females had they been viable. Therefore egg chambers with complete follicle cell clones are very interesting to study (red box).

With the lack of a Flp-DFS system for the study of maternal effect genes expressed in the follicle cells, which would allow the identification and recovery of complete follicle clones, researchers have resorted to the alternative method of indirect inference. For example, if the percentage of mutant phenotype eggs laid by mosaic females is similar to the percentage of egg chambers with follicle cell clones in dissected female siblings, the eggshell phenotype is assumed to be caused by the mutant clones, despite of the lack of direct evidence that the abnormal eggs are indeed derived from egg chambers with mutant clones (Dobens et al., 2001; Morimoto et al., 1996; Wasserman and Freeman, 1998). Therefore, it is clear that the development of a marker that would allow for the direct identification of this interesting class of eggs is needed.

A visible marker recording the genotype of homozygous mutant follicle cells into the eggshell material they secrete before degenerating has been indeed previously developed (Nilson and Schupbach, 1998). This marker is a mutant allele of the gene *defective chorion 1* (*dec-1*), present on the X chromosome in the *Drosophila* genome (Hawley and Waring, 1988). Expressed in the follicle cells of developing egg chambers, *dec-1* produces three alternatively spliced mRNAs that encode proproteins sized 106, 125, and 177 kDa. These are called fc106, fc125, and fc177, respectively. These proteins are secreted by the follicle cells and are then cleaved to generate at least five distinct protein products, which become localized within different parts of the mature chorion (Nogueron and Waring, 1995).

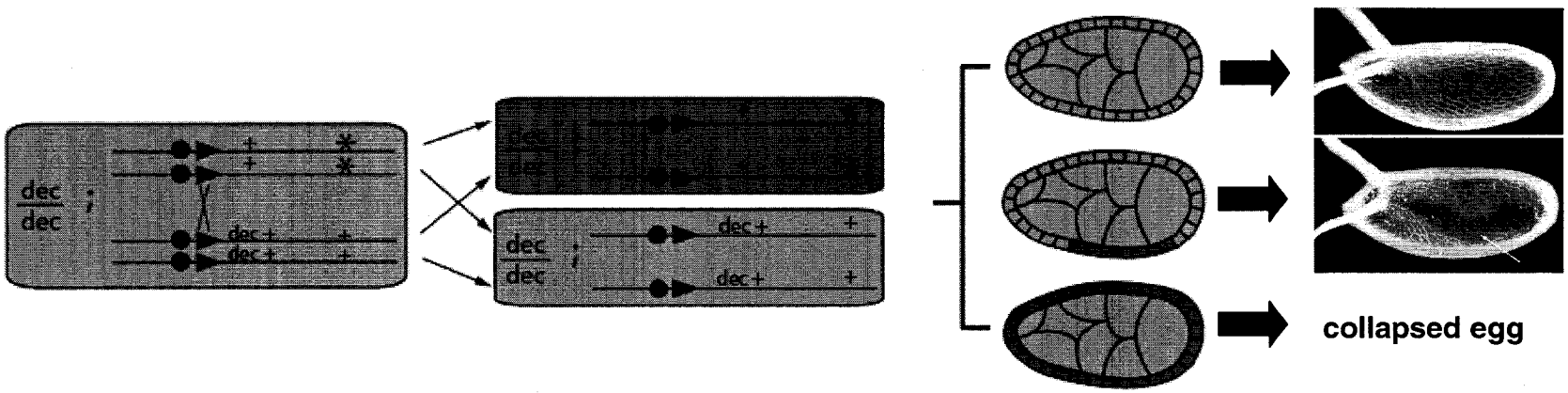
The developed marker technique relied on generating females mutant for *dec-1* on the X chromosome as well as heterozygous for the mutation of interest

and a rescuing transgene, *dec+*, each on one of the appropriate homologous *FRT* chromosomes. Because of the presence of *dec+*, these females lay eggs with normal eggshell material. When mitotic recombination is induced, stretches of follicle cells within the epithelia of some egg chambers become homozygous for the mutation of interest, and consequently lack the rescue construct. Because of the mutant *dec-1* background, these follicle cell clones hence secrete patches of faulty eggshell material, which are easily visually recognized on the surface of the eggs (Figure 6). Positional requirements of the mutation in question could consequently be worked out by studying the phenotypes of different eggs produced by egg chambers with follicle cell clones in different regions of the epithelium, as well as the embryos that develop within them. This technique proved very useful in dissecting the positional requirements for the maternal effect genes *windbeutel* and *pipe* expressed in the follicle cells, as described above (Nilson and Schupbach, 1998).

As useful as this marker technique proved to be, when the generated follicle cell clones comprise the majority of the follicular epithelium, the resulting patches of faulty eggshell material secreted become too big for the egg to remain intact. Therefore, eggs of complete follicle cells clones would collapse, making subsequent phenotype studies practically impossible (Figure 6). There was still a need for a marker technique to recover and identify such eggs to be studied.

Figure 6. A previously developed eggshell marker for follicle cell clones is not suitable for studying eggs from complete follicle cell clones.

In this system, mutant *dec-1* females are generated that are also heterozygous *in trans* for the mutation of interest (✱) as well as a *dec+* rescue construct each on one of the homologous *FRT* chromosome arms. Because of the rescue construct, these *dec-1* mutant females lay eggs with wild type eggshells. Using the Flp-FRT system, mitotic recombination (red cross) is induced at the *FRT* sites (▶) to generate daughter cells homozygous for the studied mutation and therefore lacking the *dec+* rescue construct. In the case where such homozygous mutant cells are follicle cells, the eggshell material they secrete is faulty due to also being mutant for *dec-1*; and therefore eggshell patches produced by such mutant stretches of follicle cells can be identified. However, if the entire follicular epithelium is homozygous for the studied mutation, the entire secreted eggshell will be faulty and therefore the resulting egg from such egg chambers with complete follicle cell clones would collapse. This technique is useful for the dissection of the positional requirements of maternal effect genes, but cannot be used to identify eggs from complete mutant follicle cell clones.



(Adapted from Nilson and Schupbach, 1998)

I.7. Development of a technique based on a dominant female sterile mutation for the identification of eggs from complete follicle cell clones.

During my project, I have developed a Flp-DFS system, where the DFS mutation affects the follicle cells. This system allows the recovery and identification of eggs that are products of egg chambers with complete follicle cell clones similar to the Flp-DFS system based on the ovo^{D1} mutation for the recovery of eggs from complete germline clones. The scheme relies on the use of another mutant allele of *dec-1*, developed in the lab of Gail Waring at Marquette University in Wisconsin. They created targeted deletions in the *dec-1* coding sequence to study the effects of eliminating specific protein products of the gene on the integrity of the eggshell. One of these deletions gave rise to a dominant negative form of *dec-1*, which henceforth is referred to as dec^{DN}. Transgenic dec^{DN} females were found to lay completely collapsed eggs due to flaccid eggshell material (Figure 7). These collapsed eggs cannot sustain the development of an embryo, making the dec^{DN} females sterile. Therefore this transgene acts as a DFS mutation.

The system relies on the generation of females heterozygous for the mutation of interest on the appropriate *FRT* chromosome as well as the homologous corresponding *FRT* chromosome bearing a copy of the dec^{DN} transgene. Because they have a copy of dec^{DN}, these females lay collapsed eggs, as mentioned above. However, induction of site-specific mitotic recombination using the Flp-FRT system in follicle cells produces daughter follicle cells homozygous for the mutation of interest with the copy of the dec^{DN} transgene

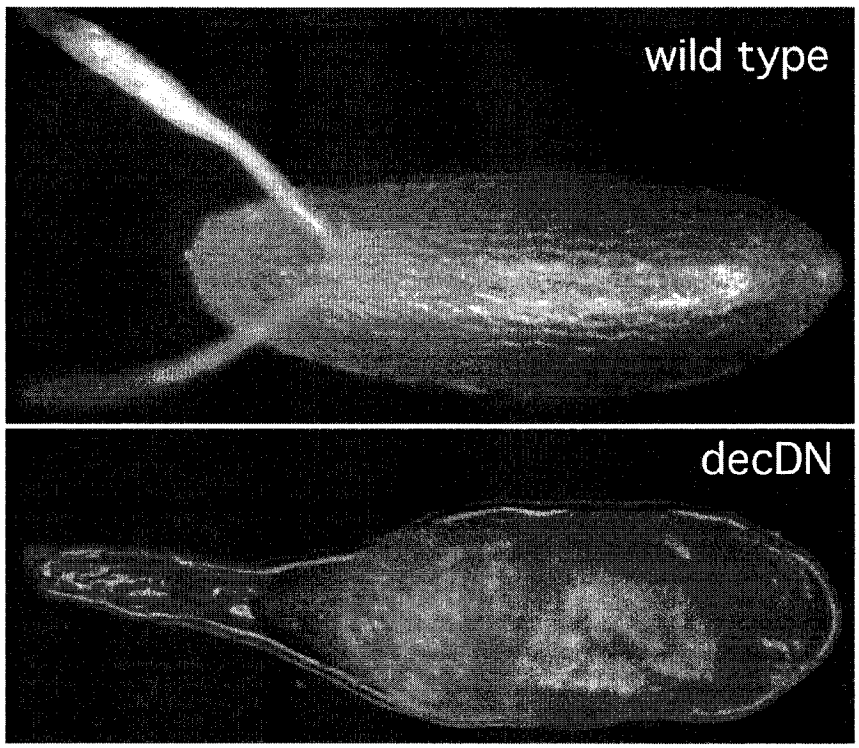


Figure 7. Examples of an egg from a wild type female and a collapsed egg from a transgenic *dec*^{DN} female.

Top: Egg from wild type female, dorsal view. When eggs are collected on apple juice plates, those from wild type females are intact with normal eggshell material.

Bottom: Eggs from *dec*^{DN} females are completely collapsed. Eggs from *dec*^{DN} females seem like deflated balloons due to their faulty flaccid eggshell material.

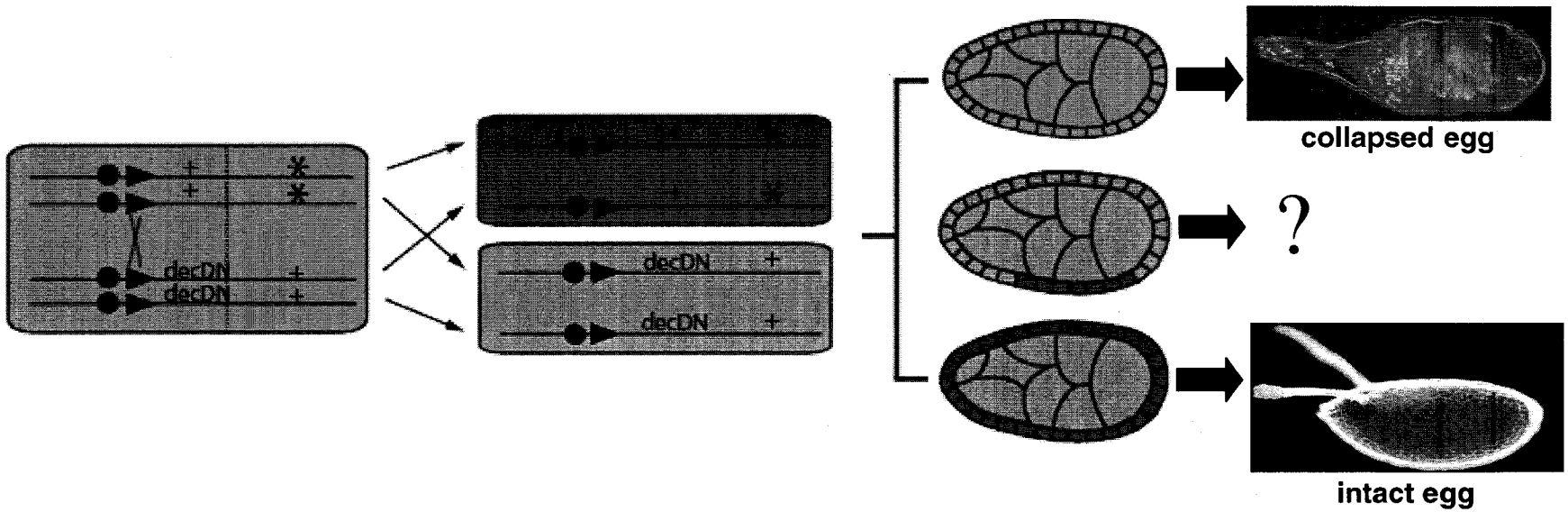
eliminated. In the case where such homozygous mutant cells comprise the entire follicular epithelia of egg chambers, normal eggshell material is secreted and the resulting laid eggs are intact amongst a collection of otherwise collapsed eggs, which are not products of such complete follicle cell clones (Figures 8 and 9).

The outcome of egg chambers with only patches of homozygous cell clones in the follicular epithelium as opposed to complete follicle cell clones was more difficult to predict. We expected a possible result to be the production of intact eggs with visible patches of faulty eggshell material due to follicle cells retaining dec^{DN}, especially in the case when such dec^{DN} follicle cells were a minority in the epithelium. Similar to the marker technique that uses the *dec+* rescue construct in a *dec-1* mutant background (Nilson and Schupbach, 1998), a possibility could have been that the proper eggshell material secreted by the non-dec^{DN} cells would be able to support such minor dec^{DN} patches, and therefore they would be visibly distinguishable just as mutant *dec-1* patches marking the eggshell stretches secreted by follicle cells homozygous for the mutation of interest were visible (see Figure 6).

The success of developing this technique provides a conclusive identification system of mutant eggs derived from egg chambers with mutant follicular epithelia, identical to eggs that would be recovered from homozygous mutant females. With this detection system, subsequent studies focusing on the roles of candidate maternal effect genes expressed in the follicular epithelium, as well as screens to identify novel ones, will be greatly facilitated.

Figure 8. Scheme showing the use of *dec*^{DN} as an eggshell marker in the developed Flp-DFS system for the identification of eggs from complete follicle cell clones.

In the developed system, females heterozygous *in trans* for a mutation of interest (✱) and the *dec*^{DN} transgene each on one of the homologous *FRT* chromosome arms are generated. These transgenic females lay collapsed eggs and are therefore sterile. Using the Flp-FRT system, mitotic recombination (red cross) is induced at the *FRT* sites (▶) to generate daughter cells homozygous for the studied mutation and therefore lacking the *dec*^{DN} transgene. In the case where the whole follicular epithelium of an egg chamber is composed of such homozygous mutant cells, the entire secreted eggshell material is wild type and therefore the produced egg stands intact and does not collapse. This developed technique allows the identification of eggs from complete mutant follicle clones to study them for patterning defects.


decDN
decDN
decDN
decDN
collapsed egg
?
intact egg

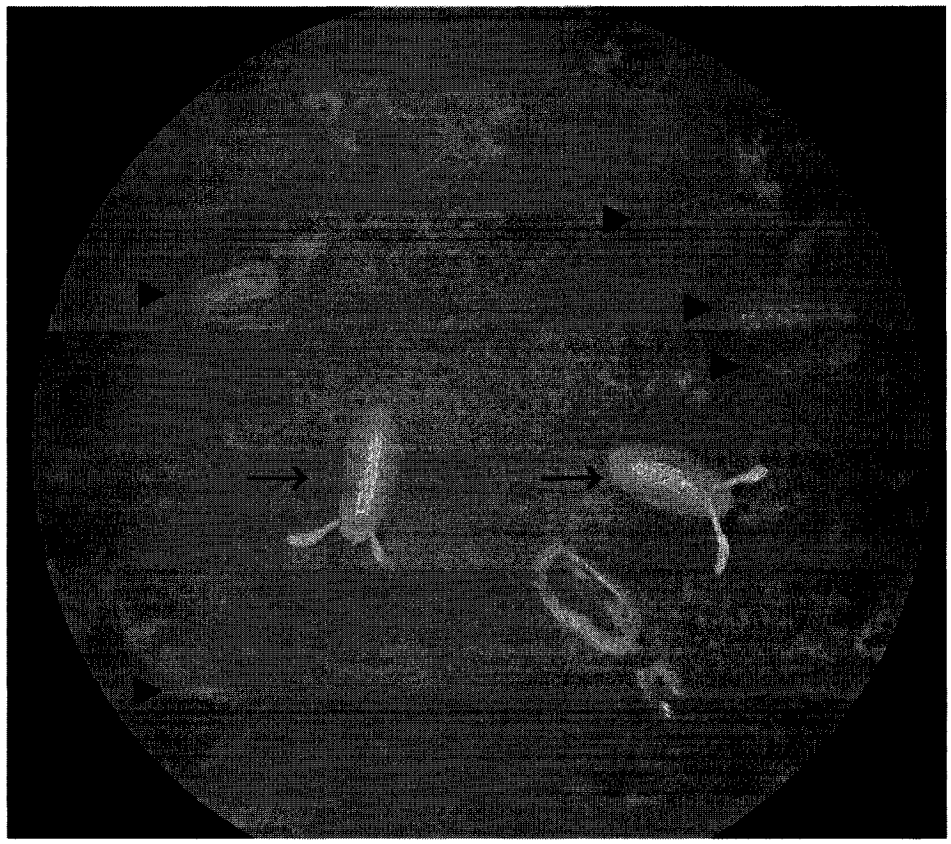


Figure 9. Mosaic *dec*^{DN} females lay intact eggs from complete follicle cell clones amongst otherwise collapsed eggs.

