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Towards a molecular genetic analysis of spermatogenesis in *Drosophila*

A thesis submitted
to the faculty of The Rockefeller University
in partial fulfillment of the requirements
for the degree of Doctor of Philosophy
by

Pierre Gönczy

5 April 1995

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ABSTRACT

Spermatogenesis is an evolutionarily conserved developmental program, which entails a sequence of fundamental biological processes: stem cell division, restricted mitotic proliferation, meiosis and morphogenetic reorganization. We have initiated a molecular genetic dissection of the early steps of spermatogenesis in *Drosophila*. We generated a collection of enhancer trap lines that identify all cell types and stages of the developmental program. We used marker lines to show that somatic cell fates are altered in the absence of germ cells, suggestive of signaling events between germ line and soma during wild-type spermatogenesis. We also used markers to examine whether *bag-of-marbles* and *benign gonial cell neoplasm*, two previously identified sterile mutations, affect the fate of the gonial daughter of the germ line stem cell. We next isolated P-element induced male-sterile mutants, which underwent mapping, complementation and phenotypic analyses. Three of these mutants were characterized in more detail. First, we showed that *chickadee* is required, presumably in stem cells, for germ cell proliferation. Second, we identified that *ms(2)916* is needed to restrict the extent of germ cell proliferation during spermatogenesis. Third, we demonstrated that *roughex* is a novel dose-dependent regulator of the second meiotic division. Finally, we designed a FLP/FRT-based screen that should allow us to directly identify loci regulating germ cell proliferation, without relying on a male-sterile phenotype. This opens up the possibility of saturating the genome for mutations affecting this key early step of spermatogenesis.

CHAPTER 1: INTRODUCTION

The production of mature sperm from an undifferentiated germ cell involves the execution of an evolutionarily conserved developmental program (reviewed by Roosen-Runge, 1977; Bellvé, 1979; Kimble and Ward, 1988; Fuller, 1993). Spermatogenesis presents biologists with a succession of fundamental processes: regulation of stem cell fate, counting of mitoses, progress through meiosis, and switch to terminal differentiation. These processes can be analyzed in *Drosophila*, where a combination of cell biological and genetic approaches may unravel the regulatory logic of this astonishing developmental program.

A late start

Aristotle reported his observations of a developing chick embryo over 2000 years ago, and the importance of eggs in the propagation of species was probably established long before his time. In contrast, the small male gametes had not been observed before the 17th century, and their role in fertilization became firmly established only in 1877.

Using the recently developed microscope, Leeuwenhoek and Hartsoeker reported in 1678 what Leeuwenhoek mistook for parasitic animals in the semen, hence the misnomer spermatozoae ("sperm animals"). The actual origin and function of spermatozoae was not recognized until 1824, when Dumas and Prevost postulated that spermatozoae might be the agents of fertilization, as they noted their universal presence in mature males, and their absence in immature and old individuals, as well as in the sterile mule. Their postulate was verified in 1877, when Hertwig and Fol observed the entry of individual sperm in sea urchin oocytes, and the subsequent fusion of pronuclei, followed by the early cleavage divisions.

Origins of the gonad

The production of functional sperm is the end-result of spermatogenesis, which takes place in testes containing both differentiating germ cells and supporting somatic cells. In most organisms, including insects and vertebrates, primordial germ cells are specified early in embryogenesis, and initially set aside from the soma (reviewed by Dixon, 1994). It is only later during embryogenesis that primordial germ cells contact specialized somatic cells, and together form a gonad that will become the site of gametogenesis.

While largely unkown in most organisms, the mechanisms responsible for the early specification of germ cells are being unraveled in the fly (reviewed by Lehmann and Ephrussi, 1994). *Drosophila* primordial germ cells -called pole cells- are formed at the posterior end of the embryo 1 hour 30 min after fertilization, prior to the cellularization of somatic cells. The posterior pole cytoplasm contains information which is both necessary and sufficient for the specification of pole cells. Thus, UV-irradiation of the posterior pole of the early embryo results in the absence of pole cells (Okada et al., 1974). Conversely, posterior cytoplasm transplanted into ectopic locations instructs other nuclei to become pole cells (Illmensee and Mahowald, 1974). Genetic analyses have identified a cascade of genes whose function is required in the mother to assemble the germ plasm at the posterior pole of the embryo. While all these genes are necessary for pole cell formation, one of them, *oskar* (*osk*; Lehmann and Nüsslein-Volhard, 1986), has been shown to be also sufficient for ectopic pole cell formation (Ephrussi and Lehmann, 1992). Further analyses of *osk* and other members of the genetic cascade should elucidate the mechanisms leading to germ line specification.

During gastrulation and germ band elongation, pole cells are transported dorsally, and then back into the embryo, where they squeeze through the gut primordium and contact lateral mesoderm (Sonnenblick, 1941; Aboïm, 1945; Mahowald, 1962; Hay et al., 1988; Warrior, 1994). Pole cells and mesodermal precursors of the somatic components

of the gonad then migrate together anteriorly to the site of gonad formation (Brookman et al., 1992; Boyle and DiNardo, 1995). About 10 pole cells are found in the newly formed gonad, and are the precursors of germ line stem cells, while the 30 or so somatic cells will give rise to all the somatic cell types found in testes and ovaries.

The migration of primordial germ cells to the site of gonad formation varies among organisms (reviewed by Gomperts et al., 1994). In birds for instance, primordial germ cells are transported through the blood, and enter the embryo proper in the vicinity of the genital ridge by squeezing through endothelial cells of the blood vessels (Dubois, 1969). In contrast, in mammals, much like in *Drosophila*, primordial germ cells translocate entirely through tissues of the developing embryo, migrating over mesenchyme towards the genital ridge (Chiquoine, 1954).

Germ cells and somatic cells in the newly formed gonad are now in place to initiate gametogenesis, which begins at a specific moment depending on the organism and the sex of the animal.

An evolutionarily conserved differentiation program

The sequence of processes that characterize spermatogenesis is remarkably conserved throughout evolution. Spermatogenesis begins as a germ line stem cell divides asymmetrically, regenerating a stem cell and producing a gonial daughter cell that undergoes a limited number of mitoses. The resulting germ cells then enter the meiotic cell cycle and execute two meiotic divisions in quick succession. Haploid germ cells finally undergo terminal differentiation to produce functional sperm. As the sequence of cellular processes is so conserved, it is likely that the regulatory logic and some of the mechanisms ensuring progress through spermatogenesis will be conserved as well.