Intact eggs (arrows) can be easily visually distinguished from collapsed eggs (arrowheads) on an egg collection apple juice plate.

Chapter II. Methods and Materials

II.1. Creating the *pCaSpeR4 - dec^{DN}* construct with the *w+* marker.

This construct was created in the Waring lab. Initially, *dec-1* was cloned and inserted into *pCaSpeR4* to be used as a rescue construct (Waring et al., 1990). This was done by first isolating an XhoI – BglII fragment of genomic DNA containing the *dec-1* gene as well as about 1kb of 3' flanking region. The vector *pCaSpeR4* was also cut with XhoI and BamHI. The DNA fragment containing the *dec-1* gene was then ligated into the vector (BglII and BamHI overhangs are compatible). A NcoI – NcoI fragment of genomic DNA that contains about 2.5kb of 5' flanking region and extends into the ORF of *dec-1* was also isolated. This was in turn inserted into a *pGEM 5Z* vector that was cut with NcoI. A fragment was then removed from the resulting *pGEM 5Z* construct using SalI and XhoI. This fragment, compatible on both ends with XhoI overhangs, was inserted into the above described *pCaSpeR4* construct after being itself cut with XhoI. Finally, ligation products were screened to identify rescue constructs with the inserted fragment in the correct orientation, since it could have also been ligated in reverse (G. Waring, personal communication).

This rescue construct was then further modified by Dan Spangenberg, a former graduate student in the Waring lab. He deleted the s20 region in the coding region of *dec-1*, which codes for the fc106 transcript product, using PCR. To do this, he designed two sets of primers, one pair to amplify the region directly 3' of the region to be deleted, and the other to amplify the region directly 5' of it. The

two primers (each belonging to a different pair) closest to the fragment targeted for deletion also contained SphI restriction sites at their 5' ends, followed by a few random bases to increase the efficiency of restriction reactions. Following PCR, the two amplified fragments were cut using SphI, producing complementary 4-base overhangs, which were subsequently blunted using the Klenow fragment treatment. The two DNA pieces were then blunt-end ligated to reconstruct the coding sequence but with the required deletion of the s20 region. The fragment was then digested with XhoI and XbaI and inserted into the *pCaSpeR4 – dec-1* rescue construct that has also been digested with XhoI and XbaI (D. Spangenberg, personal communication). The result is *pCaSpeR4 – dec-1* containing the fc106 s20 deletion, or for simplicity *pCaSpeR4 – dec^{DN}* construct, which was generously provided to us by the Waring lab.

II.2. Using PCR to confirm the mapped position of the *dec^{DN}* transgene in the *dec^{DN}* lines.

II.2.1. Primers used

To confirm the mapped cytological position of 98F of the *dec^{DN}* transgene in the *dec^{DN}* 21.1 line, I designed the forward primer F2: 5' – CCTTTCACTCGCACTTATTGC – 3' complementary to a DNA fragment within the transgene; the forward primer F19: 5' – CATTGGTATTCGGTGCGG – 3' complementary to a genomic DNA fragment 3' of the transgene, and the reverse primer B49: 5' – TTTCCTTTGCCTTCCCATC – 3' complementary to a DNA fragment 3' of both of the forward primers using MacVector software and ordered them from BioCorp Canada. The amplification of a DNA fragment of 282bp

when using F19 and B49 served as a control. The amplification of a DNA fragment of a size of 547bp when using F2 and B49 confirmed the location of the transgene (see Figure 10 A, B).

To confirm the mapped cytological position of 52B of the dec^{DN} transgene in the dec^{DN} 23.2.3 line, I designed the forward primer dec23.2.3 F1: 5' – AAGTGGATGTCTCTTGCCGACG – 3' complementary to a DNA fragment within of the transgene, the forward primer dec23.3.3 F6: 5' – GGTTGTCTGCTGTCTTTCTCCTGC – 3' complementary to a genomic DNA fragment 3' of the transgene, and the reverse primer dec23.2.3 B6: 5' – TGTGAGTGCGAATGCTCGTGTC – 3' complementary to a DNA fragment 3' of both of the forward primers using MacVector software and ordered them from BioCorp Canada. The amplification of a DNA fragment of a size of 348bp when using dec23.2.3 F6 and dec23.2.3 B6 served as a control. The amplification of a DNA fragment of 468bp when using dec23.2.3 F1 and dec23.2.3 B6 confirmed the location of the transgene (see Figure 10 A, C).

II.2.2. Genomic DNA extraction.

I extracted genomic DNA from flies of the dec^{DN} 21.1 and the dec^{DN} 23.2.3 lines on separate occasions following the Quick Genomic DNA Prep (Rubin, 2000). I collected 30 flies and froze them in a 1.5ml microcentrifuge tube. I then ground them in 400µl of Buffer A (100mM Tris-Cl pH 7.5, 100mM EDTA, 100mM NaCl, 0.5% SDS) with a tissue grinder and incubated the tube at 65°C for 30 minutes. I then added 800µl of Buffer B (200ml of 5M potassium acetate and 500ml of 6M litium chloride) and incubated the tube on ice for 30 minutes. I

followed this by two steps of centrifugation at 12,000 rpm at room temperature for 15 minutes and then transferred 1ml of the clear supernatant into a clean tube in which I precipitated the DNA by adding 600µl of isopropanol and centrifuging at 12,000 rpm at room temperature for 15 minutes. I finally washed the pellet with 70% ethanol and resuspended it in 150µl of TE (10mM Tris•Cl, 1mM EDTA).

II.3. Using irradiation to generate *FRT82B dec^{DN}*.

I crossed *w; FRT82B/TM3, Ser* (Bloomington Stock no. 2035 with *w* and *Ser* introduced) virgin females to *w; dec^{DN}w+* males from the *dec^{DN}* 21.1 line (Waring lab) and brooded the parents into new food vials every two days. I subjected vials with progeny at the second instar larval stage to a dosage of irradiation of 1500 rads from a gamma source (Animal Resources Centre at McGill University's Faculty of Medicine Building) and then reared them at 25°C. From the progeny, I collected adult *w; FRT82B/dec^{DN}w+* males with potential recombination events in their gametes and crossed them to *w* females in vials with food containing Geneticin at a concentration of 0.75mg/ml (vials were prepared according to protocol in Xu and Rubin, 1993). The *FRT* construct contains a gene, *neoR*, that imparts a Geneticin-resistance that non-*FRT* flies lack. Because *neoR* has a heat-shock promoter (*hs*), I reared the progeny at 25°C (Xu and Rubin, 1993). Progeny surviving the Geneticin selection, therefore, had the *FRT* locus and were subsequently screened for adult males with orange eyes. In total, I recovered 49 such recombinants.

II.4. Ovary Dissection and Mounting.

I dissected samples of ovaries in PBS in 20 minutes and then fixed them in 600µl heptane, 100µl PBS (Phosphate-Buffered Saline: 1l consists of 8g NaCl, 0.2g KCl, 1.44 Na_2HPO_4, 0.24g KH_2PO_4 in distilled H_2O, pH adjusted with HCl), 100µl 10% paraformaldehyde and 5µl 20% NP40 on a nutator for 20 minutes. I followed this with three rinses and then three 10-minute washes, both in PBST (PBS + 0.1% Tween-20). Finally, I mounted the samples on slides using the *SlowFade*® Antifade Kit (Molecular Probes).

II.5. Using *P*-element – induced male recombination to generate *FRT42D dec*^{DN}.

I was able to maintain the stock *w; Sp/CyO; ry Sb e Δ2-3/ TM6B, Tb*$^{+}$ (Bloomington Stock no. 3629), with a genomic transposase source, *Δ2-3*, on the third chromosome at cytological position 99B (Robertson et al., 1988) in a healthy condition. My attempts to rear and maintain another stock with the transposase source on the second chromosome (*yw; CyO, H{PΔ2-3}HoP2.1/Bc Egfr*^{E1} Bloomington Stock no. 2078) failed repeatedly. Since the technique of inducing male recombination using *P*-elements relies on the genomic transposase source being on a different chromosome than the *P*-element, I decided to use the *dec*^{DN} 23.2.3 line (Waring lab) where the *dec*^{DN} transgene was mapped and confirmed to a distal location on 2R. A frequently used *FRT* site on 2R is *FRT42D*. I set up a series of crosses to recover males in whose germline induced recombination would generate *FRT42D dec*^{DN} chromosomes.

I crossed *w; dec^{DN}w+/+; +/+* males from the *dec^{DN}* 23.2.3 line to *w; Sp/CyO; ry Sb e Δ2-3/TM6B, Tb^{+}* females (Bloomington Stock no. 3629). The progeny was reared at 18°C to minimize transposase activity in the germline of *w; dec^{DN}w+/Sp or CyO; ry Sb e Δ2-3/ +* males which I screened for and crossed to *w; FRT42D* females (Bloomington Stock no. 1802 with *w* introduced on the X chromosome). I kept these crosses at 18°C, to continue minimizing the transposase activity in the male parents, but I reared the progeny at 25°C to maximize such activity in the germline of *w; dec^{DN}w+/FRT42D; ry Sb e Δ2-3/+* males, to induce recombination events on the second chromosome resulting in gametes containing *FRT42D dec^{DN}*.

I then crossed these males, with potential recombinant gametes, to *eyFlp; FRT42D* females (Bloomington Stock no. 1802 with *eyFlp* introduced from *eyFlp, GMR-lacZ; FRT42D l(12) w+/BC* from Don van Meyel, Centre for Research in Neuroscience, McGill University). Screening the resulting progeny, I selected males with mosaic eyes with orange and white color patches, indicating their inheritance of recombinant *FRT42D dec^{DN}* chromosomes from their fathers; yet lacking the genomic transposase source *Δ2-3* to prevent further transposition events. In total, I recovered 10 such males, each from different father to ensure that they represent 10 separate recombination events, and established a stock from each. Females from only five of the lines consistently lay collapsed eggs, indicating complete penetrance of the *dec^{DN}* phenotype. The other five lines with incomplete phenotype penetrance were discarded.

II.6. Using the microinjection transgenesis technique to generate *FRT dec^{DN}* chromosome arms.

II.6.1. Microinjection of embryos.

The *pCaSpeR4 – dec^{DN} P*-element construct, with *w+* as a marker, was created and provided to us by the Waring lab. I maxipreped and purified it using the Plasmid Maxi Kit (QIAGEN). I dissolved the yield in ddH_2O at a final concentration of 690µg/ml. Beili Hu, the microinjection technician specialist, then co-injected the construct with the *P*-element transposase helper plasmid *pTurbo* at a concentration of 100-150µg/ml into the appropriate *FRT* embryos I provided. Two rounds of injections were carried out for the *w; FRT42D; FRT82B* stock (Bloomington Stock no. 8216) to yield *FRT dec^{DN}* chromosome arms for 2R and 3R. Two rounds of injections were carried out for the *w; FRT40A; FRT80B* stock (Bloomington Stock no. 8215) to yield *FRT dec^{DN}* chromosome arms for 2L and 3L. The injected embryos were allowed to develop in food vials at 25°C.

II.6.2. Screening for *FRT dec^{DN}* chromosome arms resulting from injection.

I crossed each adult injected fly individually to two *yw* adults of the opposite sex at 25°C. I carefully screened the progeny for transformant flies with rescued eye color due to the acquisition of the *w+* marker of the *pCaSpeR4 – dec^{DN}* construct. I only scored the transformant females before discarding them, since stocks could not be established from them because they were sterile due to being transgenic for *dec^{DN}*. Transformant *dec^{DN}* males, on the other hand, I individually crossed to *eyFlp; FRT* females to identify transformation events resulting in *dec^{DN}* being

incorporated distal to a functional *FRT* site. In the case when *w; FRT42D; FRT82B* embryos were injected, I crossed each transformant orange-eyed male to *eyFlp; FRT42D* females (Bloomington Stock no. 1802 with *eyFlp* introduced from *eyFlp, GMR-lacZ; FRT42D l(12) w+/BC* from Don van Meyel) and then to *eyFlp; FRT82B* females (Bloomington Stock no. 2035 with *eyFlp* introduced from *eyFlp; GMR-lacZ; FRT82B l(2) w+/ Tb,hu* from Don van Meyel). In the case when *w; FRT40A; FRT80B* embryos were injected, I crossed each transformant orange-eyed male to *eyFlp; FRT40A* females (Bloomington Stock no. 1821 with *eyFlp* introduced from *eyFlp, GMR-lacZ; FRT42D l(12) w+/BC* from Don van Meyel) and then to *eyFlp; FRT80B* females (Bloomington Stock no. 1988 with *eyFlp* introduced from *eyFlp; GMR-lacZ; FRT82B l(2) w+/ Tb,hu* from Don van Meyel). In every case, the progeny were allowed to develop at 25°C.

As adults, I screened the progeny of the transformant males for the presence of mosaic eyes with orange and white patches (see Figure 13). *eyFlp; FRT* females yielding such mosaic-eyed progeny following being crossed to a transformant male indicated that the tested transformant line had the dec^{DN} transgene successfully incorporated distal to the associated *FRT* site. Using this screening method of producing color patches in the eye due to cell clones allowed me to simultaneously test for the functionality of the *FRT* site in question and its ability to successfully mediate mitotic recombination using the Flp-FRT system. Such *FRT* dec^{DN} lines were maintained.

II.7. Testing the generated *FRT dec^{DN}* lines.

II.7.1. Testing the *FRT82B dec^{DN}* lines.

The four *FRT82B dec^{DN}* lines generated by the first round of injection of the *w; FRT42D; FRT82B* embryos were used to test the Flp-DFS system. Males from each line were crossed to *yw hsFlp; FRT82B GFP/TM6B, hu* females (Bloomington Stock no. 5188 with *yw hsFlp* introduced), *yw hsFlp; FRT82B cic^{BA53}/TM6B, hu* females (Nilson lab stock *FRT82B cic^{BA53}* with *yw hsFlp* introduced), *yw hsFlp; FRT82B cic^{U6}/TM6B, hu* females (*FRT82B cic^{U6}/TM3, Sb* from Donald Morisato with *yw hsFlp* introduced), *yw hsFlp; FRT82B pnt$^{\Delta 88}$/TM6B, hu* females (from Hannele Ruohola-Baker) and *yw hsFlp; FRT82B puc^{A251}/TM3, Sb* females (from Leonard Dobens). Adult progeny of each cross were heat-shocked for 1 hour at 37°C on three consecutive days to induce Flp-FRT site-specific mitotic recombination. Occasionally the heat-shock treatment was administered during the pupal stages. In that case, vials containing tan-colored pupae were heat-shocked following the same protocol also for three consecutive days. In each case, 7-12 mosaic females heterozygous for the two corresponding *FRT82B* chromosomes were selected and then placed in egg collection cages with an equal number of males of any genotype. Periodically, the apple juice egg collection plates were changed, and the eggs counted and examined for intact ones.

II.7.2. Testing the *FRT42D dec^{DN}* lines.

Four generated *FRT42D dec^{DN}* lines were tested: two lines generated by the *P*-element – induced male recombination method, namely lines MR-25.1 and MR-

77.1, and two lines generated by the first round of injection of the *w; FRT42D; FRT82B* embryos, namely lines inj-177.2 and inj-217.1. In each case, males from the lines were crossed to *yw hsFlp; FRT42D GFP/CyO* (from David Dansereau, Bloomington Stock no. 5626 with *yw hsFlp* introduced). Adult progeny were heat-shocked for 1 hour at 37°C for three consecutive days. The mosaic females heterozygous for the two corresponding *FRT42D* chromosomes were selected and placed in egg collection cages. The apple juice egg collection plates were changed periodically and the eggs were counted and examined for any intact ones.

Chapter III. Generation of the *FRT dec^{DN}* chromosomes.

The developed eggshell marker technique relies on the use of a DFS mutation expressed in the follicle cells to identify eggs from complete follicle cell clones. As mentioned above, this marker, *dec^{DN}*, was created by a targeted deletion of a portion of the *dec-1* coding sequence. A *P*-element construct bearing this mutant form marked with the eye color gene *miniwhite*, *w+*, was subsequently produced and then used to generate multiple independent transgenic lines. The *dec^{DN}* allele was found to act as a dominant negative form of *dec-1*, since transgenic females with a single copy of *dec^{DN}* in a wild type *dec-1* background lay completely collapsed eggs, which resemble deflated balloons on the surface of egg collection plates (see Figure 7). These collapsed eggs never hatch, rendering the *dec^{DN}* females sterile. Four transgenic lines (*dec^{DN}* 21.1, *dec^{DN}* 21.2, *dec^{DN}* 21.4 and *dec^{DN}* 23.2.3), as well as the *dec^{DN} P*-element construct, have been generously provided to us by Gail Waring.