Stem cells

Spermatogenesis is ongoing: as mature sperm leave the testis, they are replaced by a continuous supply of differentiating germ cells. Just like in other lineages that give rise to non-dividing cells with a limited life-span, spermatogenesis depends on the activity of stem cells for continued renewal. In such lineages, the rate at which stem cells and their mitotically amplifying progeny proliferate ultimately determines the number of differentiated cells produced (reviewed by Hall and Watt, 1989; Evans and Potten, 1991). Therefore, an understanding of how stem cell proliferation is controlled is central to an elucidation of how organisms maintain an adequate balance of differentiated cell types.

In vertebrates, tissues that rely on the activity of self-renewing stem cells include the blood, the epidermis, the intestinal epithelium, as well as the testis. While hematopoietic and intestinal stem cells are pluripotent, giving rise to more than one differentiated cell type, stem cells in the epidermis and the testis are unipotent, as only keratinocytes or sperm are produced, respectively.

Stem cells in these vertebrate systems are usually defined by functional assays, testing their capacity to sustain the long-term production of differentiated cells (Till and McCulloch, 1961; Whithers, 1967; Potten and Hendry, 1973). For example, germ line stem cells from a donor mouse can repopulate host testes devoid of endogenous stem cells, and thus generate a permanent supply of sperm (Brinster and Zimmermann, 1994). Except for the testis, where stem cells may be identified by their characteristic morphology and relative quiescence (Monesi, 1962; Dym and Clermont, 1970; Huckins, 1978), stem cells can not be localized with precision in most vertebrate systems. For instance, cells within the bone marrow lack a strict spatial organization, and the pluripotent stem cells can not be recognized on morphological criteria alone (Western and Bainton, 1979; Gordon et al., 1988; reviewed by Lord and Testa, 1988). In the epidermis, stem cells are located in a basal cellular layer, in contact with basement membrane (Iversen et al., 1968; Watt and Green, 1982; reviewed by Potten and Morris, 1988). However, this basal layer also contains morphologically indistinguishable mitotically

amplifying progeny cells, as well as some early keratinocytes. There are no in vivo marker specific to the epidermal stem cells (Hall and Watt, 1989). In contrast, stem cells can be readily identified in other cases, for instance in *Drosophila* ovaries, where they occupy a well-defined position at the tip of each ovariole (King, 1970; Lin and Spradling, 1993).

Normally fairly quiescent stem cells can be led to proliferate when the number of differentiated cells is insufficient. For instance, proliferation of stem cells in the rat testis increases after X-rays-induced loss of more mature germ cells (Dym and Clermont, 1970). Similarly, the division rate of epidermal stem cells or their mitotically amplifying progeny increases following wounding in the skin (Potten and Allen, 1975). An increase in the basal epidermal cell population is also observed in psoriasis, a common skin disorder (Harrison, 1983a). Misregulation of hematopoietic stem cell proliferation may also cause disease. In acute myeloid leukemia, there is a clonal overproduction of all cell types in the blood, probably resulting from deregulated proliferation of pluripotent hematopoietic stem cells (Fialkow et al., 1987; Keinanen et al., 1988). Conversely, in aplastic anemia, there is a pronounced deficit of all blood cell types, consistent with a defect in pluripotent stem cells (Harrison, 1983b).

A variety of cytokines and growth factors can modulate the proliferation of hematopoietic stem cells or their progeny in tissue culture (reviewed by Ogawa, 1993). It will be important to determine whether these modulators also control stem cell proliferation in vivo, and understand how stem cells in these various systems translate environmental cues to modify their rate of cell cycle progression.

Asymmetric cell division

Stem cells divide asymmetrically, in that the two daughters of the division have distinct fates (Horvitz and Herskowitz, 1992). While one daughter remains a self-renewing stem cell, the other has a different fate, that of leading to the production of

differentiated cells. Understanding how this initial asymmety is established and transduced at a cellular level is key in understanding stem cell lineages.

Asymmetric cell divisions can be either intrinsically determined, or influenced by extrinsic factors, coming for instance from neighbouring cells. One particular striking example of cell intrinsic mechanism occurs during mating type interconversion in *S. pombe*. The division of fission yeast is asymmetric, as one daughter is competent to switch mating-type, while the other is not (Miyata and Miyata, 1981). This difference in switching competence is not due to cytoplasmic factors, but rather to chromosomal differences at the mating-type locus, as homologous chromosomes in diploid cells can switch independently (Egel, 1984). It appears that, in haploids, one of the two DNA strands at the mating-type locus is modified, for instance allowing switching to occur. As only one of the daughter cells gets such a modified mating-type locus, only one daughter is able to switch, distinguishing it from it's sister (Klar, 1987; Klar, 1990). Thus, marking one DNA strand in a parental cell can generate asymmetry among daughters.

In another instance, unequal segregation of a determinant among daughter cells causes them to adopt distinct fates. *numb* was first identified genetically to be required for the asymmetric division of a set of sensory organ precursor (SOP) cells (Uemura et al., 1989). numb protein is asymmetrically distributed at the plasma membrane of the SOP cell, and is unequally partitioned into the two daughters, thus presumably establishing their distinct fates (Rhyu et al., 1994).

Cell extrinsic mechanisms determine other asymmetric cell divisions. For instance, in the 4 cell stage *C. elegans* embryo, the ABp blastomere requires a signal from its P2 neighbour in order to adopt a fate different from its sister blastomere ABa (Mello et al., 1994). ABp similarly adopts the fate of its sister in embryos lacking *glp-1* or *apx-1* function (Mello et al., 1994). *glp-1* encodes a member of the Notch/lin-12 family of transmembrane receptors, while *apx-1* encodes its likely ligand, as it is homologous to Delta, a ligand of *Drosophila* Notch (Austin and Kimble, 1989; Yochem et al., 1989;

Fehon et al., 1990; Mello et al., 1994). *glp-1* protein is expressed at the surface of the ABp blastomere (Evans et al., 1994), and is likely to receive the apx-1 signal emanating from the P2 neighbour. Thus, cell-cell interactions can establish an asymmetry between two potentially equivalent sisters.

Close to nothing is known of the mechanisms that determine asymmetric cell fates among the daughters of long-term, self renewing stem cells. An observation that may suggest a role for cell extrinsic mechanisms is that such stem cells are usually located near a basal lamina, while differentiating cells are not. For instance, germ line stem cells in the mammalian testis are in contact with the outer basement membrane of the sex cords (reviewed by Bellvé, 1979). Epithelial stem cells are also in close contact with the underlying basement membrane (reviewed by Potten and Morris, 1988). Interestingly, purified human epidermal stem cells can be separated from their mitotically amplifying progeny on the basis of $\beta 1$-integrin levels, and adhesiveness to extra-cellular matrix proteins (Jones and Watt, 1993). Moreover, epidermal cells continue to proliferate only when they are placed on a cellular substratum (Rheinwald and Green, 1975a,b). Though correlative, these observations suggest that factors secreted by neighbouring cells or deposited in the extra-cellular matrix may maintain the fate of self-renewing stem cells.