As described above, in the scheme for using the Flp-DFS system based on *dec^{DN}*, eggs from complete follicle cell clones can be identified for being intact (see Figures 8 and 9). Therefore, I tested the penetrance of the *dec^{DN}* collapsed-egg phenotype to ensure that *dec^{DN}* transgenic females do not occasionally lay random intact eggs, which could potentially be confusing when the mutant intact eggs are ultimately recovered. The *dec^{DN}* phenotype was found to be completely penetrant in the four *dec^{DN}* lines (Table 1).

Since the proposed technique is a combination of both the Flp-FRT system and the DFS system as described earlier, the first crucial step in its development

day	decDN 21.1		decDN 21.2		decDN 21.4		decDN 23.2.3	
	Total	Intact	Total	Intact	Total	Intact	Total	Intact
1	210	0	30	0	284	0	129	0
3	261	0	40	0	166	0	150	0
5	100	0	63	0	43	0	71	0
7	30	0	37	0	46	0	57	0
8	127	0	26	0	15	0	31	0
10	70	0	10	0	48	0	87	0
12	86	0	27	0	37	0	85	0
14	77	0	23	0	29	0	29	0

Table 1. The collapsed egg phenotype of *dec*^{DN} is completely penetrant.

Transgenic *dec*^{DN} females of the four *dec*^{DN} lines from Gail Waring lay completely collapsed eggs, without the detection of any sporadic intact eggs. The phenotype was found to be persistent over successive days of egg collection.

was the generation of functional *FRT* dec^{DN} chromosomes, where a copy of the dec^{DN} allele would be found distal to the *FRT* site on a given chromosome arm. Typically, the process of combining two loci onto the same chromosome arm is accomplished by exploiting the phenomenon of meiotic recombination, which generates recombinant chromosomes in the gametes of adults. To generate specific recombinant chromosomes, adults heterozygous *in trans* for the two loci each on one of two homologous chromosome arms are generated, and their progeny are subsequently screened for the inheritance of a recombinant chromosome bearing both of the desired loci. However, in *Drosophila*, meiotic recombination events take place exclusively in females (Spradling, 1993). Therefore, for our purposes such meiotic recombination events cannot be exploited, since dec^{DN} females are sterile. Any resulting recombinant chromosomes generated by the process of meiotic recombination in dec^{DN} females cannot be recovered since no embryos develop within the collapsed eggs. Consequently we had to resort to alternative approaches to generate the *FRT* dec^{DN} chromosome arms necessary for the development of the technique.

III.1. Unsuccessful Approach: inducing mitotic recombination using irradiation.

III.1.1. Background and Rationale

Unable to rely on meiotic recombination to recover *FRT dec*^{DN} chromosome arms from the sterile *dec*^{DN} transgenic females, the initial alternative method we decided to pursue was to induce recombination events in males using irradiation. The use of irradiation treatments has been shown to have a number of effects on the genome including the induction of random mutations (Ashburner, 1989). However, another important observed outcome is the induction of crossover events during the process of mitosis (for review see Wurgler, 1991). This is believed to take place as a result of the treatment's generation of random breaks that lead to exchange events between homologous chromosomes (Ashburner, 1989). Previously, this phenomenon was routinely utilized to generate homozygous cell clones from heterozygous mother cells before the development of the Flp-FRT system, as described earlier. Although the frequency of recombination events is low, the procedure induces such events even in males, where they are normally absent (Perrimon, 1984). Indeed, during the development of the Flp-DFS system for maternal effect genes expressed in the germline, where a similar problem of dealing with a DFS mutation, *ovo*^{D1}, was faced, irradiation treatments were successfully utilized to recover recombinant *FRT ovo*^{D1} chromosome arms from irradiated heterozygous males (Chou and Perrimon, 1996).

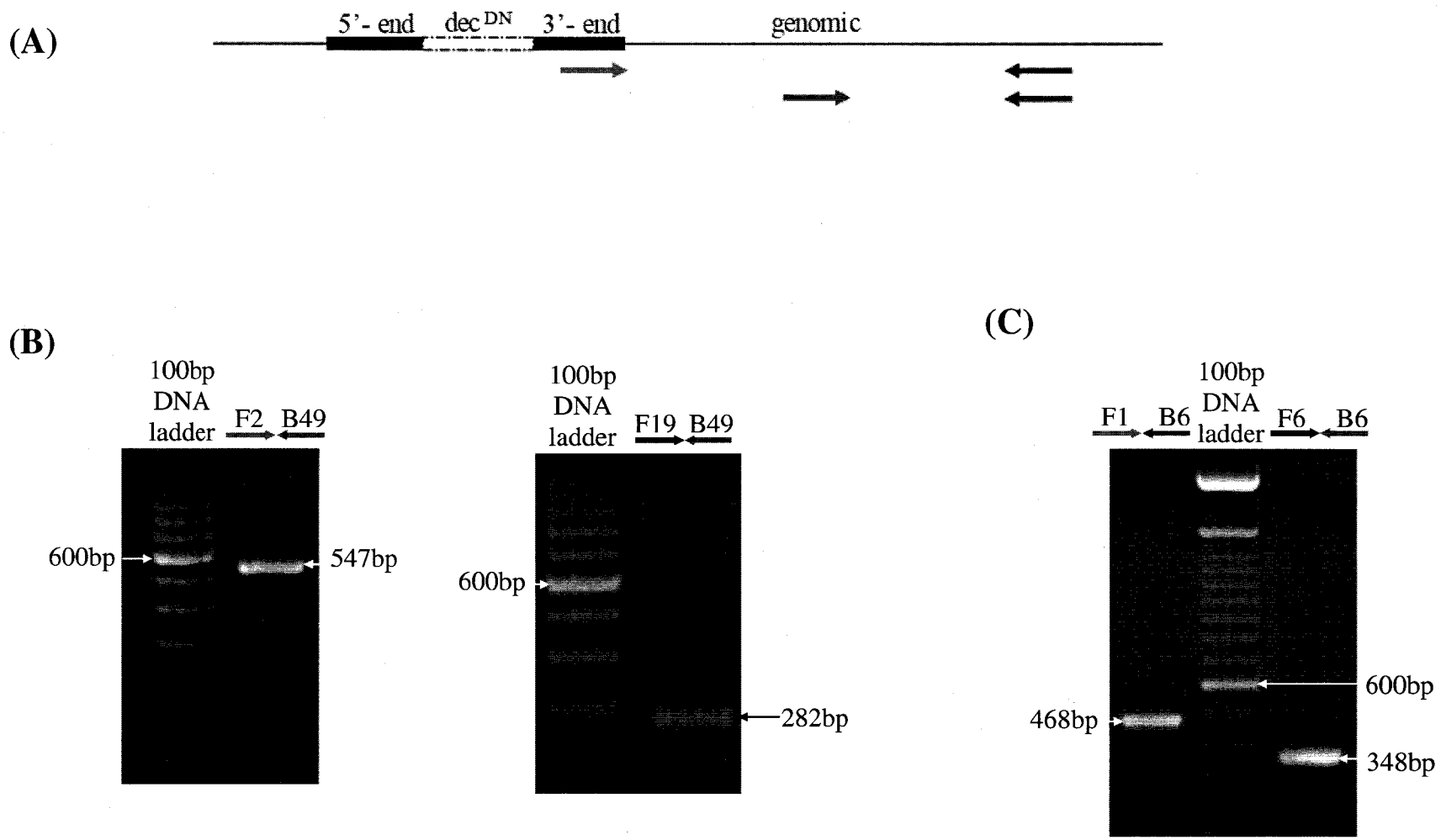
III.1.2. Confirming the *dec*^{DN} mapped position on 3R in the *dec*^{DN} 21.1 line using PCR.

The position of the *dec*^{DN} transgene in the *dec*^{DN} 21.1 line from the Waring lab was mapped (M. Walczak and L. Nilson, unpublished) using the inverse PCR technique (Rubin, 2000). It was mapped to a distal location on the right arm of the third chromosome (3R), namely to a cytological position of 98F. I then confirmed this mapped location using PCR. I designed one forward primer complementary to a DNA fragment within the transgene, another forward primer complementary to a genomic DNA fragment 3' of the transgene and a single reverse primer also complementary to a genomic DNA fragment 3' of both forward primers. The same reverse primer was used in separate PCR reactions with either of the two forward oligonucleotides (Figure 10A).

The rationale for using this method as a confirming technique is that only if the transgene is indeed where it had been initially mapped by inverse PCR would have the first forward primer and the reverse primer resulted in the amplification of a DNA fragment of an expected size, in this case 547bp. However, regardless of whether or not the transgene is indeed where it had been mapped, the second forward primer with the reverse primer would have yielded an amplified DNA fragment of an expected size, in this case 282bp. This latter reaction served as an internal positive control for the reaction parameters (Figure 10 A, B).

Figure 10. Using PCR to confirm the mapped location of the *dec*^{DN} transgene in the lines *dec*^{DN} 21.1 and *dec*^{DN} 23.2.3.

(A) For each case, a forward primer complementary to a DNA fragment in the transgene (→), another forward primer complementary to a genomic DNA fragment 3' of the transgene (→) and a reverse primer complementary to a DNA fragment 3' of both forward primers (←) were designed. Only if the transgene is indeed where it has been mapped will → and ← amplify a DNA fragment. The pair → and ← served as a control for PCR parameters. **(B)** For the *dec*^{DN} 21.1 line, F2 and B49 amplified a fragment of the expected 547bp size, confirming its location on 3R. **(C)** For the *dec*^{DN} 23.2.3 line, F1 and B6 amplified a DNA fragment of the expected 468bp size, confirming its location on 2R.


(A)
5'- end
dec DN
3'- end
genomic
(B)
100bp
DNA
ladder
F2
B49
600bp
547bp
100bp
DNA
ladder
F19
B49
600bp
282bp
(C)
F1
B6
100bp
DNA
ladder
F6
B6
600bp
468bp
348bp

III.1.3. Generating *FRT82B dec^{DN}* chromosomes using irradiation.

Having confirmed the location of the transgene in line *dec^{DN}* 21.1 to a distal location on 3R, I set up crosses to generate males heterozygous *in trans* for the *dec^{DN}* transgene and the corresponding *FRT* site of 3R on the homologous chromosome arm. The available *FRT* site on 3R is located at cytological position 82B and is therefore referred to as *FRT82B*. I irradiated these flies during larval stages to induce mitotic recombination events in their germline. It has been previously reported that the earlier the treatment is administered the higher the frequency of inducing clones (Perrimon, 1984). Accordingly, second instar larvae were irradiated to generate the *FRT ovo^{D1}* chromosome arms (Chou and Perrimon, 1996). After recovering the irradiated heterozygous males as adults, I screened their progeny to identify any potential recombinants containing the desired *FRT82B dec^{DN}* chromosomes. I recovered 49 such potential recombinants and established a stock from each.

III.1.4. Loss of the *dec^{DN}* phenotype penetrance and *FRT* functionality in recombinants generated by irradiation.

Testing for the ability of all 49 recombinant lines to be used in the Flp-DFS system to recover intact eggs from complete follicle cell clones would have been technically difficult. Therefore, I chose five random lines: lines 4B, 11B, 13D, 26A and 33B. Before starting with testing the Flp-DFS system, however, I needed to test whether the *dec^{DN}* collapsed egg phenotype was still completely penetrant to ensure that recombinant females do not lay occasional random intact eggs,

which could be confusing when the mutant intact eggs are ultimately recovered. To do that, I set up egg collection cages to recover eggs from recombinant females of each of these lines. Although these females were not mosaic, in each case I occasionally observed intact eggs that sometimes hatched amongst otherwise collapsed eggs (Table 2). This indicated that the irradiation treatment somehow disturbed the dec^{DN} phenotype, causing it to lose its full penetrance that is otherwise observed in each of the four dec^{DN} transgenic lines from the Waring lab (see Table 1).

An additional problem observed with the recombinant lines was an inability to generate complete follicle cell clones. This was observed when I generated female flies with a source of Flipase that were heterozygous for the recombinant *FRT82B* dec^{DN} chromosome of line 11B as well as a wild type homologous chromosome *FRT82B GFP*. I calculated the frequency of generating stage 10 egg chambers with complete follicle cell clones in their ovaries on successive days after the induction of mitotic recombination using the Flp-FRT system. The frequency remained zero in contrast to the control mosaic females, with two wild type *FRT82B* chromosomes, *FRT82B GFP* and *FRT82B*, where it increased to reach 6% on day 19 after the induction of mitotic recombination (Figure 11). In fact, although cell clones were observed in the follicular epithelia of some of the egg chambers in recombinant ovaries, they were usually small, rarely large enough to comprise the majority of the epithelium (Figure 12).

A possible explanation at the time was that the generated homozygous cells in the mosaic *FRT82B* dec^{DN}*/FRT82B GFP* females suffer from impaired viability, which in turn affects their ability to divide to give rise to a growing

Table 2. Loss of the *dec*^{DN} phenotype penetrance in the five tested recombinant lines from irradiation.

Egg collection cages were set up for females from each of the five randomly chosen recombinant *FRT dec*^{DN} lines recovered from irradiation. In each case, the total number of eggs was counted and the number of intact eggs was scored on successive days. As shown above, occasional intact eggs (numbers in red) were observed amongst otherwise collapsed eggs. Such unexpected intact eggs from *dec*^{DN} females indicated a loss in the *dec*^{DN} phenotype penetrance.

day	rec 4B	
	Total	Intact
7	41	0
8	70	0
9	91	0
10	60	0
11	65	0
12	49	0
13	120	0
14	150	0
15	50	0
16	100	0
17+18+19	129	13
20	150	2
21	38	12
22	22	0
23	61	7
24+25	67	5
26+27	50	7
TOTAL	**1313**	**46**

day	rec 11B	
	Total	Intact
6	72	0
7	52	0
8	69	0
9	116	0
10	78	0
11	72	0
12	141	0
13	103	1
14	92	1
15	85	1
16	105	0
17	89	2
18	124	0
19+20	116	0
21	81	0
22	52	2
23	69	1
24	84	0
25	40	0
TOTAL	**1640**	**8**

day	rec 13D	
	Total	Intact
7	137	0
8	122	0
9+10+11	226	0
12	200	0
13	200	0
14	150	0
15	150	0
16+17+18	282	10
19	55	5
20	41	4
21	32	0
22	94	0
23	28	0
TOTAL	**1717**	**19**

day	rec 26A	
	Total	Intact
6	109	0
7	134	0
8	161	0
9+10+11	200	0
12	82	0
13	21	0
14	175	7
15	59	5
16	89	11
17	36	0
18	57	3
19	43	6
TOTAL	**1166**	**32**

day	rec 33B	
	Total	Intact
8+9+10	164	0
11	110	0
12	56	0
13	77	0
14+15+16	64	0
17	175	0
18	110	0
19	72	0
20	73	0
21	85	1
22	157	7
TOTAL	**1143**	**8**

Figure 11. Inability to generate complete follicle cell clones in mosaic females with irradiated recombinant chromosome.

The percentage of stage 10 egg chambers with complete follicle cell clones in females heterozygous for the 11B recombinant chromosome and the homologous *FRT82B GFP* wild type chromosome is consistently zero (blue line). In control females heterozygous for the same *FRT82B GFP* wild type chromosome as well as *FRT82B*, another wild type chromosome, the percentage of such egg chambers increases to reach 6% at 19 days after the induction of mitotic recombination (pink line).

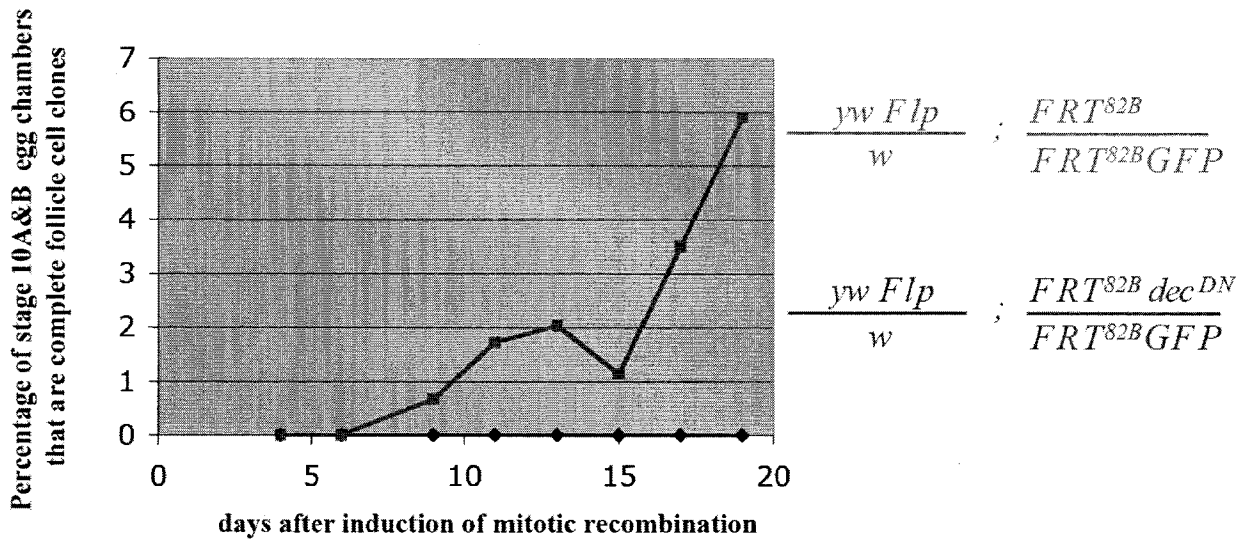
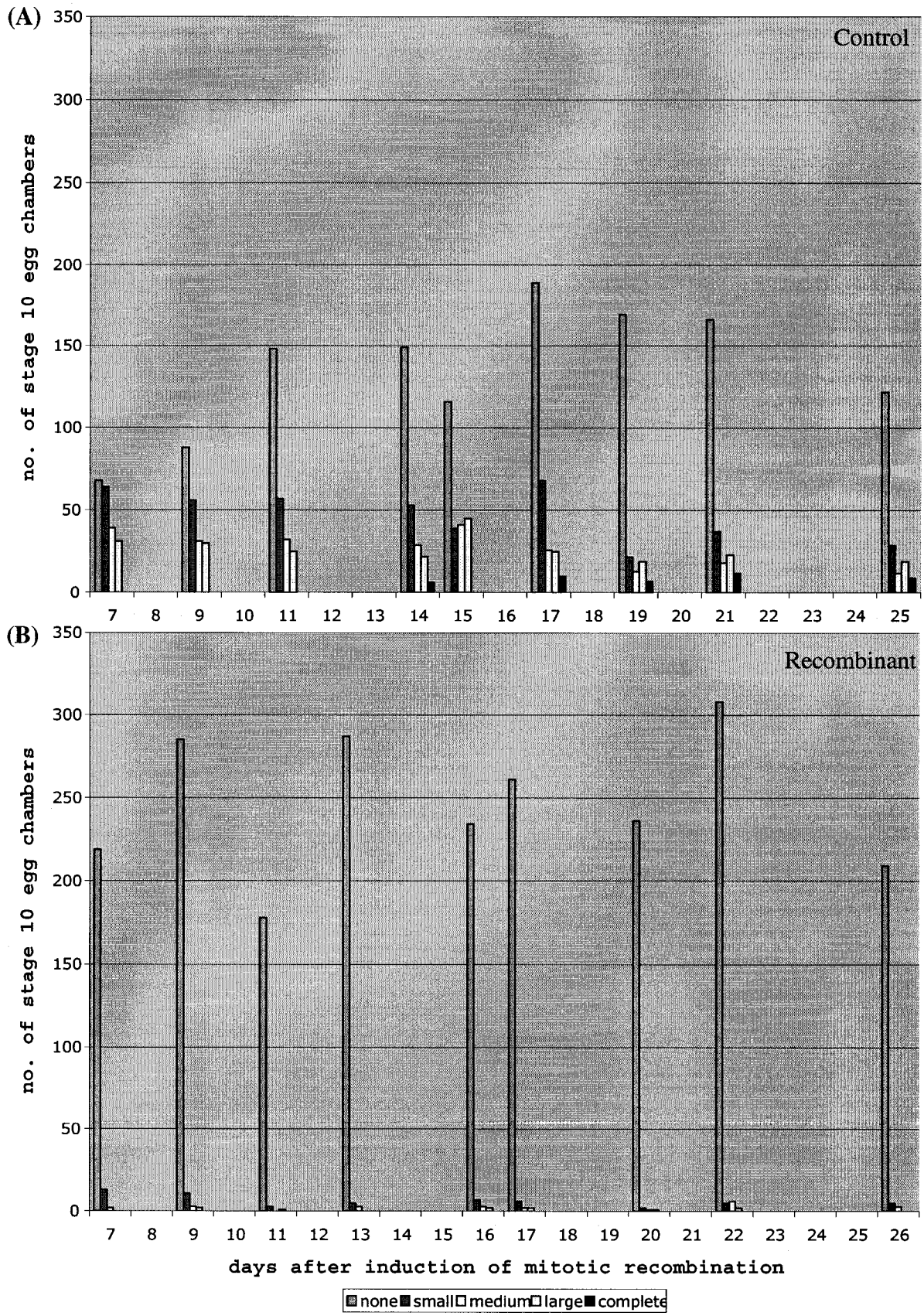

Percentage of stage 10A&B egg chambers that are complete follicle cell clones
7
6
5
4
3
2
1
0
0
5
10
15
20
days after induction of mitotic recombination
yw Flp / w ; FRT82B / FRT82B GFP
yw Flp / w ; FRT82B dec DN / FRT82B GFP

Figure 12. Inability to generate many larger cell clones in mosaic recombinant females.

(A) Dissected control females heterozygous for *FRT82B GFP* and *FRT82B*, both wild type chromosomes, contained many stage 10 egg chambers with larger follicle cell clones, sometimes even comprising the whole epithelium. **(B)** Dissected mosaic females heterozygous for *FRT82B GFP* and the *FRT82B* dec^{DN} chromosome from recombinant 11B did not contain many larger follicle cell clones. (none = no clone, small = 1-5 cells, medium = 6-20 cells, large = >20 cells but not complete, complete = whole epithelium).


(A)
Control
no. of stage 10 egg chambers
350
300
250
200
150
100
50
0
7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25
(B)
Recombinant
no. of stage 10 egg chambers
350
300
250
200
150
100
50
0
7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26
days after induction of mitotic recombination
none small medium large complete

population of homozygous cell clones. The genotype of these generated cells is either homozygous for *FRT82B GFP* or for *FRT82B* dec^{DN}. There is no reason to suspect that the *FRT82B GFP/FRT82B GFP* cell population would have a growth disadvantage, since they are homozygous for a wild type chromosome. However, the *FRT82B* dec^{DN}/*FRT82B* dec^{DN} cells could have been suffering from such a disadvantage if the irradiated recombinant chromosome also harbored an induced homozygous lethal mutation. And since the scoring of cell clones of all sizes, including the complete follicle cell clones, was done by screening for non-GFP – expressing homozygous *FRT82B* dec^{DN} cells, this indeed could have been an explanation for the observation. Screening for the homozygous *GFP* cells is not routinely done since it is more difficult to reliably detect clones of cells expressing GFP at double the intensity in a background of heterozygous *GFP* cells.

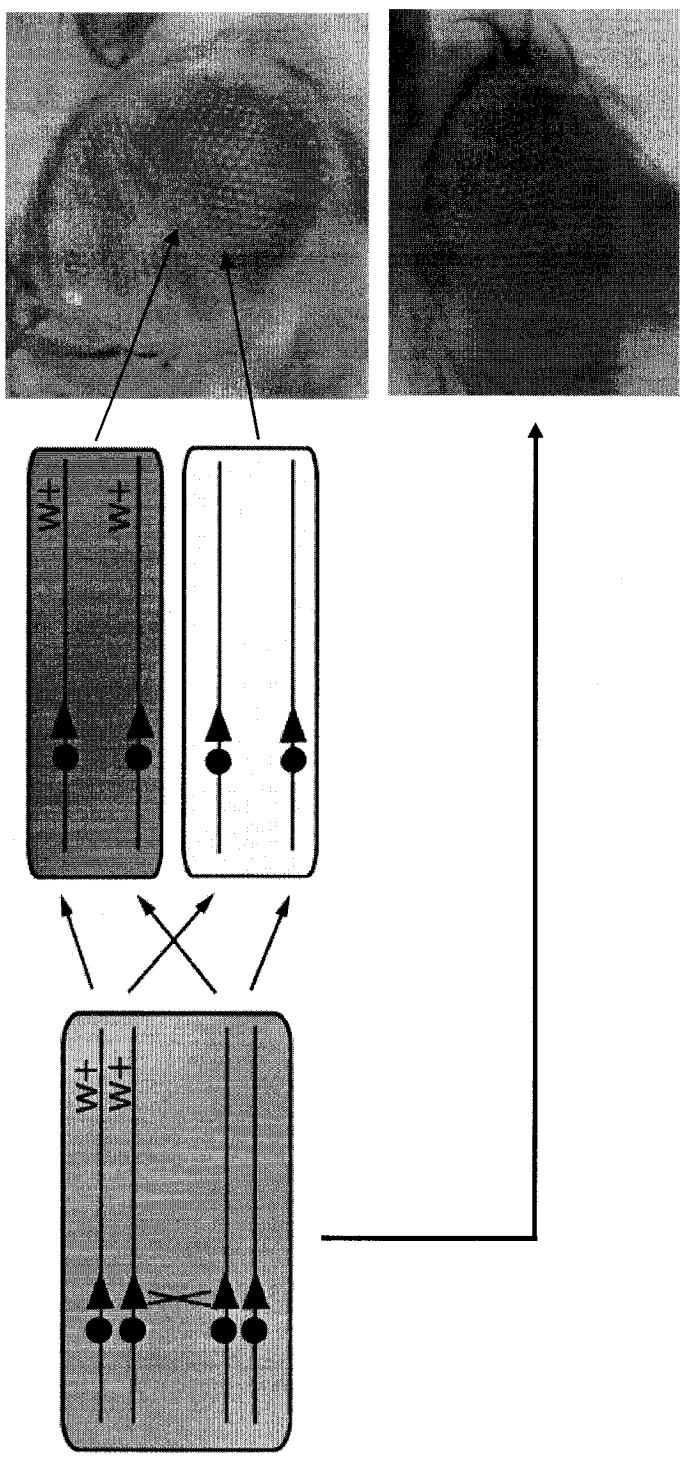
If that were the case, and only the homozygous *FRT82B* dec^{DN} cells of the twin spots exhibited survival and/or division defects, then the recombinant chromosome would have still been useful for our purposes. In the scheme for using dec^{DN} as an eggshell marker, the goal is to identify eggs from egg chambers with the entire follicular epithelium homozygous for a specific mutation (see Figure 8). Such egg chambers would lack follicle cells with *FRT* dec^{DN}. Therefore any growth disadvantage for cells homozygous for the *FRT* dec^{DN} chromosomes theoretically should not pose a problem. On the contrary, it might give the homozygous mutant cell clones an advantage in populating the follicular epithelium.

I proceeded with further attempts to test the functionality of the *FRT* sites on the recombinant chromosomes. I generated adult flies of the genotype *eyFlp; FRT82B GFPw+ / FRT82B*, where the expression of the Flipase (*Flp*) is under the control of the *eyeless* (*ey*) promoter, driving its expression in the eye tissue exclusively. Therefore, such control flies have mosaic adult eyes with bright orange sectors as well as white patches due to generated eye cell clones having two copies of *w+* and twin spots lacking *w+*, respectively (Figure 13). However, when I generated recombinant flies of the genotype *eyFlp; FRT82B* dec^{DN}*w+/ FRT82B*, where mitotic recombination being induced in the eyes should have also resulted in such mosaic eyes with color patches, none at all were observed. All 49 putative recombinants were tested using this latter method, yet none successfully generated mosaic eye colors. Instead, the flies had homogenous orange eyes (Figure 13).

In reference to the above explanation, if indeed homozygous *FRT82B* dec^{DN} cells had a growth disadvantage, then the observation should have been eyes predominantly white, due to the decreased viability of the dec^{DN}*w+/*dec^{DN}*w+* cells. Therefore, the only possible explanation is that the recombinant *FRT82B* dec^{DN} chromosomes from irradiation had faulty *FRT* sites that could not efficiently mediate mitotic recombination events induced by the Flp-FRT system. This explanation is consistent with the observation that only small clones were generated in the ovaries of mosaic recombinant females. Since the *FRT* on the irradiated chromosome is not fully functional, mitotic recombination events occur so rarely and detected clones are smaller than normal.

Figure 13. Using the Flp-FRT system, mosaic eyes with orange versus white color patches are produced in flies heterozygous for a w+ distal to one of the *FRT* sites.

Flies with the *w+* marker have homogenous orange eyes. When mitotic recombination events (red cross) are mediated by Flipase active in the eye tissue of flies heterozygous for a *w+* distal to one of the two *FRT* sites (▶) on homologous chromosomes, cell clones with double *w+* and twin spots lacking *w+* are generated. This results in bright orange patches as well as white patches respectively in the adult eye.


w+
w+
w+
w+

Although irradiation was successfully used to generate the *FRT ovo*^{D1} chromosomes, in fact both of the above outlined problems, the loss of phenotype penetrance as well as the compromised functionality of the *FRT* sites, were reported (Chou and Perrimon, 1996). The authors describe that they had to screen their recombinant lines to successfully isolate ones with fully penetrant *ovo*^{D1} dominant female sterile phenotype that also successfully generated mitotic recombination events at a high frequency. They report how such lines were two of forty for 2L (5%), two of nine for 2R (22%), four of eighty for 3L (5%) and two of seventy for 3R (2.9%) on separate attempts of using irradiation to generate *FRT ovo*^{D1} for each chromosome arm (Chou and Perrimon, 1996). They apparently overcame the problem by performing numerous rounds of irradiation.

Given these observations, it seemed that many more rounds of irradiation treatments would be required to recover potentially useful recombinants that would then be screened for phenotype penetrance and *FRT* functionality. We therefore decided to try alternative methods to generate *FRT dec*^{DN} chromosomes arms. The two attempted methods are outlined and discussed below.

III.2. Successful Approach: inducing mitotic recombination in males using P-elements.

III.2.1. Background and Rationale

Male recombination events, which do not occur in *Drosophila*, were surprisingly observed at a rate of around 1% in a wild strain in Texas (Hiraizumi, 1971). It was initially thought to be due to previously missed meiotic crossover events (Hiraizumi, 1971), until it was later shown to be taking place mainly during premeiotic divisions (Engels, 1979; Hiraizumi, 1979; Hiraizumi et al., 1973). It was reported to be one of a number of aberrant outcomes, such as chromosomal rearrangements and sterility, of a syndrome that was termed hybrid dysgenesis. Hybrid dysgenesis was shown to occur exclusively in progeny generated from crossing males of particular strains, referred to as P strains, to females of other specific strains, referred to as M strains. It was later discovered that the P strains contain functional *P*-elements that are only activated when found in the permissive cytoplasmic environment of the M strains (Kidwell, 1977).

Since then, numerous studies have shed light on what is today a major tool in the field of *Drosophila* molecular genetics (Ryder and Russell, 2003). A number of *P*-elements were shown to contain conserved internal 2.9kb structures as well as terminal inverted repeats (O'Hare and Rubin, 1983), both necessary for their own transposition (Spradling and Rubin, 1982) as well as that of other smaller defective *P*-elements that lack the 2.9kb internal region, yet contain the conserved termini (Rubin and Spradling, 1982). This suggested that the internal region is required to supply the transposase (Karess and Rubin, 1984), and the

termini are essential as recognition target sites. Later a stable genomic source of transposase was generated, which is capable of mobilizing defective *P*-elements but not itself (Robertson et al., 1988).

Studies in which such stable genomic sources were combined *in trans* with the chromosome containing the *P*-element have led to a deeper understanding of the induced male recombination phenomenon, although the exact explanation for its occurrence is still not well understood. It was shown that the great majority of such induced crossover events take place within the 2kb stretch on either side of the *P*-element in the genome. Two thirds of the recovered recombinants exhibit structural rearrangement events, half of which are accompanied by duplication events. The other half have deletions typically from a few base pairs to over 100 kb (Preston et al., 1996), a phenomenon that is utilized today as a mutagenesis tool (Ryder and Russell, 2003). The rest of the recombinants, namely a third of the total, are products of simple recombination events (Preston and Engels, 1996; Preston et al., 1996). This latter observation caused researchers to employ *P*-elements as a tool to induce male recombination (Ryder and Russell, 2003). The ability to induce crossover events in males using this technique prompted us to attempt to use it to generate *FRT* dec^{DN} recombinants from males heterozygous *in trans* for the dec^{DN} transgene and the corresponding *FRT* site on the homologous chromosome.

It is important to note that just as the dec^{DN} transgene is a *P*-element, so are the *FRT* sites (Chou and Perrimon, 1996). Therefore for our purposes two different *P*-elements are involved, each present on a specific location on one of two homologous chromosomes. Although this detail did not imply that the

technique should be avoided, it meant that we could not predict the expected frequency of recovering recombinants. To date, reported studies have only focused on flies heterozygous or homozygous for a specific *P*-element chromosome and not heterozygous for two different chromosomes with a different *P*-element on each, as in our case.