Mitotically amplifying cells

The departing daughter of a stem cell does not directly turn into a differentiated cell. Instead, it is first mitotically amplified, and only then do the resulting cells initiate terminal differentiation. The number of amplifiying mitotic divisions is typically invariant within a given lineage. For instance, commited precursors in chick skeletal muscle undergo 4 divisions (Quinn et al., 1985), while keratinocytes in culture divide less than 15 times (Barrandon and Green, 1987). During mammalian spermatogenesis, 5 or 6 amplifying divisions occur in the rat and the mouse, while there are 10 in the golden hamster (reviewed by Bellvé, 1979). In *Drosophila* spermatogenesis, the number of

amplificatory mitoses is species-specific: there are 3 in *D. hydei*, 4 in *D. melanogaster*, and 5 in *D. pseudoobscura* (Liebrich, 1984). A particularity of spermatogenesis at this amplificatory step is that many germ cells degenerate before progressing to subsequent stages of the developmental program (reviewed by Roosen-Runge, 1977). As much as two thirds of amplified germ cells may degenerate in mammals (Huckins, 1978), while the extent of this phenomenon has not been quantified in *Drosophila*. In mammals, the degenerating germ cells undergo programmed cell death (Allan et al., 1992). Although the reason for such an elimination is not understood (but discussed by Oakberg, 1956; Clermont, 1962; Huckins, 1978), it clearly impacts on the final number of differentiated germ cells.

What sets the number of amplifying divisions has been only investigated in a few cases. For example, during erythropoiesis, erythopoietin produced by the kidney induces the mitotic amplification of commited erythrocyte precursors (reviewed by Adamson and Brown, 1978). Erythropoietin production is itself regulated by the presence of terminally differentiated erythrocytes. Thus, a hormonal feedback mechanism modulates mitotic amplification in this instance. In the developing rat optic nerve, bipotential 02A precursor cells undergo a limited number of divisions before differentiating into either oligodendrocytes or type-2 astrocytes (Raff et al., 1985; Temple and Raff, 1986). Platelet-derived-growth factor (PDGF) is secreted by neighbouring type-1 astrocytes and can allow 02A cells in culture to undergo a set number of mitoses (Raff et al., 1988). Therefore, localized expression of a growth factor can determine the extent of mitotic amplification.

Although the signals that determine the counting of mitoses in transient amplifying cells are mostly poorly understood, they will undoubtly feed into the basic components that drive cell cycle progression in all eukaryotic cells. Negative and positive regulators of cell-cycle progression can influence a cell until a restriction point late in G1 called Start (reviewed by Pardee, 1989). After Start, cells are commited to undergoing S phase

and progress through another cell cycle. In recent years, there has been a tremendous increase in the knowledge of the basic cellular components allowing passage through Start, as well as of the mechanisms by which these components are modulated in physiological and pathological conditions (reviewed by Sherr, 1994). Briefly, passage through Start is mediated by cyclin-dependent-kinases (Cdk's), composed of a catalytic Cdk moiety, associated with a G1 cyclin regulatory subunit (reviewed by Reed, 1992; Sherr, 1994). The activity of Cdk's is further regulated by a series of mechanisms, including modifications of their phosphorylation status and association with inhibitory partners (Cdi's).

Modulation of cell cycle progression can be achieved by altering the careful balance of components determining Cdk activity (reviewed by Hunter and Pines, 1994). For instance, overexpression of one of the mammalian G1 cyclins, cyclin D1, is likely responsible for certain parathyroid adenomas. In this case, the gene encoding cyclin D1 comes under the control of the parathyroid hormone gene promoter following a chromosomal inversion, and is thus upregulated in a tissue-specific manner (Motokura et al., 1991; Inaba et al., 1992; Xiong et al., 1992). Cdk4, the main Cdk partner of cyclin D1, can also be a target of modulation. Cdk4 protein is downregulated following transforming-growth-factor $\beta 1$ (TGF$\beta 1$)-induced cell cycle arrest in Mv1Lu cells, and constitutive Cdk4 expression can overcome this block to cell cycle progression (Ewen et al., 1993). Finally, Cdi's can also be targets of modulation. For instance, p21, a Cdi which inhibits the activity of a variety of Cdk's, is induced by p53 following DNA damage, and thereby arrests cell cycle progression (El-Deiry et al., 1993; Xiong et al., 1993; Dulic et al., 1994; El-Deiry et al., 1994).

Modulation of cell-cycle progression can thus be accomplished by a series of regulatory mechanisms, and it is likely that variations on these same themes determine the rate of stem cell proliferation and allow for precise counting of mitotically amplifying divisions.

The meiotic cell cycle

During spermatogenesis, mitotically amplified germ cells next enter the meiotic cell cycle, undergo premeiotic S phase, progress through G2, and then execute two meiotic divisions in quick succession to generate a set of haploid cells.

The decision to enter the meiotic cell cycle has been best analyzed in *S. cerevisiae*, where it is governed by both environmental and cell-intrinsic factors (reviewed by Mitchell, 1988; Malone, 1990). First, nitrogen starvation of diploid cells results in down-regulation of a cAMP-dependent signal transduction cascade, thereby causing G1 cell cycle arrest. Several components of this nutritional control cascade have been identified, including adenylate cyclase, RAS1 and RAS2, as well as cAMP-dependent protein kinase subunits (Matsumoto et al., 1983; Kataoka et al., 1984; Tatchell et al., 1985; Toda et al., 1985; Toda et al., 1987; Toda et al., 1987). Second, diploid cells must be heterozygous at the mating-type locus in order to enter meiosis. In vegetatively growing cells, a negative regulator, *rme1*, prevents entry into meiosis through the inhibition of an inducer of meiosis, *ime1* (Mitchell and Herskowitz, 1986; Kassir et al., 1988; Smith and Mitchell, 1989). Entry into meiosis is initiated as the MATa1/MATα2 products of the mating-type locus repress RME1 transcription, thereby resulting in IME1 expression. IME1 in turn is the first component of a transcriptional cascade which leads to the expression of a set of meiosis-specific genes.

The meiotic cell cycle differs from the mitotic cell cycle in several ways. For instance, while commitment to progress through the mitotic cell cycle is made at Start, this is not so during the meiotic cell cycle. Indeed, yeast cells can return to the vegetative cycle even from prophase of meiosis I, if challenged with growth medium (reviewed by Esposito and Klapholz, 1981). This is not only true of *S. cerevisiae*, but also of other organisms, including *Lillium* and *Aspergillus* (reviewed by Baker et al., 1976). Perhaps reflecting this commitment difference, mutations in *cdc7*, *cdc28* or *cdc39*, while blocking mitotically dividing cells at Start, do not arrest cells in meiosis until after DNA

replication and spindle pole body separation (Schild and Byers, 1978; Schuster and Byers, 1989).