III.2.2. Confirming the dec^{DN} mapped position on 2R in the dec^{DN} 23.2.3 line using PCR.

The transposase stock that I could maintain in a healthy state has the stable genomic source on the third chromosome, *Δ2-3(99B)*. Reported experiments to study *P*-element – induced male recombination were routinely set up to generate flies with the genomic source of transposase and the *P*-element on different chromosomes. I therefore decided to use the transgenic line dec^{DN} 23.2.3, in which the transgene was mapped using inverse PCR (Rubin, 2000) to a distal location on the right arm of the second chromosome, namely to cytological position 52B (M. Walczak and L. Nilson, unpublished). I confirmed this mapped location using the two forward one reverse primers PCR method previously discussed (see Figure 10 A, C). With the transgene on 2R, we decided to recombine it with the commonly used *FRT* site of that chromosome arm. It is found at cytological position 42D and is therefore referred to as *FRT42D*.

III.2.3. Generating *FRT42D* dec^{DN} chromosomes using *P*-element – induced male recombination.

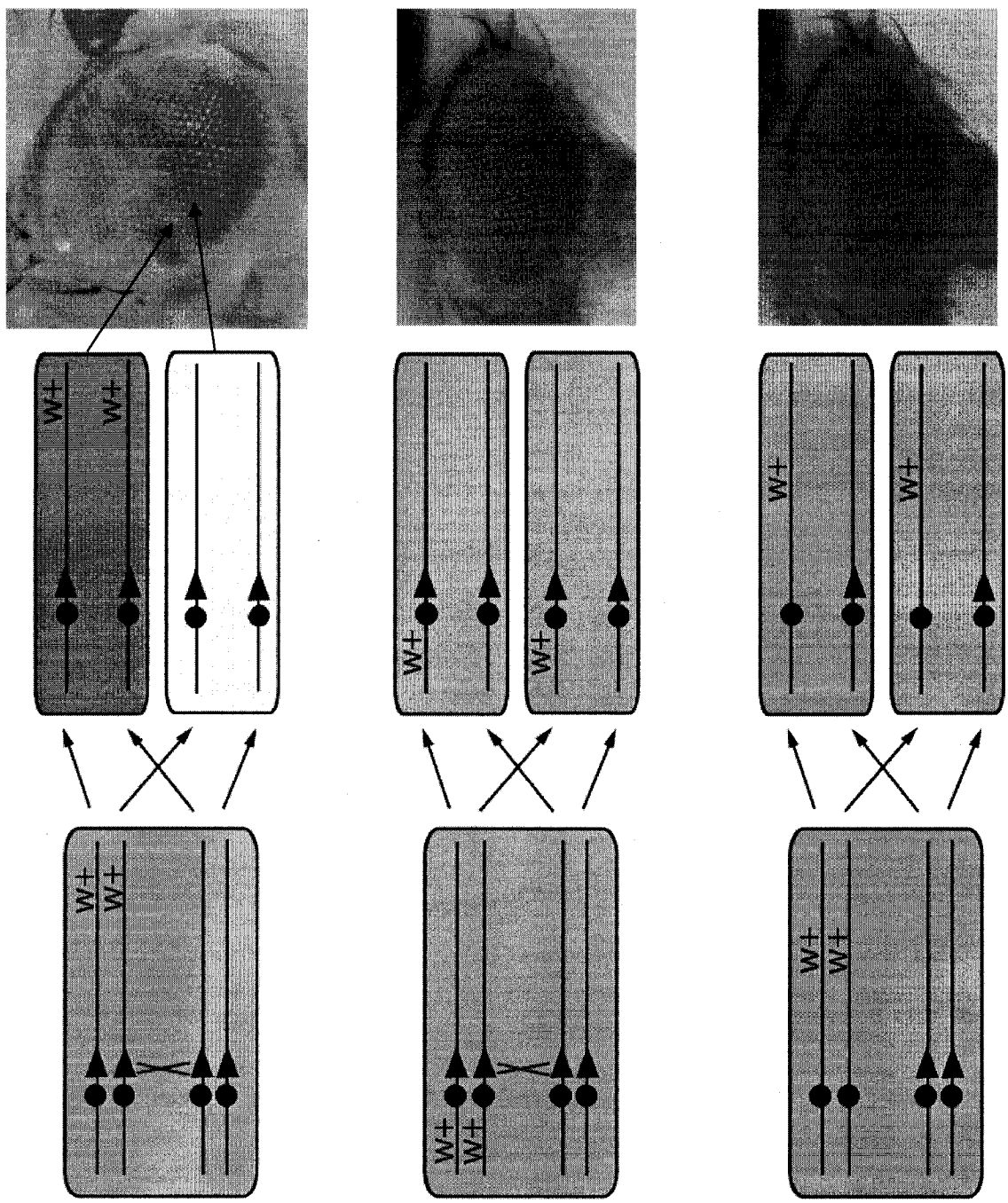
To generate males where *P*-element – induced recombination would potentially result in *FRT42D* dec^{DN} recombinant gametes, I first crossed males from the

dec^{DN} 23.2.3 line to females from the transposase stock. The progeny were reared at 18°C to minimize the activity of the transposase in male progeny with both the transposase and the *dec*^{DN} *P*-element in their genome, and therefore minimize the mobilization of *dec*^{DN} at this point (Preston et al., 1996). Then I crossed these males in turn to *FRT42D* females, keeping the parents at 18°C but rearing the progeny at 25°C. At this step the activity of the transposase was to be maximized to increase the chances of recovering recombinant *FRT42D dec*^{DN} gametes in progeny males containing the genomic transposase source on the third chromosome and heterozygous for the *dec*^{DN} and the *FRT42D* second chromosomes (Preston et al., 1996).

Finally, these males with potential recombinant gametes were crossed to *eyFlp; FRT42D* females. Male progeny of this cross would thus have a copy of the *eyFlp* on the first chromosome, as well as an *FRT42D* second chromosome from their mothers. Only if they also inherit recombinant *FRT42D dec*^{DN}*w+* chromosomes from their fathers, where the *FRT* site is still functional and the *dec*^{DN} marked with the *w+* gene is still distal to cytological position 42D would they develop eyes that have eye color patches of orange and white (Figure 14). If, however, the *FRT* site was mobilized such that it was no longer at 42D, Flp-FRT–mediated mitotic recombination events would not have been possible, and therefore no such mosaic eyes would have been detected. Similarly, if the *dec*^{DN}*w+ P*-element was mobilized to another chromosome arm, no mosaic eyes would have been produced. Although mitotic recombination would have still taken place using the Flp-FRT system, the absence of the *w+* from a distal

Figure 14. Screening for functional *FRT dec*^{DN} chromosomes by testing for the ability to produce mosaic eyes with color patches.

(A) Only when the *FRT* site (▶) in the recombinant chromosome is fully functional, and the *w+* gene remains distal to it, will mitotic recombination events (red cross) be properly mediated to produce eyes with color patches. **(B)** If the *w+* is mobilized to another chromosome arm (shown here on the left arm of the same chromosome for simplicity), then recombination events (red cross) cannot generate color patches. The resultant eyes are homogenously orange. **(C)** If the *FRT* site is mobilized or loses its functionality, then inducing mitotic recombination using the Flp-FRT system is no longer possible, and the resultant eye is homogenously orange.


W+
W+
W+
W+
W+
W+
W+
W+
W+
W+
W+
W+

position to the *FRT* sites would have resulted in such recombination events not having an effect on the color of the developing eyes (Figure 14). This screening technique therefore simultaneously guaranteed that the *FRT* sites in the recovered recombinants are fully functional, since it is the proper mediation of mitotic recombination events by the Flp-FRT system that results in the color patches. Screening the progeny of a total of 294 separate males with potential recombinant gametes, I recovered 10 adult males with mosaic eyes. Each of these was derived from a different father, ensuring that they represent unique recombination events.

Next, I tested the penetrance of the dec^{DN} phenotype in the recovered lines. It was penetrant in only five of the total of ten, namely: MR-25.1, MR-77.1, MR-143.1, MR-184.1 and MR-202.1. Females from the other five lines laid occasional intact eggs that also hatched. One possible explanation for this loss of penetrance in some lines is that the recombination events that gave rise to these chromosomes were accompanied by deletions within the dec^{DN} *P*-element, affecting the dec^{DN} expression. Another explanation could be that, due to recombining the dec^{DN} *P*-element onto a novel chromosome, its expression became affected within this new genomic context. This could be because of potentially different regulatory elements found locally within the neighboring region on the chromosome, or due to the new genetic background in general.

The five lines that retained full penetrance of the dec^{DN} collapsed egg phenotype were maintained for further testing of the system.

III.3. Successful Approach: using the microinjection transgenesis technique.

III.3.1. Background and Rationale

To generate transformant *Drosophila* lines with a particular transgene integrated in their genome, a frequently used method is the microinjection transgenesis technique. *P*-element plasmids, incapable of transposition independently, are created so as to contain the transgene as well as a marker, which would in turn be used to identify the flies in whose genome the transgene has been successfully incorporated. Dechorionated eggs, with developing embryos within, are then microinjected with this *P*-element plasmid as well as a helper plasmid that supplies the *P*-element transposase required for the incorporation of the transgene. The helper plasmid itself is defective to prevent it from being incorporated within the genome. The injection is done into the posterior of the embryos; into a region where the pole cells that ultimately give rise to the germline stem cells are known to develop (Okada, 1998), with the goal of achieving stable integrations of the transgene into the genome of the future gametes. The injected embryos are then allowed to develop into adult flies with potential transgenic germline cells. When adults, they are crossed and their progeny are screened for the expression of the marker to identify any transformants that have inherited the transgene from their injected parent.

A widely used marker is *miniwhite*, *w*+, which partially rescues the eye color in the transformant adult flies. When *w*+ is used, the injected embryos should be mutant for the eye color gene *w*, which causes them to develop white

eyes as opposed to the wild type bright red eyes. When adults, these injected flies are crossed to *w* mutant flies as well, and therefore any resulting progeny with non-white rescued eye color are transformants that have inherited *w+*, as well as the transgene it marks, from the injected parent. The eyes of transformant flies can be different shades of orange/red depending on the genomic context in which the transgene, and in turn the *w*, was incorporated. (For more details on using microinjection as a transgenesis technique see Chia, 2002).

Indeed, the transgenic dec^{DN} lines from the Waring lab were generated using the microinjection technique. A *P*-element *pCaSpeR4* construct was created to contain both the dec^{DN} allele, generated by a targeted deletion within the coding sequence of the *dec-1* gene, as well as the *w+* marker. This injected construct was generously provided to us.

Since the first step in developing the eggshell marker technique for complete follicle cell clones relying on the use of dec^{DN} was to generate *FRT* dec^{DN} chromosome arms, the option of injecting the dec^{DN} *P*-element construct into *FRT* embryos presented itself. This idea was not immediately pursued since, for our purposes, there was a major drawback that led us to believe that its use would be highly inefficient. As explained above, the incorporation of the injected *P*-element construct into the genome of the pole cells of the embryos is achieved by co-injecting a helper plasmid, which is a source of the *P*-element transposase that mediates the steps involved in the mobilization of the *P*-element construct. Although the transposase can derive *P*-element integration into the genome, it can also derive *P*-element excision and/or its re-integration into another location. It is important to note that the *FRT* sites are themselves *P*-elements that have been

integrated using the microinjection transgenesis technique. Therefore, in the proposed scheme of injecting the dec^{DN} *P*-element into *FRT* flies, the transposase from the helper plasmid needed for the integration of dec^{DN} would also recognize the *P*-element *FRT* sites as targets and potentially mediate their mobilization. The removal of the *FRT* sites and/or their integration elsewhere directly interferes with the functionality of the Flp-FRT system, since these sites need to be found on identical cytological positions on homologous chromosomes for the technique to succeed. We expected the risk of disrupting the *FRT* sites to be so high that the scheme would not be significantly efficient to generate the *FRT* dec^{DN} chromosome arms, and therefore we initially refrained from pursuing it, and initially pursued the alternative approaches described above.

An attractive option was to use the microinjection technique where the helper plasmid supplies a transposase that would not recognize the *FRT* sites as targets for mobilization. Therefore, this transposase could not be a *P*-element specific transposase, but rather that of another transposable element. A promising scheme was to use *piggyBac*, a transposable element discovered and characterized in the cabbage looper moth, *Trichoplusia ni* (Cary et al., 1989). After its successful use for gene transfer in a variety of insects such the Mediterranean fruit fly *Ceratitis capitata* (Handler et al., 1998), it was finally shown to function in *Drosophila melanogaster* (Handler and Harrell, 1999). Recently, the necessary and sufficient sequences needed for its use in transformation assays have been identified allowing for the construction of the smallest possible *piggyBac* vectors (Li et al., 2005; Li et al., 2001) to be used in conjunction with *piggyBac*-specific transposase.

We therefore decided to explore the possibility of subcloning the dec^{DN} allele out of the *pCaSpeR4 P*-element construct into a *piggyBac* vector and injecting it along with a *piggyBac* transposase helper plasmid into *FRT* embryos, a scheme that would not pose a risk onto the *P*-element *FRT* sites, and that was indeed reported to be successfully used to transform *FRT* flies (Hacker et al., 2003). I planned to subclone dec^{DN} into the *piggyBac* construct *pXL-BacII-ECFP*, which contains only the minimal sequences needed for *piggyBac*-mediated transformation as well as *enhanced cyan fluorescent protein* as a marker. I ordered the chosen vector along with the helper plasmid *pCaSpeR-hs-orf* from Malcolm J. Fraser at the University of Notre Dame. However, the shipping was quite delayed due to a variety of factors, and after a fruitless long waiting period, we decided to perform a pilot for injecting the dec^{DN} *P*-element into *FRT* embryos to test the efficiency of dec^{DN} insertion on chromosomes retaining the *FRT* transgene at its original location.

III.3.2. Injection of dec^{DN} *P*-element construct into *FRT* flies.

Injection of the *pCaSpeR4* – dec^{DN} *P*-element construct along with the *P*-element transposase helper plasmid *pTurbo* was done by Beili Hu, the microinjection technician in the Biology Department. I then screened the recovered transformant males (the transformant dec^{DN} females are sterile), for the ability to produce progeny with mosaic eyes, a technique, which I used to identify transformation events where the *pCaSpeR4* – $dec^{DN}w+$ construct was integrated distal to an *FRT* site that also retained its functionality (see Figure 4).

III.3.2.1. Injecting *w; FRT42D; FRT82B* embryos to recover *FRT42D dec^{DN}* on 2R and *FRT82B dec^{DN}* on 3R.

Two rounds of injections were performed on *w; FRT42D; FRT82B* embryos. Orange-eyed transformant males were individually crossed to *eyFlp; FRT42D* females and then to *eyFlp; FRT82B* females. In each case, the progeny was screened for mosaic eyes with orange and white patches (see Figure 13).

When such progeny were recovered from *eyFlp; FRT42D* mothers, they must have inherited an *FRT42D dec^{DN}w+* chromosome from their transformant father. Similarly, mosaic-eyed progeny recovered from *eyFlp; FRT82B* mothers must have inherited an *FRT82B dec^{DN}w+* chromosome from their transformant father for the color patches to be generated. In the case when the transformant male did not have the *pCaSpeR4 – dec^{DN}w+* construct incorporated distal to the *FRT* site being tested or if the *FRT* site being tested had been mobilized to a new position compromising its functionality, then such mosaic-eyed progeny would not have been generated (see Figure 14).

In the first round of injection, a total of 367 injected flies were recovered. 75 died and 101 were sterile and therefore generated no progeny. A total of 50 orange-eyed transformant males were recovered from 11 of the fertile ones, while the remaining 180 did not produce transformants. Using the explained scheme to identify functional *FRT dec^{DN}* chromosomes, 32 of the 50 transformant males were found to not have either functional *FRT42D dec^{DN}* or *FRT82B dec^{DN}* chromosomes. Only four had functional *FRT42D dec^{DN}* chromosomes and therefore generated mosaic-eyed progeny when crossed to *eyFlp; FRT42D* females: lines inj-177.2, inj-217.1, inj-217.2 and inj-217.5.

Nine had functional *FRT82B dec*^{DN} chromosomes, generating mosaic-eyed progeny when crossed to *eyFlp; FRT82B* females. However the mosaic-eyed progeny of five of them had a rough eye phenotype. Cell clones in the mosaic eyes were either homozygous for the *FRT82B* chromosome or for the *FRT82B dec*^{DN} chromosome. There is no reason to believe that cells homozygous for the wild type *FRT82B* chromosome would have an aberrant phenotype. Therefore, it is very likely that the *FRT82B dec*^{DN} chromosomes in these lines harbored an induced mutation, possibly resulting from the *pCaSpeR4 – dec*^{DN} construct being integrated at a site that disrupts the expression of a certain gene resulting in the eye cell clones homozygous for this created mutation to develop into rough patches. These five lines were discarded. Therefore a total of four *FRT82B dec*^{DN} lines were maintained from the first round of injection: lines inj-140.1, inj-140.2, inj-140.6 and inj-140.7.

In the second round of injection, a total of 259 injected flies were recovered. One died and 55 were sterile and therefore generated no progeny. A total of 94 orange-eyed transformant males were recovered from 15 of the fertile ones, while the remaining 189 did not produce transformants. 82 of the 94 transformant males did not have either functional *FRT42D dec*^{DN} or *FRT82B dec*^{DN} chromosomes. Only seven had functional *FRT42D dec*^{DN} chromosomes: lines inj-2.27.7, inj-2.129.4, inj-2.129.5, inj-2.221.9, inj-2.246.1, inj-2.246.4, and inj-2.255.1; while only five had functional *FRT82B dec*^{DN} chromosomes: lines inj-2.129.3, inj-2.129.6, inj-2.142.2, inj-2.142.13 and inj-2.142.14.

III.3.2.2. Injecting *w; FRT40A; FRT80B* embryos to recover *FRT40A dec^{DN}* on 2L and *FRT80B dec^{DN}* on 3L.

Another two rounds of injections were performed on *w; FRT40A; FRT80B* embryos. In this case, orange-eyed transformant males were individually crossed to *eyFlp; FRT40A* females and then to *eyFlp; FRT80B* females; then the progeny were screened for mosaic eyes with orange and white patches. Mosaic-eyed progeny recovered from *eyFlp; FRT40A* mothers must have inherited from their transformant father an *FRT40A* chromosome where the *pCaSpeR4 – dec^{DN}w+* construct has been integrated distal to the *FRT40A* site. And similarly, mosaic-eyed progeny recovered from *eyFlp; FRT80B* mothers must have inherited an *FRT80B* chromosome where the *pCaSpeR4 – dec^{DN}w+* construct has been integrated distal to the *FRT80B* site (see Figure 13). Transformant males with chromosomes where the *pCaSpeR4 – dec^{DN}* construct was integrated elsewhere in the genome and/or the *FRT P*-element was mobilized from its position, could not generate such mosaic-eyed progeny (see Figure 14).

In the first round of injection, a total of 218 injected flies were recovered. 25 died and 60 were sterile and therefore generated no progeny. None of the fertile flies had transformant progeny. This was quite surprising. Fearing that the *pCaSpeR4 – dec^{DN}w+* construct aliquot that was given to Beili Hu, the microinjection technician, had been damaged or that its label was confused, a new aliquot was given to her for a second round of injections.

In the second attempt, a total of 190 injected flies were recovered. 22 died and 32 were sterile and therefore generated no progeny. Only a total of 4 orange-eyed transformant males were recovered from 3 of the fertile ones, while the

remaining 133 did not produce transformants. This was a much lower number of transformants than expected based on the numbers recovered from the two injection rounds for the *w; FRT42D; FRT82B* embryos. Screening their progeny from crosses with *eyFlp; FRT40A* and *eyFlp; FRT80B* females did not result in the identification of lines with functional *FRT40A* $dec^{DN}w+$ or *FRT80B* $dec^{DN}w+$ chromosomes.

The failure to recover a significant number of transformants from injected *w; FRT40A; FRT80B* embryos remains a mystery. Beili Hu had mentioned that she had found the adult flies not as healthy as flies usually are, which resulted in her obtaining fewer eggs from the usual number of adults. 71% and 28% of the recovered adults died or were sterile in the first and second rounds of injection, respectively. In the case of *w; FRT42D; FRT82B* embryos, 48% and 22% died or were sterile in the first and second rounds of injection, respectively. Comparing these numbers, it seems that the failure to recover transformants from the *w; FRT40A; FRT80B* stock is not simply due to most of the injected embryos giving rise to sick or sterile adults.

A possible explanation is that in the instances when transgenesis was successful and the *P*-element transposase integrated the *pCaSpeR4* – dec^{DN} construct into the genome of pole cells of the injected *w; FRT40A; FRT80B* embryos, this was accompanied by the *FRT* sites also being frequently targeted by the transposase, resulting in many events of *FRT* mobilization that eventually killed the injected embryo. In this case, the number of transformants was significantly less than expected as a result of the injected embryos with integrated dec^{DN} failing to develop into adults. This could be due to one or both of the *FRT*

sites in the *w; FRT40A; FRT80B* stock being more susceptible to mobilization than the *FRT* sites in the *w; FRT42D; FRT82B* stock. Keeping track of the percentage of injected embryos that successfully developed into adults in both cases would have helped address this hypothesis. Also another way to test this, and hopefully recover functional *FRT* dec^{DN} chromosome arms for 2L or 3L in the process, is to inject embryos with only one of the two *FRT* sites; i.e. *w; FRT40A* embryos and *w; FRT80B* embryos.

Chapter IV. Testing the Flp-DFS technique for the study of complete follicle cell clones.

As explained, *FRT dec*^{DN} chromosomes for arms 2R and 3R were successfully generated using the methods of *P*-element – induced male recombination and microinjection. In each case, the location of the *dec*^{DN} construct was mapped to a distal location to the *FRT* site in question, using the screening technique that is based on creating mosaic eyes described above. The technique also simultaneously tested the functionality of the *FRT* site, since adults with such mosaic eyes with color patches can only be generated when the tested chromosome has an *FRT* site that is capable of mediating Flp-FRT – induced site-specific mitotic recombination. I also showed that the *dec*^{DN} phenotype was completely penetrant for each recovered and maintained *FRT dec*^{DN} line; all the females consistently laid collapsed eggs and no occasional intact eggs.

Five *FRT42D dec*^{DN} lines were generated using the *P*-element – induced male recombination method; namely lines MR-25.1, MR-77.1, MR-143.1, MR-184.1 and MR-202.1. The microinjections of *w; FRT42D; FRT82B* embryos resulted in the recovery of a total of 11 *FRT42D dec*^{DN} lines: inj-177.2, inj-217.1, inj-217.2, inj-217.5, inj-2.27.7, inj-2.129.4, inj-2.129.5, inj-2.221.9, inj-2.246.1, inj-2.246.4, and inj-2.255.1; and a total of 9 *FRT82B dec*^{DN}*w+* lines: inj-140.1, inj-140.2, inj-140.6, inj-140.7, inj-2.129.3, inj-2.129.6, inj-2.142.2, inj-2.142.13 and inj-2.142.14.

The next crucial step after successfully recovering these lines was to test if indeed such generated *FRT dec*^{DN} lines can be used to recover intact eggs from

mosaic females bearing follicle cell clones, as previously discussed. Such intact eggs would develop from egg chambers where the entire follicular epithelium is homozygous mutant for the gene being studied and therefore lacking the expression of dec^{DN} (see Figure 8).

Although an ultimate use of this system is to screen for novel zygotically required maternal effect genes expressed specifically in the follicle cells, for the initial testing we chose already well characterized such genes whose effect on the eggs is known. If the system functions as predicted, all intact eggs recovered from mosaic females would exhibit the expected phenotypes. The recovery of intact eggs showing no or other phenotypes would have meant that they are not indeed products of egg chambers with homozygous mutant follicular epithelia.

IV.1. Testing the *FRT82B dec^{DN}* lines.

We decided to start the testing phase with the *FRT82B dec^{DN}* lines, because we had characterized mutations available in zygotically required maternal effect genes on 3R known to be required in the follicle cells during oogenesis. I used the four *FRT82B dec^{DN}* lines from the first round of injection for this purpose, namely lines inj-140.1, inj-140.2, inj-140.6 and inj-140.7, since the second round of injection had not been completed at this stage.

For each of these four lines, I generated females with a source of Flipase under the control of a heat-shock promoter (*hs*) to induce site-specific mitotic recombination at elevated temperatures. These females were also heterozygous for the *FRT82B dec^{DN}* chromosome and a corresponding *FRT82B* chromosome bearing the characterized mutation. I then placed these generated mosaic females with males in egg cages to collect their eggs on apple juice plates over successive days and screen for any recovered intact eggs amongst the collection of collapsed ones. In the case where such intact eggs were recovered, they were carefully examined to determine if they exhibit the expected phenotype characteristic of eggs from egg chambers with homozygous mutant follicular epithelia.

IV.1.1. Using a wild type *FRT82B* chromosome to generate wild type intact eggs.

The first corresponding *FRT82B* chromosome I used was a wild type *FRT82B GFP* chromosome that does not bear any mutation. My expectation was to recover occasional intact eggs that appeared wild type, and indeed that was my observation (see Figure 9). I counted the total number of eggs as well as the

number of intact ones, all of which were wild type, found on plates of successive days after the induction of mitotic recombination. A subset of the intact eggs hatched (Table 3), which confirms that the intact eggs were able to sustain the development of an embryo.

The other chosen corresponding *FRT82B* chromosomes I used in the testing procedure had well characterized mutations in zygotically required maternal effect genes distal to the *FRT* site. Therefore, all these mutations are homozygous lethal. As described, the rationale was to generate egg chambers with the entire follicular epithelium homozygous mutant, which would in turn produce intact eggs with the expected mutant phenotype.

IV.1.2. Using *FRT82B* chromosomes bearing *cic* alleles to generate intact dorsalized eggs.

Similarly, I generated females mosaic for two independently generated loss of function alleles of the gene *capicua* (*cic*). *Cic* is known to be expressed in the follicle cells, where it is required for the proper patterning of the D-V axis both in the eggshell and the developing embryo (Goff et al., 2001). Viable females can be recovered if heterozygous for two weak mutant *cic* alleles. The eggs these females lay are dorsalized: the dorsal domain is expanded resulting in a shift of the positions of the dorsal appendages causing them to be more lateral than in wild type, and also in the presence of a ring of appendage material at base of the appendages at the anterior circumference (Goff et al., 2001) (Figure 15B).

The two alleles I used, cic^{BA53} and cic^{U6}, are strong hypomorphs, and therefore we expected the egg chambers with complete homozygous mutant follicle cell clones to give rise to strongly dorsalized eggs. Indeed all the

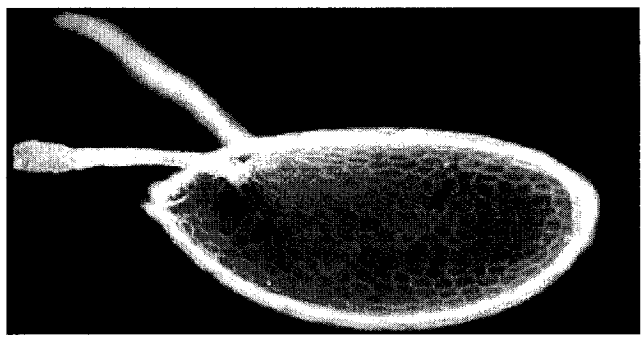
day	inj-140.1		inj-140.1		inj-140.4		inj-140.7	
	Total	Intact	Total	Intact	Total	Intact	Total	Intact
7	144	**2(1)**			42	0		
8	62	**1**	46	0	43	0	59	0
9	47	0	24	0	41	0	75	0
10	47	0	34	0	66	0	43	0
11	53	0	30	0	21	0	21	0
12	42	0	39	0	40	0	25	0
13	51	0	38	0	36	0	32	0
14	39	0	44	0	32	0	42	0
15	47	0	49	0	50	**2(1)**	76	0
16	47	0	23	0	53	**1**	28	0
17	43	0	49	0	67	**8(3)**	20	0
18	46	0	37	0	40	**5(2)**	20	0
19	41	**5(1)**	38	**1(1)**	24	**4(1)**	25	0
20	65	**1(1)**	44	**1**	47	**7**	28	0
21	122	**3(1)**	73	**2**	25	**4**	16	0
22	37	**2(1)**	43	**2**	28	**3(1)**	32	0
23	42	**3(1)**	53	0	43	**4(3)**	30	0
24	41	**5(1)**	19	**7(3)**	27	**5**	12	**1**
25	133	**2**	51	**3**	23	**6(4)**	39	**2**
26	84	0	51	0	35	**7(5)**	39	0
27	46	**11(9)**	45	**9(1)**	45	**6(1)**	34	**11**
28	47	**11(6)**	19	**2(2)**	26	**5(1)**	49	0
29	60	**14(9)**	35	**3(1)**	33	**3(1)**	36	**6(3)**
30	61	**18(10)**	35	**3**	33	**3(1)**	36	**3**
TOTAL	1447	**78(41)**	919	**33(8)**	**920**	**73(24)**	**817**	**23(3)**
%	5.39		3.59		7.93		2.82	
Average %	4.93							

Table 3. The recovery of intact wild type eggs from mosaic females heterozygous for the tested *FRT82B dec^{DN}* chromosomes as well as the wild type chromosome *FRT82B GFP*. Eggs laid by mosaic *yw hsFlp; FRT82B dec^{DN}/FRT82B GFP* females were collected on apple juice plates on successive days after the induction of mitotic recombination events. In each case, the total number of eggs on the plate was counted and they were examined for intact eggs. When observed, these intact eggs were always wild type (number in red) and sometimes a few of them even hatched (number in parentheses). The table also shows the total number of eggs and the total percentage of intact eggs recovered from each group of females.

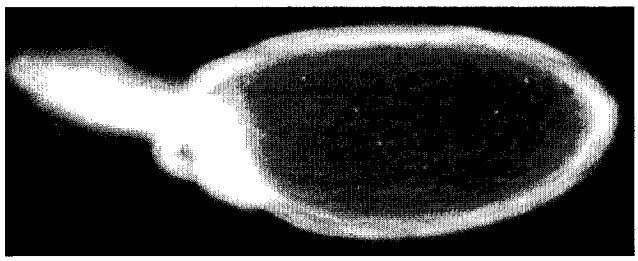
Figure 15. Examples of eggs from complete homozygous mutant follicle cell clones.

(A) Wild type egg from a wild type female. **(B)** A dorsalized egg produced from egg chambers with homozygous mutant *cic* follicle cell clones. The dorsal domain is expanded resulting in a lateral shift in the position of the dorsal appendages as well as a ring of appendage material around the anterior circumference. **(C)** An egg with a single thick appendage reportedly produced from egg chambers with homozygous $pnt^{\Delta 88}$ mutant follicle cells clones. The thick appendage is said to be due to the loss of the midline region, as opposed to simply the fusion of the two appendages. **(D)** A small egg with shortened appendages reportedly produced from egg chambers with homozygous puc^{A251} mutant follicle cells clones.

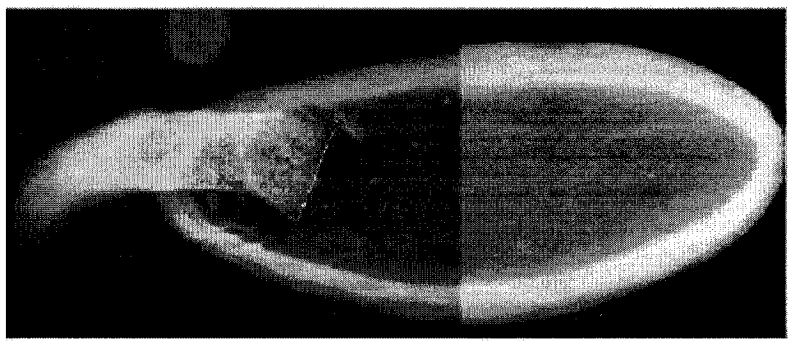
(A)



(B)

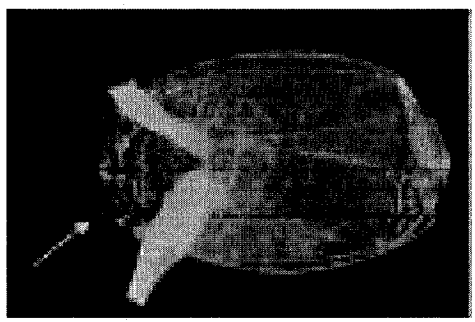


(C)



(From Morimoto et al., 1996)

(D)



(From Dobens et al., 2001)

recovered intact eggs from the generated mosaic cic^{BA53} and cic^{U6} females were dorsalized, with laterally shifted appendages and a ring of appendage material at the anterior circumference, just as expected (Figure 16).

In the case of cic^{BA53}, the percentage of intact eggs from mosaic females was much lower than that recovered from the wild type control chromosome when mitotic recombination events were induced in the pupal stages (Table 4A and see Table 3). A proposed explanation for this observation was that pupae with large homozygous mutant clones did not survive, and therefore the only viable mosaic females recovered had small and less frequent clones, in turn giving rise to fewer intact eggs due to the fewer egg chambers with complete follicle cell clones. A proposed solution was to induce recombination events in the adults. With all stages of development already completed, homozygous *cic* clones will only be generated in mitotically active tissues and should not have an effect on the adults' viability. However, in the case of cic^{BA53}, the percentage of observed intact eggs remained low even when recombination was induced in adults (Table 4B).