Premeiotic S phase also differs from S phase in the mitotic cell cycle. Premeiotic S lasts longer, for instance about twice as long in the case of mammalian spermatogenesis (reviewed by Bellvé, 1979). Moreover, premeiotic S is partially under separate genetic control, as some *S. cerevisiae* mutants, such as *mei-1* and *spo-9*, are specifically impaired in premeiotic S (reviewed by Esposito and Klapholz, 1981).

During meiosis I, chromosome separation is different than during mitosis, since homologous chromosomes pair and then segregate to opposite poles of the anaphase spindle. By contrast, during meiosis II, sister chromatids segregate to opposite poles just as they do during mitosis. Thus, the mechanics of chromosome segregation must be uniquely regulated at meiosis I. Accordingly, loci specifically required for the execution of meiosis I have been identified, both in yeast and flies. Some of these, such as *Drosophila ord* and *meiS-322*, are required for sister chromatid cohesion during meiosis I (reviewed by Baker and Hall, 1976).

Studies in a variety of organisms demonstrated that the meiotic divisions during oogenesis are controled by modifications of the universal mechanisms that drive cells through M phase (reviewed by Nurse, 1990; Hunt and Murray, 1993a). Briefly, the G2/M transition is dependent on the activation of a cdc2 kinase, composed of a cdc2 catalytic subunit and a G2 cyclin regulatory subunit. This complex is maintained inactive during G2 by phosphorylation of the cdc2 moiety (Gould and Nurse, 1989; Solomon et al., 1990; Krek and Nigg, 1991; Norbury et al., 1991). Entry into M is triggered by the cdc25 phosphatase, which removes the inhibitory phosphorylation, turning the complex into an active cdc2 kinase (Dunphy and Kumagai, 1991; Gautier et al., 1991; Millar et al., 1991; Strausfeld et al., 1991). cdc2 kinase is subsequently inactivated by the abrupt degradation of the cyclin subunit via a ubiquitin mediated process (Murray et al., 1989; Ghiara et al., 1991; Glotzer et al., 1991). Inactivation of the cdc2 kinase is a prerequisite for exit from

M, and cells with a nondegradable form of cyclin remain arrested at metaphase, with condensed chromosomes and an intact nuclear envelope. Oocytes in most organisms become arrested at metaphase of one of the two meiotic divisons, with high levels of cdc2 kinase (reviewed by Hunt and Murray, 1993b). In frogs for instance, oocyte remain arrested in meiosis II owing to a cytostatic factor, which prevents cyclin degradation. Fertilization induces an increase in intracellular calcium, which somehow relieves the block to cyclin proteolysis, resulting in the inactivation of cdc2 kinase and release from meiotic arrest.

Additional modifications of the archetypical cell cycle must exist during the meiotic divisions in all organisms. First, whereas feeback controls normally ensure that M phase only takes place if S phase has been completed (reviewed by Hartwell and Weinert, 1989; Murray, 1992), such mechanisms must be modified to allow meiosis II to be coupled to meiosis I without an intervening S phase. Second, a true interphase does not always occur between the two meiotic divisions. In oocytes of different organisms, chromosomes remain condensed and the nuclear envelope does not reform, despite a drop in cdc2 kinase activity (Dorée et al., 1983; Gerhart et al., 1984; Draetta et al., 1989). Therefore, it has been postulated that another kinase activated at meiosis I may prevent a full interphase by maintaining some cdc2 substrates phosphorylated between the two meiotic divisions (Hunt and Murray, 1993b). During spermatogenesis in several *Drosophila* species, although not in *D. melanogaster*, germ cells similarly fail to return to a true interphase configuration between the two meiotic divisions (Cooper, 1950). This suggests that modifications of the cdc2 kinase cycle may exist in this instance as well.

Terminal differentiation and morphogenesis

After execution of the two meiotic divisions during spermatogenesis, haploid germ cell exit the cell cycle and embark on a program of terminal differentiation. There is remarkable morphological conservation in the resulting spermatozoae among different

organisms: an axoneme is assembled, cytoplasmic constituents are reorganized and eventually discarded, the DNA is compacted, and an acrosome is positioned in front of the nucleus. In almost all organisms, the mature sperm has essentially become a haploid rod-shaped nucleus with an efficient flagellar propulsion system (see Nath, 1965). In a few organisms, including many species of worms and crustaceans, the terminal differentiation program is somewhat different, as sperm are not flagellar, and use pseudopods instead to move towards the oocyte (see Nath, 1965; Kimble and Ward, 1988).

The mechanims ensuring proper cellular reorganization during post-meiotic differentiation are not understood, but are likely to partially rely on regulated translational control. Autoradiographic studies show that the bulk of transcription during spermatogenesis occurs during the growth phase preceeding the meiotic divisions, while translation occurs both then and post-meiotically (reviewed by Bellvé, 1979; Fuller, 1993). Therefore, mechanisms must be in place to prevent the translation of a set of mRNA's until terminal differentiation. For example, this is the case for the mouse protamine mRNA, whose translation is prevented by a sequence in the 3'UTR, which also delays translation of an heterologous mRNA (Braun et al., 1989). This is also the case of a family of male-specific transcripts in *Drosophila*, which are transcribed prior to the meiotic divisions, but not translated until later stages, owing to a negative regulatory element in the 5'UTR (Schäfer et al., 1990).

Spermatogenesis in *Drosophila*

Thanks to the work of numerous electron microscopists in the 1960's and 70's, developmental biologists studying spermatogenesis in *Drosophila* today can rely on an extensive knowledge of the sequence of cellular events occuring during this 10 day long developmental program (Anderson, 1967; Bairati, 1967; Shoup, 1967; Tates, 1971; Stanley et al., 1972; Tokuyasu et al., 1972a; Tokuyasu et al., 1972b; Tokuyasu, 1974a;

Tokuyasu, 1974b; Tokuyasu, 1974c; Tokuyasu, 1975a; Tokuyasu, 1975b; Hardy et al., 1979; reviewed by Lindsley and Tokuyasu, 1980).

D. melanogaster males have two testes, each a blind tube 2 mm in length and 0.1 mm in diameter (refer to Fig. 1 throughout the description). The closed end of the testis is at the apical tip, which contains early germ cells, while the open end is at the base, where the testis connects to the seminal vesicle, into which mature sperm exit at the end of spermatogenesis.

Going from the tip to the base, each steady-state testis comprises all stages of spermatogenesis displayed in a developmental gradient. At the very tip, five to nine germ line stem cells and twice as many somatic cyst progenitor cells are anchored around a hub of post-mitotic somatic cells (refer also to Fig. 2 for the early stages). The plasma membrane of both germ line stem cells and cyst progenitor cells contacts the basal lamina surrounding the cells of the hub. Spermatogenesis begins when a germ line stem cell and two neighbouring somatic cyst progenitor cells, also acting as stem cells, divide in concert. The division of these three cells is asymmetric, in that it gives rise to pairs of daughters with distinct fates. Those daughters which remain in contact with the hub regenerate the parental self-renewing stem cells, while the three others take on the fate of progressing through subsequent steps of the developmental program. These three daughters are one primary gonial cell, coming from the germ line stem cell, and two cyst cells, resulting from the two cyst progenitor cells. The two cyst cells surround the primary gonial cell, thus forming a cyst, the fundamental unit of spermatogenesis, which matures as it moves from the tip towards the base of the testis.

While the cyst cells no longer divide, the primary gonial germ cell undergoes four amplificatory mitotic divisions, still close to the testis tip. Due to incomplete cytokinesis at each of these divisions, the resulting 16 primary spermatocytes are interconnected by intercellular bridges. Germ cells then enter an extended growth phase, during which their volume increases 25 fold. As they progress through the growth phase, cysts move down

to about the first coil of the testis, where germ cells execute the two meiotic divisions in quick succession.

The 64 haploid spermatids then initiate a dramatic program of terminal differentiation, during which cellular organelles are restructured, the nucleus is compacted and the flagellar tail assembled. Tail elongation occurs at about twice the speed of downwards cyst movement, so that sperm heads, which are at the front of the cyst, approach the base of the testis at the same time that the corresponding tails approach the tip. Sperm bundles eventually become 1.8 mm long, extending from the base to almost the tip of the testis. During post-meiotic stages, the two cyst cells become structurally distinct. The head-cyst cell remains compact, surrounding and interdigitating with sperm heads, whereas the tail-cyst cell becomes a long thin cell as it elongates with sperm tails. At the completion of spermatid differentiation, the head-cyst cell is entrapped by a specialized epithelial cell in the base of the testis. The process of individualization follows, during which previously syncitial germ cells become individualized by the investment of plasma membrane. During individualization, which starts at the front of the sperm bundle and moves towards the tip of the testis, a cystic bulge collects superfluous organelles and cytoplasmic remnants, leaving individualized spermatids stripped of cytoplasm. Coiling ensues, during which the bundle of spermatids collapses down to the base of the testis. The specialized terminal epithelial cell then phagocytozes the head cyst cell, as well as the collapsed tail cyst cell and waste bag, which contains material collected by the cystic bulge. Spermatozoae are released and transit to the seminal vesicle, where they remain stored until needed.

Genetics of spermatogenesis in *Drosophila*

The genetic analysis of spermatogenesis in *Drosophila* began with the discovery that the Y chromosome, although dispensible for viability, is absolutely required for male fertility (Bridges, 1916). Six separate Y chromosome loci were subsequently shown to be

required for fertility (reviewed by Fuller, 1993). Cloning of regions corresponding to fertility factors in *D. melanogaster* and *D. hydei* revealed that they contain mostly repetitive DNA (Lifschytz et al., 1983; Vogt and Hennig, 1986a,b; Huijser et al., 1988; Bonaccorsci et al., 1990). These observations, combined with the fact that Y-fertility factor transcription during the growth phase results in lampbrush loops, has led to the postulate that Y fertility factors may serve as ribonucleic storage sites for proteins prior to the meiotic divisions (Hulsebos et al., 1984; Hackstein et al., 1990). However, a dynein heavy chain gene appears to be located in one of the Y fertility factors (Goldstein et al., 1982; Hays, cited in Fuller, 1993), which may thus turn out to be conventional loci buried in repetitive DNA.

A large number of male-sterile mutants have identified loci on other chromosomes that are required for spermatogenesis (reviewed in Lifschytz, 1987; Fuller, 1993). Although most of these mutations affect post-meiotic aspects of the differentiation program, a few block progress through the early steps of spermatogenesis.

First, mutations in either *benign gonial cell neoplasm* (*bgcn*) or *bag-of-marbles* (*bam*) result in the accumulation of early germ cells during both spermatogenesis and oogenesis (Gateff, 1982; McKearin and Spradling, 1990). *bam* and *bgcn* are thus crucial for progress past one of the early stages of gametogenesis. However, their phenotypic analysis during spermatogenesis is rudimentary, and the exact fate of the accumulating germ cells has not been examined.

Second, in *ms(3)spermatocyte arrest*, *cannonball* and *always early*, germ cells do not progress through the meiotic cell cycle past the early stages of chromosome condensation (Fuller, 1993). Their further characterization should provide clues about what governs the transition from the late growth phase into meiosis I.

Third, *twine*, a germ line specific cdc25 homologue, is required slightly later, at the G2/M transition of meiosis I (Alphey et al., 1992; Courtot et al., 1992). *twine* mutant germ cells remain 4N cells, but nevertheless undergo most aspects of the terminal

differentiation program, such as elongation of sperm tails and nuclear reshaping. Importantly, this indicates that proper execution of the meiotic divisions is not a prerequisite for post-meiotic differentiation.

Other male-sterile mutants affect the mechanics of the meiotic divisions, and identify loci required for chromosome segregation, spindle formation or cytokinesis (Lifschytz and Meyer, 1977; reviewed by Fuller, 1993). ß2-tubulin is a structural protein specific to spermatogenesis, which is required in microtubule-based processes during the meiotic divisions and in post-meiotic germ cells (Kemphues et al., 1982). Germ cells carying a ß2-tubulin null fail to assemble a spindle at meiosis I (cited in Fuller, 1993). However, although chromosomes are incapable of segregating, they nevertheless decondense during the brief meiotic interphase, and recondense again on schedule for meiosis II. Interestingly, this reveals that the feeback mechanisms that usually arrest cells in metaphase if the spindle is inappropriately assembled (Hoyt et al., 1991; Li and Murray, 1991), are either modified or absent during the meiotic divisions during *Drosophila* spermatogenesis.

Notwithstanding the analysis of these and other select loci, not enough is known about the genetic circuitry controlling progress through spermatogenesis. There are several reasons for this lack of knowledge.