Similarly, in the case of mosaic females with homozygous cic^{U6} cell clones, the percentage of intact eggs remained low when the recombination events were induced at the adult stage compared to the pupal stage (Table 5A, B).

IV.1.3. Using an *FRT82B* chromosome bearing $pnt^{\Delta 88}$ allele to generate intact eggs with a single thick appendage.

Another mutation I used to test the technique was in another zygotically required maternal effect gene known to be expressed in the follicle cells, namely *pointed* (*pnt*). Coding for a transcription factor, *pnt* has been shown to be required in the

Figure 16. The recovery of intact dorsalized eggs amongst otherwise collapsed eggs from mosaic females.

Mosaic females heterozygous for the *FRT82B* dec^{DN} chromosome as well as the homologous *FRT82B cic* chromosome lay occasional intact eggs that are visible dorsalized (arrow) amongst a collection of otherwise collapsed eggs (arrowheads). These eggs are products of egg chambers with complete homozygous mutant follicle cells.

Table 4. The recovery of intact dorsalized eggs from mosaic females heterozygous for the tested *FRT82B dec^{DN}* chromosomes as well as *FRT82B cic^{BA53}*.

Eggs laid by mosaic yw *hs-Flp; FRT82B dec^{DN}/FRT82B cic^{BA53}* females were collected on apple juice plates on successive days. In each case, the total number of eggs (T) on the plate was counted and they were examined for intact eggs (I). When observed, these intact eggs were always dorsalized (in red). The table shows the total number of eggs and the total percentage of intact eggs recovered from each group of females. The percentage of intact eggs recovered was not seen to increase when adults were heat shocked (B) instead of pupae (A).

(A)

day	inj-140.1		inj-140.1		inj-140.1		inj-140.4		inj-140.4		inj-140.4		inj-140.4		inj-140.4		inj-140.6		inj-140.7	
	T	I	T	I	T	I	T	I	T	I	T	I	T	I	T	I	T	I	T	I
6			155	0	57	0	170	0					58	0	80	0	151	0	15	0
7	92	0	123	0	46	0	99	0	161	0			45	0	90	0	80	0	9	0
8	78	0	33	0	47	0	64	0	41	0			44	0	52	1	40	0	27	0
9	48	0	58	0	57	0	40	0	35	2	80	0	33	0	25	0	23	0	14	0
10	33	0	59	0	62	0	36	0	36	0	75	0	10	0	26	0	23	1	12	0
11	36	0	56	0	51	0	33	0	27	0	95	0	35	0	24	0	23	0	18	0
12	56	0	83	0	59	0	66	0	29	0	85	0	25	0	22	0	35	0	17	0
13	54	0	18	0	62	0	70	0	44	0	57	0	43	0	13	0	9	0	32	0
14	47	0	39	0	32	0	72	0	36	0	57	0	41	0	19	0	20	0	59	0
15	59	0	62	0	28	0	62	0	36	0	69	0	23	0	31	0	28	0	12	0
16	57	0	39	0	26	0	67	0	25	0	95	0	22	0	40	0	29	0	17	0
17	53	0	82	0	68	0	60	0	44	0	71	0	56	0	27	0	35	0	43	0
18	89	0	83	0	44	0	57	0	28	0	62	0	47	0	27	0	37	0	26	0
19	54	0	152	0	40	0	58	0	13	0	80	0	36	0	25	0	62	0	18	0
20	55	0	50	0	40	0	66	0	21	0	80	0	35	0	25	0	39	0	19	0
21	25	0	81	0	42	0	56	0	38	0	73	0	41	0	27	0	23	0	57	0
22	26	0	44	0	42	0	25	0	17	0	88	0	40	0	33	0	18	0	29	0
23	37	0	12	0	32	0	24	0	17	0	37	0	41	0	44	0	15	0	29	0
24	38	0	13	0	32	0	46	0	38	0	65	0	42	0	18	0	17	0	30	0
25	35	0	43	0	27	0	47	0	40	0	86	0	15	0	19	0	25	0	20	0
26	35	0	56	0	27	0	39	0	13	0	62	0	17	0	14	0	17	0	21	0
27	51	0	51	0	37	0	38	0	13	0	46	0	31	0	2	0	11	0	30	0
28	52	0	27	0	12	0	15	0	57	0	48	0	10	0	7	0	17	0	35	0
29	31	0	59	0	13	0	15	0			63	0	12	0	22	0	17	0	10	0
30	31	0	19	0	14	0	14	0			32	0	12	0	48	0	10	0	15	0
TOTAL	1172	0	1497	0	997	0	**1339**	**0**	**809**	**2**	**1506**	**0**	**814**	**0**	**760**	**1**	804	**1**	**614**	**0**
%	0		0		0		0		0.25		0		0		0.13		0.12		0	
Average %	0.17																			

(B)

day	inj-140.1		inj-140.1		inj-140.1		inj-140.1		inj-140.4		inj-140.4		inj-140.4		inj-140.4		inj-140.6		inj-140.6		inj-140.6		inj-140.7		inj-140.7		inj-140.7	
	T	I	T	I	T	I	T	I	T	I	T	I	T	I	T	I	T	I	T	I	T	I	T	I	T	I	T	I
5+6	64	0	142	0	76	0	119	0	103	0	173	0	76	0	139	0	166	0			100	0	140	0			180	0
7+8	74	0	88	0	112	0	95	0	91	0	200	0	27	0	79	0	147	0			94	0	162	0	222	0	183	0
9+10	149	0	141	0	96	0	65	0	147	0	220	0	50	0	128	0	119	0			155	0	94	0	134	0	80	0
11+12	131	0	148	0	80	0	83	0	175	0	121	0	38	0	84	0	96	0			86	0	37	0	129	0	84	0
13+14	110	0	140	0	65	0	146	0	153	0	114	0	19	1	83	0	56	0	77	0	52	0	27	0	160	0	120	0
15+16	122	0	158	0	98	0	112	0	140	1	139	0	32	1	76	0	43	0	14	0	42	0	99	0	108	0	132	0
17+18	91	0	144	0	71	0	123	0	112	0	130	0	15	0	128	0	57	0	0	0	89	0	161	0	123	0	102	0
19+20	65	0	71	0	87	0	123	0	132	0	69	0	30	0	80	0	64	0	25	0	93	0	89	0	180	0	108	0
21+22	80	0	68	0	87	0	119	0	61	0	87	0	17	0	25	0	62	0	17	0	64	0	21	0	153	0	93	0
23+24	50	0	75	0	61	0	99	0	104	0	104	0	25	0			55	1	19	0					64	1	43	0
25+26	23	0	27	0	40	0	79	0	96	0	20	0					37	0	19	0					57	0	64	0
27+28	19	0	46	0	49	0	104	0	53	0	19	0					30	0	4	0							33	0
29+30							110	0	53	0							14	0	6	0								
TOTAL	978	**0**	1248	**0**	922	**0**	1267	**0**	**1420**	**1**	**1396**	**0**	**329**	**2**	**822**	**0**	956	**1**	181	**0**	775	**0**	**830**	**0**	**1330**	**1**	**1222**	**0**
%	0		0		0		0		0.07		0		0.61		0		0.1		0		0		0		0.08		0	
Average %	0.215																											

Table 5. The recovery of intact dorsalized eggs from mosaic females heterozygous for the tested *FRT82B dec^{DN}* chromosomes as well as *FRT82B cic^{U6}*.

Eggs laid by mosaic *yw hs-Flp; FRT82B dec^{DN}/FRT82B cic^{U6}* females were collected on apple juice plates on successive days. In each case, the total number of eggs (T) on the plate was counted and they were examined for intact eggs (I). When observed, these intact eggs were always dorsalized (in red). The table shows the total number of eggs and the total percentage of intact eggs recovered from each group of females. Similar to the case of *cic^{BA53}*, the percentage of intact eggs recovered was not seen to increase when adults were heat shocked **(B)** instead of pupae **(A)**.

(A)

day	inj-140.1		inj-140.4		inj-140.6		inj-140.7	
	Total	Intact	Total	Intact	Total	Intact	Total	Intact
6+7	139		247	0	174	0	294	0
8+9	112	0	147	0	286	0	208	0
10+11	72	0	159	0	175	0	238	0
12+13	39	0	92	0	152	0	262	1
14+15	32	0	93	0	113	0	191	0
16+17	29	0	76	0	141	0	214	3
18+19	11	0	165	10	127	2	215	4
20+21	31	0	117	0	131	0	133	2
22+23	23	0	161	0	145	1		
24+25	30	0	106	5	141	0		
26+27	23	0	37	0	45	0		
TOTAL	**541**	**0**	**1400**	**15**	**1630**	**3**	**1755**	**10**
%	0		1.07		0.18		0.57	
Average %	0.61							

(B)

day	inj-140.1		inj-140.1		inj-140.1		inj-140.4		inj-140.6		inj-140.6		inj-140.7		inj-140.7	
	T	I	T	I	T	I	T	I	T	I	T	I	T	I	T	I
5+6	150	0	219	0			343	0	291	0	182	0	264	0	277	0
7+8	118	0	173	0			176	0	229	0	141	0	190	0	268	0
9+10	109	0	124	0			217	0	205	0	188	0	244	0	256	0
11+12	60	0	70	0			201	0	209	0	138	0	151	0	251	0
13+14	26	0	97	0	139	0	192	0	145	0	150	2	149	0	181	0
15+16	54	0	82	0	123	0	182	1	180	2	59	0	96	0	271	3
17+18	60	0	66	0	129	3	206	2	276	2	106	1	187	0	198	2
19+20	27	0	94	0	102	1	222	0	145	3	95	0	85	0	188	0
21+22	50	0	35	0	69	0	103	0	183	4	43	0			285	1
23+24	28	0	39	0	70	1	117	0	102	0	43	0			147	3
25+26	20	0	39	0	41	0	56	0	68	2	55	0			176	0
27+28			20	0	41	0	91	2	79	0	30	0			76	0
29+30			31	0	31	0										
TOTAL	**702**	**0**	**1089**	**0**	**745**	**5**	**2106**	**5**	**2112**	**13**	**1230**	**3**	**1366**	**0**	**2574**	**9**
%	0		0		0.67		0.24		0.62		0.24		0		0.35	
Average %	0.424															

follicular epithelia of egg chambers for the proper development of the dorsal appendages. It is thought to act in the dorsal midline to negatively regulate the EGF receptor pathway, allowing for the midline to develop (Morimoto et al., 1996). Eggs developing from egg chambers with clones homozygous for the allele $pnt^{\Delta 88}$ in the midline region reportedly develop with one thick appendage (see Figure 15C). Because the width of the appendage is more than the sum of the widths of the two appendages in wild type, this phenotype is thought to result from the loss of the midline fate as opposed to simply a fusion of the two individual appendages (Morimoto et al., 1996). To date there is no direct evidence that complete $pnt^{\Delta 88}$ follicle cell clones, which would consistently comprise the entire midline region, would generate eggs with a single thick appendage.

We decided to use the same $pnt^{\Delta 88}$ allele to test the four *FRT82B* dec^{DN} lines. I generated mosaic females heterozygous for the *FRT82B* dec^{DN} chromosome being tested as well as the corresponding *FRT* chromosome with the $pnt^{\Delta 88}$ mutation. As expected, intact eggs with a single thick appendage were occasionally observed. (Table 6A, B).

Table 6. The recovery of intact eggs with a single thick appendage from mosaic females heterozygous for the tested *FRT82B dec^{DN}* chromosomes as well as *FRT82B pnt$^{\Delta 88}$*.

Eggs laid by mosaic *yw hs-Flp; FRT82B dec^{DN}/FRT82B pnt$^{\Delta 88}$* females were collected on apple juice plates on successive days. In each case, the total number of eggs (T) on the plate was counted and they were examined for intact eggs (I). When observed, these intact eggs always had a single thick appendage, as expected (in red). The table shows the total number of eggs and the total percentage of intact eggs recovered from each group of females when the heat-shock treatment was administrated during the adult stage **(B)** compared to during the pupal stage **(A)**.

(A)

day	inj-140.1		inj-140.6		inj-140.7	
	Total	Intact	Total	Intact	Total	Intact
6+7	187	0	165	0	135	0
8+9	190	0	252	0	138	0
10+11	182	0	219	0	143	0
12+13	289	0	140	0	81	0
14+15	257	0	123	**1**	160	0
16+17	223	**1**	111	0	147	0
18+19	207	**3**	116	**1**	116	**1**
20+21	89	0	166	0	153	0
22+23	137	0	172	**1**	99	0
24+25	119	0	1135	0	100	0
26+27	112	0	92	0	57	0
TOTAL	**1992**	**4**	**2691**	**3**	**1329**	**1**
%	0.2		0.11		0.08	
Average %	0.13					

(B)

day	inj-140.1		inj-140.1		inj-140.4		inj-140.4		inj-140.6		inj-140.6		inj-140.7		inj-140.7	
	T	I	T	I	T	I	T	I	T	I	T	I	T	I	T	I
6+7	172	0	181	0	229	0	239	0	179	0	261	0	148	0	129	0
8+9	144	0	192	0	199	0	217	0	147	0	268	0	122	0	226	0
10+11	190	0	210	0	191	0	252	0	120	0	234	0	131	0	134	0
12+13	198	0	289	0	173	0	187	0	126	0	190	0	123	0	207	0
14+15	213	0	225	0	216	0	168	0	96	0	203	0	102	0	202	0
16+17	117	0	160	**1**	133	**1**	216	0	155	0	148	**1**	96	0	158	**1**
18+19	111	**1**	237	**1**	192	0	155	0	98	0	200	0	139	**2**	176	0
20+21	144	**1**	137	0	137	**2**	121	0	96	0	251	**1**	160	**1**	181	0
22+23	123	**4**	154	0	115	**1**	159	**1**	58	0	160	0	111	0	134	0
24+25	167	**1**	132	**3**	110	0	185	**1**	80	0	154	0	91	0	122	0
26+27	100	**3**	102	0	79	0	135	**1**	90	0	95	0	63	0	95	**2**
28+29	112	**3**	71	0	81	**3**	86	0	72	0	146	**2**	46	**1**	143	0
30+31							95	0	54	0					85	**1**
TOTAL	**1791**	**13**	**2090**	**5**	**1855**	**7**	**2215**	**3**	**1371**	**0**	**2310**	**4**	**1332**	**4**	**1992**	**4**
%	0.73		0.24		0.38		0.14		0		0.17		0.3		0.2	
Average %	0.31															

IV.1.4. Using an *FRT82B* chromosome bearing puc^{A251} allele to generate intact small eggs with shortened appendages.

The last mutation we decided to test affects the gene *puckered* (*puc*). Known to be involved in the JNK pathway, one of its studied functions is to downregulate the activity of Jun kinase in the process of dorsal closure during *Drosophila* embryonic development (Martin-Blanco et al., 1998). It has also been reported to have a possible role in the regulation of follicle cell morphogenesis during the process of oogenesis. The study claims that it is most probably involved in the signaling pathways that bring about the movements and rearrangements in the cells of the follicular epithelium during the process of dorsal appendage formation and elongation, as well as the process of nurse cell dumping when the nurse cells deliver most of their contents into the growing oocyte, as they get smaller in size and finally degenerate (Dobens et al., 2001).

It has been reported that occasional small eggs with shortened dorsal appendages have been recovered from mosaic females heterozygous for the allele puc^{A251} (see Figure 15D). Therefore they assumed that these eggs were products of egg chambers with complete or large follicle cell clones homozygous for puc^{A251}. Lacking a cell marker for eggs from complete follicle cell clones, a direct association between the observed egg phenotype and mutant egg chambers could not have been made (Dobens et al., 2001). We decided to use puc^{A251} in our testing technique to find out if intact eggs with the reported phonotype could indeed be recovered from mosaic females heterozygous for puc^{A251}.

I did not observe any intact eggs from the generated mosaic females. This was the observation both when mitotic recombination events were induced at

pupal stages and at the adult stage (Table 7A, B). Initially this was quite puzzling since it meant that, contrary to the published data on the *puc* mosaic eggshell phenotype (Dobens et al., 2001), egg chambers with complete follicle cell clones were not being generated. However, direct inspection of ovaries with follicle cell clones homozygous for the puc^{A251} allele, marked by the absence of a conventional cell marker, revealed that egg chambers with complete follicle cell clones were never detected in the ovaries of dissected mosaic females, and that any clones detected were consistently small (C. Laplante, personal communication). This observation is consistent with the lack of intact eggs observed with the dec^{DN} system and suggests that the abnormal eggs reported previously (Dobens et al., 2001) may have arisen sporadically, and were likely not caused by puc^{A251} mutant follicle cell clones.

IV.1.5. No evidence that the intact eggs are products of egg chambers with patches of homozygous follicle cell clones in the follicular epithelium.