A very large number of loci, probably over 1000, can mutate to male-sterility (discussed by Lindsley and Tokuyasu, 1980; Lifschytz, 1987; Fuller, 1993). Some of these loci may be truly spermatogenesis-specific, just like ß2-tubulin, and have a male-sterile null phenotype. On the other hand, other loci may be also required for processes outside of spermatogenesis, and the male-sterile mutation may be a weak or spermatogenesis-specific allele of an essential genes. Consistent with this view, 15 % of a set of conditional lethal mutations became male-sterile when shifted to the restrictive temperature, in addition to 18 % which were sterile even at the permissive temperature (Shellenbarger and Cross, 1979). In addition, in one study, 3 out of 4 male-sterile strains,

mapping to a genetically well defined region of the X chromosome, turned out to be mutations in essential genes (Geer et al., 1983). Similarly, in females, germ line clones for many zygotic lethal mutations on the X chromosome result in sterility (Perrimon et al., 1989). Essential loci that can mutate to a male-sterile phenotype can be separated into two groups. First, the locus may be generally required for some aspect of cellular metabolism, and the frequent isolation of alleles affecting fertility could reflect the sensitivity of the developing germ cells to alteration of basic cellular functions (discussed by Lindsley and Tokuyasu, 1980; Lifschytz, 1987; Fuller, 1993). Alternatively, a locus could be required both during spermatogenesis and for other specific developmental processes. Such a locus could be interesting, and reveal fundamental aspects of the regulatory logic of spermatogenesis.

A second difficulty associated with a genetic dissection of spermatogenesis, in particular of the post-meiotic stages, stems from the fact that the vast majority of male-sterile mutants affecting terminal differentiation do not display a tight arrest phenotype (discussed by Lifschytz, 1987; Fuller, 1993). While accumulation of assembly intermediates permitted the ordering of gene function in the case of bacteriophage T4 assembly (reviewed by Wood and King, 1979), this approach is unlikely to be fruitful for analyzing post-meiotic morphogenesis. Indeed, various aspects of the terminal differentiation program, such as axoneme elongation, nuclear reshaping or mitochondrial derivative maturation, proceed on parallel and independent pathways. Moreover, even at the electron microscopic level, most mutants do not accumulate wild-type looking intermediates, and instead have a non-descript post-meiotic phenotype. Moreover, the large number of male-sterile mutants affecting terminal differentiation, as well as their pleiotropy, renders a thorough genetic analysis quite laborious. Ordering gene action can be difficult without knowing the null phenotype; in the case of male-sterile mutants, obtaining null alleles might often yield lethal mutations whose phenotype during spermatogenesis could be studied only in mosaic animals.

Finally, historical reasons also explain why not enough is known about what regulates the orderly sequence of processes during spermatogenesis. The field is small, and earlier work was driven primarily with the hope of understanding the mechanics of the meiotic divisions and the execution of morphogenesis, rather than the regulation of the developmental program. Orcein stains allowed one to follow the chromosomes during the meiotic division, and electron microscopy was used to analyze post-meiotic assembly, while no reagents were developed for examining earlier stages of spermatogenesis, or for following the somatic cells. Moreover, most of the male-sterile mutants that have been generated have not been mapped, were not taken through complementation analysis, and were not kept for further analysis.

Aims of this work

We wanted to investigate the molecular genetics of the early stages of spermatogenesis and begin addressing the following questions. How is stem cell fate maintained and daughter cell fate established ? Does the counting of mitotic divisions matter, and how is it regulated ? What causes germ cells to switch from the mitotic division program to the meiotic cell cycle ? How is their progress through the meiotic divisions controlled ? In what way may somatic and germ cells interact to ensure proper progress through spermatogenesis ?

Our project encompassed the following specific points, which are detailed in Chapters 2 through 7:

- we generated and characterized enhancer trap lines that identify all cell types and stages during spermatogenesis, in order to facilitate the molecular analysis of the developmental program at the light microscope level (Chapter 2)
- we used agametic testes and enhancer trap lines expressing lacZ in specific somatic cells, as well as other reagents, to investigate whether germ cells may signal to the somatic component of the testis during early spermatogenesis (Chapter 3)

- we used enhancer trap lines to explore the requirement for *bam* and *bgcn* function during the very early stages of spermatogenesis (Chapter 4)

- we generated a collection of P-element induced male-sterile mutations, in order to identify loci required for early processes during spermatogenesis, and to allow for their potential rapid molecular characterization (Chapter 5)

- we investigated the phenotype of two mutants from the collection, *chickadee* and *ms(2)916*, which alter germ cell proliferation (Chapter 6)

- we designed a FLP/FRT-based screen to identify novel loci that can mutate to a phenotype of overproliferating germ cells (Chapter 7)

- we analyzed another locus identified in the collection of male-sterile mutants, *roughex*, which is a novel dose-dependent regulator of the second meiotic division (Chapter 8)

CHAPTER 2: DESCRIBING SPERMATOGENESIS WITH ENHANCER TRAP LINES

INTRODUCTION

The sequence of cellular events that both germ cells and somatic cells undergo during the course of spermatogenesis has been extensively described at the ultrastructural level by electron microscopy (reviewed by Lindsley and Tokuyasu, 1980).

Ultrastructural analysis has been extremely valuable in defining all morphologically distinct cell types and stages present in testes. However, electron microscopy is too cumbersome to allow effective screening through mutant strains, and can not be easily linked to the molecular genetic tools developed by the *Drosophila* community. Therefore, as a complementary approach to the ultrastuctural work, we sought to analyze spermatogenesis at the light microscope with P-element enhancer traps (O'Kane and Gehring, 1987; Bellen et al., 1989; Bier et al., 1989).

Enhancer traps contain a weak promoter fused to the lacZ reporter gene. Upon insertion of the P-lacZ element in the genome, the weak promoter can be brought under the influence of a neighbouring enhancer, resulting in spatially and temporally restricted ß-galactosidase activity. It has been shown that several of the enhancers detected in this manner control the transcription of a neighbouring gene in a similar pattern (Fasano and Kerridge, 1988; Bier et al., 1989; Wilson et al., 1989).

P-element enhancer traps can be utilized in different ways to probe spermatogenesis. First, enhancer trap lines provide indispensable molecular markers for each of the labeled cell types and stages during spermatogenesis. Such marker lines are crucial in following the fate of specific cells in wild-type and in mutant backgrounds.

Second, enhancer traps can serve to generate male-sterile lines by insertional mutagenesis. Compared to a classical approach, this provides the advantage of indicating where the mutated gene might be normally expressed. Third, enhancer trapping is the method of choice to identify genes that may play a role in spermatogenesis but that are not easily recovered in male-sterile screens. For instance, lethal P-insertions lines can be examined as heterozygotes to determine whether a given essential gene is expressed in testes. Viable and fertile lines expressing lacZ during spermatogenesis can serve to identify genes that are redundant in function or whose mutant phenotype is not male-sterility. Moreover, viable and fertile lines can also identify genes that are essential for male fertility in cases where the insertion event has not led to disruption of gene function.

We began our analysis by looking at patterned lacZ expression to identify enhancer trap lines that could serve as molecular markers during spermatogenesis. In this Chapter, we describe marker lines that label germ cells, somatic cyst cells and all other somatic cells present in testes. Some lines label germ cells or cyst cells in a stage-specific manner during their differentiation program. These expression patterns reveal transient identities for the cyst cells that had not been previously recognized by morphological criteria. Some markers label early stages of male but not female germ cell differentiation and prove useful in the analysis of germ line sex-determination. Other lines label the hub of somatic cells around which stem cells are anchored. We also describe how marker lines enable us to identify presumptive cells in the embryonic gonadal mesoderm before they give rise to morphologically distinct cell types. Finally, this collection of marker lines will allow the characterization of genes expressed in the germ line or the soma during spermatogenesis.

RESULTS

We first examined testes of flies that did not contain an enhancer trap to assess levels of eukaryotic ß-galactosidase (Fig. 3A). No X-gal staining was observed, except in occasional degenerating cysts (Fig. 3A, arrow) and in waste bags (Fig. 3A, arrowhead), which contain superfluous cytoplasmic material released during the final stages of post-meiotic differentiation. The low level and sporadic nature of this endogeneous activity could not be confused with the reproducible expression patterns generated by individual enhancer trap lines. Moreover, eukaryotic ß-galactosidase could easily be distinguished from lacZ expression by using an antibody directed specifically against the bacterial ß-galactosidase (see for instance Fig. 6H, arrowheads).

We examined testes in males from three different sets of P-element enhancer trap lines. The first comprised 700 homozygous viable and fertile strains (see Materials and Methods). The frequencies of staining patterns in the germ line and the soma (excluding the epithelial cells connecting the testis to the seminal vesicle) were determined by close examination of 120 lines in this first set. 55 % expressed lacZ in the germ line only, 5 % in the soma only, while 13 % labeled both germ cells and somatic cells. 27 % did not give rise to any staining pattern in testes.

The second set comprised 77 male-sterile strains that we identified, and in which spermatogenesis was defective (see Materials and Methods). In 71 of these lines, ß-galactosidase expression was observed in germ cells or some somatic cells in the testis. We found a striking enrichment for one expression pattern among the lines defective for spermatogenesis: staining in all germ cells of the testis. Among the 77 lines, 21 expressed ß-galactosidase in the germ line beginning in stem cells and persisting through the post-meiotic stages (see Fig. 3H and 3I). By comparison, only two lines from the first set of 700 had expression throughout the male germ line.

Finally, we examined a set of 3500 strains that we generated in the course of a P-element mediated mutagenesis (see Chapter 5 and Materials and Methods). For this third set, we pre-screened the lines at the dissecting microscope and kept for further analysis only those that represented novel staining patterns, as well as those that labeled cell types at the tip of the testis.

Combining strains from these three sets, Figures 3 through 8 describe the different types of staining patterns observed during spermatogenesis. Table 1 gives summarized information about the marker lines presented in the text and the figures, and about additional strains that were kept for further study in our laboratory.

Germ line

Most lines labeling the germ line initiated lacZ expression during the spermatocyte growth phase and displayed ß-galactosidase activity until after the meiotic divisions (Fig. 3B). Germ cells could be easily identified by their characteristic morphology and numbers. X-gal-positive cells in the growth phase region were typical of maturing spermatocytes, with a large nucleus and prominent nucleolus (Fig. 3C, arrow). X-gal positive cells were organized in groups of 16 large cells up to the meiotic divisions (Fig. 3D, arrow) and 64 smaller cells after that (Fig. 3D, arrowhead). Post-meiotically, X-gal positive nuclei became elongated and eventually rod-shaped (Fig. 3E, arrow), as expected from maturing spermatids undergoing nuclear reshaping. The germ line nature of the labeled cells was further confirmed by crossing two such marker lines to *oskar*^{301} (*osk*^{301}) females (Lehmann and Nüsslein-Volhard, 1986). Progeny resulting from these crosses were agametic and carried a copy of the enhancer trap. As expected, lacZ expression was absent from these agametic testes (data not shown).

Although most strains staining in the germ line initiated lacZ expression at the begining of the growth phase (see Fig. 3C), a few lines did so at a later time (Fig. 3F). In such strains, germ cells were not staining during most of the growth phase (Fig. 3G,

arrow), and ß-galactosidase activity was first evident only just prior to the meiotic divisions, and persisted after that (Fig. 3G, arrowhead).

A few lines, most of them male-sterile, expressed lacZ in germ cells of all stages, beginning in stem cells and persisting through post-meiotic stages (Fig. 3 H and 3I).

Other lines exhibited a staining pattern that was restricted to particular stages of germ cell differentiation. Among these, some expressed lacZ in stem cells (Fig. 4A, arrow), and to a lesser extent in their daughters. Some lines had a similar staining pattern in the germ line (Fig. 4B, arrow), but also expressed lacZ in the somatic cells of the hub (Fig. 4B, arrowhead). Yet other lines expressed lacZ in stem cells as well as in germ cells in the subsequent proliferation region (Fig. 4C, arrow), but not in later stages (Fig. 4C, arrowhead). Finally, one line expressed lacZ in a "stripe" of germ cells at the border between the proliferation and the growth phase regions (Fig. 4D, arrow), but not in germ cells before or after that (Fig. 4D, arrowhead and thin arrowhead).

Male germ cell identity

For those lines that expressed lacZ in germ cells during the early stages of spermatogenesis (Fig. 5A and 5E, arrows), we also determined the X-gal staining pattern during the early stages of oogenesis. In some strains, there was no corresponding staining in early female germ cells (Fig. 5B and 5F, arrowheads). Thus, these lines are markers of early male germ cell identity.

We next investigated whether these marker lines would respond to changes in the germ line sex determination pathway. *snf*^{1621} (fs(1)1621) (Gans et al., 1975) and *Sxl*^{f4} (Lindsley and Zimm, 1992) are two mutations which interfere with this pathway in females. In both cases, mutant ovaries fill up with undifferentiated germ cells that resemble spermatogonia, presumably as a result of a defect in germ line sex-determination (Salz et al., 1987; Oliver et al., 1988; Steinmann-Zwicky, 1988; Bopp et al., 1993; reviewed by Pauli and Mahowald, 1990; Steinmann-Zwicky, 1992). The two

male-specific marker lines that we tested expressed lacZ in *snf* and Sxl^{f4} mutant ovaries, reflecting the sexual transformation of these mutant XX germ cells (Fig. 5C, 5D, 5G, 5H, arrows). This demonstrates that such marker lines can serve to probe early male germ cell identity.

We obtained a marker line expressing lacZ in the early stages of oogenesis (Fig. 5J, arrow; Fasano and Kerridge, 1988), and showed that it failed to express lacZ in the corresponding stages of spermatogenesis (Fig. 5I, arrowhead). However, lacZ expression was still maintained in *snf* and Sxl^{f4} mutant ovaries (Fig. 5K and 5L, arrows). Thus, this marker line does not reflect the sexual transformation occuring in these mutants, and it is not useful to probe early female germ cell identity.