As previously described, the scheme allows egg chambers with complete follicle cell clones to give rise to intact eggs. However, it was difficult to predict the outcome of egg chambers with patches of homozygous follicle cell clones that do not comprise the entire epithelium. We expected the follicle cell dec^{DN}/dec^{DN} twin spots to secrete faulty eggshell material that would be visibly distinguished from otherwise intact eggshell material from the mutant follicle cells. This expectation was based on the outcome of the marker technique that uses the *dec+* rescue construct in a *dec-1* mutant background, where the faulty eggshell patches secreted by the homozygous mutant follicle cells that have lost the rescue

Table 7. The inability to recover of intact eggs from mosaic females heterozygous for the tested *FRT82B dec^{DN}* chromosomes as well as *FRT82B puc^{A251}*.

Eggs laid by mosaic *yw hs-Flp; FRT82B dec^{DN}/FRT82B puc^{A251}* females were collected on apple juice plates on successive days. In each case, the total number of eggs (T) on the plate was counted and they were examined for intact eggs (I). Eggs from mutant *puc^{A251}* follicle cells were previously reported to be smaller than usual with shortened appendages. However, intact eggs were never observed when mitotic recombination was induced in the pupal stage **(A)** or in the adult stage **(B)**.

(A)

day	inj-140.1		inj-140.1		inj-140.4		inj-140.4		inj-140.4		inj-140.6		inj-140.6		inj-140.7		inj-140.7		inj-140.7	
	T	I	T	I	T	I	T	I	T	I	T	I	T	I	T	I	T	I	T	I
6+7	174	0	180	0	273	0	199	0	123	0	175	0	136	0			403	0	127	0
8+9	129	0	288	0	144	0	183	0	127	0	82	0	161	0	123	0	247	0	116	0
10+11	96	0	220	0	183	0	264	0	102	0	121	0	172	0	153	0	236	0	188	0
12+13	44	0	288	0	250	0	244	0	153	0	138	0	127	0	118	0	191	0	162	0
14+15	55	0	137	0	80	0	271	0	112	0	102	0	169	0	225	0	238	0	189	0
16+17	107	0	299	0	141	0	117	0	167	0	107	0	103	0	127	0	239	0	107	0
18+19	81	0	90	0	135	0	238	0	21	0	103	0	140	0	204	0	220	0	99	0
20+21	29	0	130	0	170	0	101	0	136	0	65	0	85	0	60	0	284	0	196	0
22+23	84	0	112	0	122	0	202	0	80	0	81	0	91	0	149	0	193	0	140	0
24+25	40	0	132	0	123	0	150	0	61	0	67	0	61	0	139	0	267	0	154	0
26+27	39	0	95	0	125	0	17	0	83	0	85	0	73	0	137	0	228	0	95	0
28+29	14	0	109	0	86	0	168	0	60	0	88	0	50	0	99	0	126	0	99	0
30+31			151	0			159	0	64	0	57	0								
TOTAL	**892**	**0**	**2231**	**0**	**1832**	**0**	**2313**	**0**	**1289**	**0**	**1271**	**0**	**1368**	**0**	**1534**	**0**	**2872**	**0**	**1672**	**0**
%	0		0		0		0		0		0		0		0		0		0	

(B)

day	inj-140.1		inj-140.1		inj-140.4		inj-140.4		inj-140.6		inj-140.6		inj-140.7		inj-140.7	
	T	I	T	I	T	I	T	I	T	I	T	I	T	I	T	I
4+5	252	0	254	0	88	0	177	0	50	0	245	0	998	0	253	0
6+7	199	0	64	0	145	0	135	0	39	0	196	0	118	0	133	0
8+9	179	0	125	0	34	0	180	0	62	0	211	0	103	0	175	0
10+11	124	0	96	0	53	0	136	0	41	0	172	0	98	0	150	0
12+13	117	0	56	0	43	0	148	0	26	0	206	0	125	0	118	0
14+15	147	0	82	0	66	0	168	0	20	0	194	0	71	0	151	0
16+17	199	0	90	0	48	0	149	0	49	0	122	0	95	0	125	0
18+19	142	0	72	0	76	0	172	0	27	0	206	0	60	0	116	0
20+21	214	0	115	0	30	0	104	0	14	0	81	0	131	0	153	0
22+23	98	0	48	0	37	0	134	0	9	0	95	0	101	0	135	0
24+25	115	0	28	0	42	0	98	0	6	0	90	0	135	0	127	0
26+27	85	0	31	0	36	0	79	0			63	0	89	0	126	0
28+29	90	0	26	0	31	0	81	0					65	0	76	0
TOTAL	**1961**	**0**	**1087**	**0**	**729**	**0**	**1761**	**0**	**343**	**0**	**1881**	**0**	**2189**	**0**	**1838**	**0**
%	0		0		0		0		0		0		0		0	

construct were visibly distinguishable (Nilson and Schupbach, 1998). However, no such eggs with different eggshell make-up were ever detected.

IV.1.6. Frequency of recovered intact eggs with *FRT82B* chromosome arms bearing mutations is lower than expected.

It is clear that the frequencies of obtaining intact eggs with the chromosomes *FRT82B* cic^{BA53}, *FRT82B* cic^{U6} and *FRT82B* $pnt^{\Delta 88}$ are significantly lower than that of recovering wild type intact eggs when using the wild type *FRT82B GFP* chromosome. This may be due to mutant eggs being generally less intact than wild type, regardless of the dec^{DN} system. For instance, mutant *cic* eggs have been generally observed to be slightly less intact than wild type eggs, most probably as a secondary effect of the mutation (L. Nilson, personal communication).

However, this low frequency is not foreseen as a future problem when the Flp-DFS system relying on dec^{DN} is used in screens for instance, since the obtained information from the intact eggs is unambiguous, and the clear difference between collapsed and intact eggs makes the screening process not too hard to do.

IV.2. Testing the *FRT42D dec^{DN}* lines.

We decided to also test a number of the recovered *FRT42D dec^{DN}* lines, namely MR-25.1 and MR-77.1 generated by the *P*-element – induced male recombination technique, and inj-117.2 and inj-217.1 generated by the microinjection transgenesis technique. We chose a subset of the lines to test because of the practical difficulty of testing each individual line.

As in the case of testing the *FRT82B dec^{DN}* lines, I generated females with a source of Flipase under the control of a heat-shock promoter, which were also heterozygous for the *FRT42D dec^{DN}* chromosome and a corresponding *FRT42D* chromosome. Eggs on egg collection plates were periodically examined for intact eggs of the expected phenotype depending on the corresponding *FRT42D* chromosome being used. I tested four lines using a wild type *FRT42D* chromosome for the generation of mosaics.

IV.2.1. Using a wild type *FRT42D* chromosome to generate wild type intact eggs.

The corresponding *FRT42D* chromosome I used was a wild type *FRT42D GFP* chromosome that does not bear any mutation. My expectation was to recover occasional intact eggs that appeared wild type, just as when testing the *FRT82B dec^{DN}* lines with a wild type *FRT82B GFP* chromosome. Indeed, I observed the recovery of occasional wild type intact eggs (see Figure 9) that sometimes hatched, amongst a collection of otherwise collapsed eggs (Table 8). The hatching of some of the intact eggs confirms that the generated intact eggs could sustain the development of an embryo.

day	MR-25.1		MR-77.1		MR-77.1		inj-177.2		inj-217.1		inj-217.1	
	T	I	T	I	T	I	T	I	T	I	T	I
5+6	308	0	231	0	247	0	259	0	154	0	300	0
7+8	172	0	130	0	213	0	143	0	140	0	69	**1**
9+10	124	0	139	0	81	0	70	0	84	0	77	**1(1)**
11+12	90	**1**	85	**2(2)**	33	0	33	0	73	0	22	0
13+14	31	0	29	0	22	0	58	0	53	**3(1)**	27	**1(1)**
15+16	54	**2(1)**	21	**3**	30	0	57	0	69	**3**	13	**2**
17+18	38	**3(1)**	21	**1(1)**	32	**1(1)**	51	**2**	39	**5(1)**		
19+20	22	0	30	**6(3)**			67	**3(2)**	21	**5(3)**		
21+22	68	**10(5)**	17	**2(2)**					37	**8(5)**		
23+24	30	**4(2)**	12	0					17	**2(1)**		
25+26	15	**3(3)**	22	**4(2)**					14	**1(1)**		
TOTAL	952	**23(12)**	**737**	**18(10)**	**658**	**1(1)**	738	**5(2)**	**701**	**27(12)**	**481**	**4(2)**
%	2.42		2.44		0.15		0.68		3.85		0.83	
Average %	1.73											

Table 8. The recovery of intact wild type eggs from mosaic females heterozygous for the tested *FRT42D dec^{DN}* chromosomes as well as the wild type chromosome *FRT42D GFP*.

Eggs laid by mosaic *yw hsFlp; FRT42D dec^{DN}/FRT42D GFP* females were collected on apple juice plates on successive days after the induction of mitotic recombination events. In each case, the total number of eggs on the plate was counted and they were examined for intact eggs. When observed, these intact eggs were always wild type (number in red) and sometimes a few of them even hatched (number in parentheses). The table shows the total number of eggs and the total percentage of intact eggs recovered from each group of females.

Chapter V. Future Directions

V.1. Using *FRT42D wind* to test the system.

As in the case of testing the *FRT82B dec*^{DN} lines, we initially planned to test the *FRT42D dec*^{DN} lines with corresponding *FRT42D* chromosomes bearing mutations. The plan was to use *wind*, which is found on 2R. However, due to practical obstacles and time constraints, the generation of *FRT42D wind* fly stocks was not completed in due time to be included in the testing phase.

As described above, *wind* is a maternal effect gene required in the follicle cells and known to be involved in the events of establishing the D-V axis of the developing embryo. It is expressed in all follicle cells where it is involved in ER trafficking (Konsolaki and Schupbach, 1998), however it has been reported to be required specifically in the ventral follicle cells for the proper localization of Pipe in the Golgi. In the absence of Wind, Pipe is retained in the ER (Sen et al., 2000).

Although *wind* is a maternal effect gene expressed in the follicle cells, it is not zygotically required. Therefore, homozygous mutant *wind* flies are viable. Homozygous *wind* females lay eggs with a wild type eggshell pattern, but the embryos that develop within them lack ventral fates. This could be easily observed by dechorionating the deposited eggs and examining the features of the developing embryo. Wild type embryos exhibit characteristic ventral structures called ventral denticles. Embryos in eggs from homozygous *wind* females lack these structures. Knowing the exact phenotype of the eggs and embryos produced by egg chambers with the entire follicular epithelium homozygous mutant for

wind alleles makes it a potentially useful gene to test the *FRT42D dec*^{DN} lines with.

An especially useful application of using *FRT42D wind* chromosomes in the testing process would be to directly address the issue of whether the recovered intact eggs from mosaic females are indeed due to complete follicle cell clones as opposed to simply very large ones. Using the loss of the *dec+* rescue construct as an eggshell marker in females otherwise mutant for *dec-1* showed that partial homozygous *wind* follicle cell clones have localized dorsalizing effects on the developing embryo. Only the sections of the developing embryo that correspond to partial *wind* clones on the ventral side become dorsalized. The other parts of the embryo not surrounded by such *wind* mutant follicle cell clones were found to be properly patterned (Nilson and Schupbach, 1998). Therefore, by using *wind* one can examine intact eggs from mosaic females. If indeed all the recovered intact eggs are consistently completely dorsalized, then they are strictly products of complete follicle cell clones. However, observing developing embryos that are only dorsalized in some of their segments would argue that the developed Flp-DFS system based on *dec*^{DN} allows for egg chambers with large follicle cell clones that do not necessarily comprise the entire epithelium to produce intact eggs.

V.2. Using *P*-element – induced male recombination to recover *FRT dec*^{DN} lines for 2L and 3L.

Since using the *P*-element – induced male recombination technique has been successful in generating *FRT dec*^{DN} lines on 2R, it can be used to recover

recombinant lines on 2L and 3L. In this case, dec^{DN} stocks where the transgene is incorporated on each of these chromosome arms, preferably at a distal position to increase the chance of recovering the required recombination events, are needed. The locations of the dec^{DN} transgene on the remaining two dec^{DN} lines from the Waring lab could be mapped using inverse PCR. If neither of them has dec^{DN} on appropriate positions on 2L or 3L, then such lines can be generated by injecting the *pCaSpeR4* – dec^{DN} construct into *yw* embryos.

The stock with the genomic transposase source, Δ *2-3*, on the third chromosome, which has been used for *FRT42D* dec^{DN} can similarly be used to recover *FRT40A* dec^{DN} recombinant lines on 2L. However, to generate *FRT* dec^{DN} lines for 3L, a stock with the genomic transposase source, Δ *2-3*, on the second chromosome is needed.

V.3. Using the microinjection transgenesis technique to recover *FRT* dec^{DN} lines for 2L and 3L.

As explained above, the scheme of subcloning dec^{DN} into a *piggyBac* vector and then co-injecting it with *piggyBac*-specific transposase into *FRT* embryos promises to be highly efficient (Hacker et al., 2003).

Alternatively, recovering *FRT* dec^{DN} lines is possible by directly injecting the *pCaSpeR4* – dec^{DN} *P*-element into *FRT* embryos, as I have done. Since the injection into *w; FRT40A; FRT80B* embryos only yielded a few transformants, injecting into embryos with only either one of the two *FRT* sites: *w; FRT40A* or *w; FRT80B* might be fruitful.

V.4. Using the technique to study existing mutations and/or to identify novel ones using screens.

So far the frequency of recovered intact eggs from mosaic females is not high enough to use the system to screen for new mutations. Ideally, it should be increased, possibly by following different heat-shock regimens, such as changing the stage at which the flies are heat-shocked or the duration of the treatment, to alter the details of the induction of site-specific mitotic recombination events. Also ideally, the intact eggs should be somehow shown to be products of complete follicle clones for the system to be of optimum use. If these two issues are successfully addressed, then the system can ultimately be used to study candidate maternal effect genes required in the follicle cells, or as a basis for screens to identify novel ones, such as the gene coding for the substrate of Pipe.

Chapter VI. Summary

In *Drosophila*, maternal effect genes responsible for the proper patterning of the egg, and consequently the embryo, are expressed both in the germline and the follicular epithelium of egg chambers. To identify these genes, screens are typically set up where the eggs and embryos of females homozygous for randomly induced mutations are examined for patterning defects. However, such homozygous females cannot be generated when the mutation affects zygotically required genes. To identify maternal effect genes that are also zygotically required; i.e. they are expressed both during embryonic development and later in females during oogenesis; mosaic females are typically recovered using the Flp-FRT system that induces site-specific mitotic recombination events, resulting in homozygous cell clones. When studying maternal effect genes expressed in the follicular epithelium, eggs produced from egg chambers with complete follicle cell clones are most interesting to study, since their follicular epithelia are identical to those of egg chambers in homozygous females, had they been viable. However, to date, it has not possible to distinguish these eggs from amongst a collection of eggs laid by mosaic females.

We have developed an eggshell marker based on a dominant negative allele, dec^{DN}, of the gene *dec-1*, that allows us to identify these eggs from mosaic females. *dec-1* is expressed in the follicle cells, coding for a number of structural proteins that become integrated in different locations of the secreted eggshell material. Females with a copy of the dec^{DN} transgene lay collapsed eggs that never hatch, making them sterile. In our developed Flp-DFS technique, mosaic females

are generated that are heterozygous for *FRT* dec^{DN} chromosomes as well as homologous *FRT* chromosomes bearing the studied mutation. Complete homozygous mutant follicle cell clones do not express dec^{DN} and therefore the secreted eggshell material is not faulty. Eggs produced from this class of egg chambers are intact and can therefore be identified from amongst a collection of otherwise collapsed eggs laid by the mosaic females.

Because the technique relies on the Flp-FRT system to generate follicle cells, a crucial step was to generate *FRT* dec^{DN} chromosome arms. The routinely used method of meiotic recombination could not be used for our purposes since it takes places exclusively in female *Drosophila*, and dec^{DN} females are sterile. Therefore alternative methods were utilized. The method of irradiation, reportedly used by another team to create *FRT* chromosomes bearing another dominant female sterile mutation, was not successful. However, *FRT* dec^{DN} chromosomes for the right arms of the second and third chromosomes were indeed generated using the *P*-element – induced male recombination method and the microinjection transgenesis method. The generated lines were finally used to demonstrate that intact eggs with expected phenotypes were recovered from mosaic females heterozygous for *FRT* dec^{DN} as well as corresponding homologous *FRT* chromosomes bearing characterized mutations.

The developed Flp-DFS system based on dec^{DN} will allow, for the first time, the recovery and identification of eggs from complete follicle cell clones. This system will facilitate the study of candidate maternal effect genes required in the follicle cells as well as contribute to the success of screens for novel ones.

Chapter VII. References

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