Cyst cells

We identified marker lines that stain the somatic cyst cells. Several lines expressed lacZ starting in the growth phase region and in all cyst cells past that stage (Fig. 6A). The lacZ-expressing cells were identified as cyst cells because each cyst of developing germ cells was associated with two X-gal-positive nuclei at its periphery (Fig. 6B, arrows), the number and location expected for cyst cells. The identity of these cells was further ascertained by showing that ß-galactosidase-positive nuclei (Fig. 6C, arrows) were located within cytoplasm containing ß3-tubulin(Fig. 6D, arrows), a ß-tubulin isotype specifically expressed in cyst cells in male gonads (Kimble et al., 1989).

Several lines labeled cyst cells in a stage-specific manner. For instance, some labeled mainly early cyst cells (Fig. 6E), including cyst progenitor cells (Fig. 6E, arrows). As expected from the ultrastructural data, cyst progenitor cell nuclei were smaller and not as spherical as those of neighbouring germ line stem cells (Fig. 6E, thin arrowhead), and were located further away from the apical cells of the hub (Fig. 6E, arrowhead). Other lines labeled cyst cells at the border between the proliferation and the growth phase regions (Fig. 6F), but not during later stages of differentiation. Yet others labeled cyst

cells only in the growth phase region (data not shown). These transient expression patterns indicate that cyst cells adopt distinct identities in the period prior to the meiotic divisions that had not been revealed by morphological criteria.

Some lines labeled cyst cells only in post-meiotic stages of the differentiation program (Fig. 6G, arrows), an observation confirmed by the lack of staining in larval gonads (data not shown), where only premeiotic stages are represented. A few lines expressed lacZ in both tail- and head-cyst cells. The head-cyst cell nuclei could be identified by their position at the base of the testis and their close association with sperm heads (Fig. 6H, arrowheads and corresponding arrows). One line expressed lacZ specifically in head-cyst cells, but not in tail-cyst cells (data not shown). Reciprocally, two lines labeled tail-cyst cells but not head-cyst cells (Fig. 6I, arrows). The tail-cyst cell nuclei could be easily recognized by their invariable location, squeezed against the periphery of elongated sperm tail bundles (Fig. 6J, arrow).

Apical cells of the hub

At the apical tip of the testis, a group of 12 to 16 small somatic cells form the hub around which stem cells are anchored (Hardy et al., 1979). We identified marker lines that express lacZ either exclusively in these apical cells (Fig. 7A, arrow) or in these cells as well as in germ line stem cells (see Fig. 4B) or early cyst cells (see Fig. 6E). The identity of the labeled cells was suggested by their clustering (Fig. 7B, arrow) and by the radial position of germ line stem cells (Fig. 7B, arrowheads) around the cluster. Additional evidence that the labeled cells indeed constitute the apical hub was obtained by showing that the cluster of ß-galactosidase positive nuclei (Fig. 7D, arrow) also expressed fasciclin III (Fig. 7E, arrow), a marker specific for the apical cells of the hub in male gonads (Brower et al., 1981).

By morphological criteria, the apical cells can be identified as early as the first instar larval gonad (Aboïm, 1945). We examined one line at this developmental stage to

determine whether it was a faithful marker for these cells. Indeed, we observed strong lacZ expression in the apical cells of the first instar larval gonad (data not shown). Furthermore, in 13 to 15 hour embryos, a cap of ß-galactosidase positive cells was observed at the anterior end of the newly formed gonad (Fig. 7G, arrow). Thus, this line represents an early molecular marker for the presumptive apical cells in the gonadal mesoderm.

Other somatic cells

At the base of the testis, the sheath includes a layer of epithelial cells that fuse with the seminal vesicle. We identified marker lines that label most epithelial cells of the terminal region (Fig. 8A, from the arrow to the arrowhead). The labeled cells were identified by the fact that the stain reaches the seminal vesicle (Fig. 8A, arrowhead) and by the location of the labeled nuclei within the epithelium of the testis (Fig. 8B, arrows), rather than the lumen (Fig. 8B, arrowhead).

Some lines specifically labelled what appears to be the specialized epithelial cells that entrap the head-cyst cells prior to the coiling of spermatids (Tokuyasu et al., 1972b). We infer this because the labelled cells are the subset of terminal epithelial cells located furthest away from the seminal vesicle (Fig. 8C, arrows, compare with Fig. 8B), in an area with many coiled sperm bundles. Other lines labelled the reciprocal subset of cells in the terminal epithelial cells, those closest to the seminal vesicle (Fig. 8D, arrow).

Finally, we obtained marker lines that identify the two cell types forming the sheath of the testis, pigment cells and muscle cells (Bairati, 1967). Both pigment cell and muscle cell nuclei were easily identified by their location within the sheath of the testis and their typical appeareance. Pigment cell nuclei were large and sparse (Fig. 8E and 8F, arrows), and were located in the outer-most layer of the sheath (data not shown), as expected from the ultrastructural data (Bairati, 1967). Muscle cell nuclei were numerous and small (Fig.

8G and 8H, arrows) and were found in the sheath slightly below pigment cell nuclei (data not shown).

Fig. 1 Spermatogenesis in Drosophila. **(A)** Phase contrast view of an adult testis. The apical end (api), or tip, is left in all figures; ter: terminal end, or base. Bar=50 μm in this and all other figures. **(B)** Schematic representation of 5 stages of spermatogenesis. Arrows pointing to part (A) indicate where approximately in the testis each stage begins; cells are displaced from the tip towards the base as they mature. Germ line stem cells and somatic cyst progenitor cells are anchored around a hub of somatic cells (hub) at the tip of the testis. Only one germ line stem cell (ste) and two cyst progenitor cells (cyp) are represented for clarity. asy: asymmetric divisions of one germ line stem cell and two neighbouring cyst progenitor cells give rise to one primary gonial cell (spg) and two cyst cells (cyc), respectively. mit: the primary gonial cell undergoes 4 mitotic divisions, while the two cyst cells no longer divide. gro: the resulting 16 spermatocytes (spc) progress through an extended growth phase. mei: the two meiotic divisions occur in quick succession. mor: the 64 haploid spermatids (spt) undergo dramatic morphological changes. Only 6 spermatids are shown for clarity. The two cyst cells become structurally distinct, the head-cyst cell (cyh) being associated with the sperm heads and the tail-cyst cell (cyt) elongating with the sperm tails. The head-cyst cell then becomes entrapped by a specialized epithelial cell (tec) at the base of the testis. Coiling of the sperm bundle and release of motile spermatozoae (spz) into the seminal vesicle ensues. Only one spermatozoa is shown for clarity. See text for additional information.

Figure 1

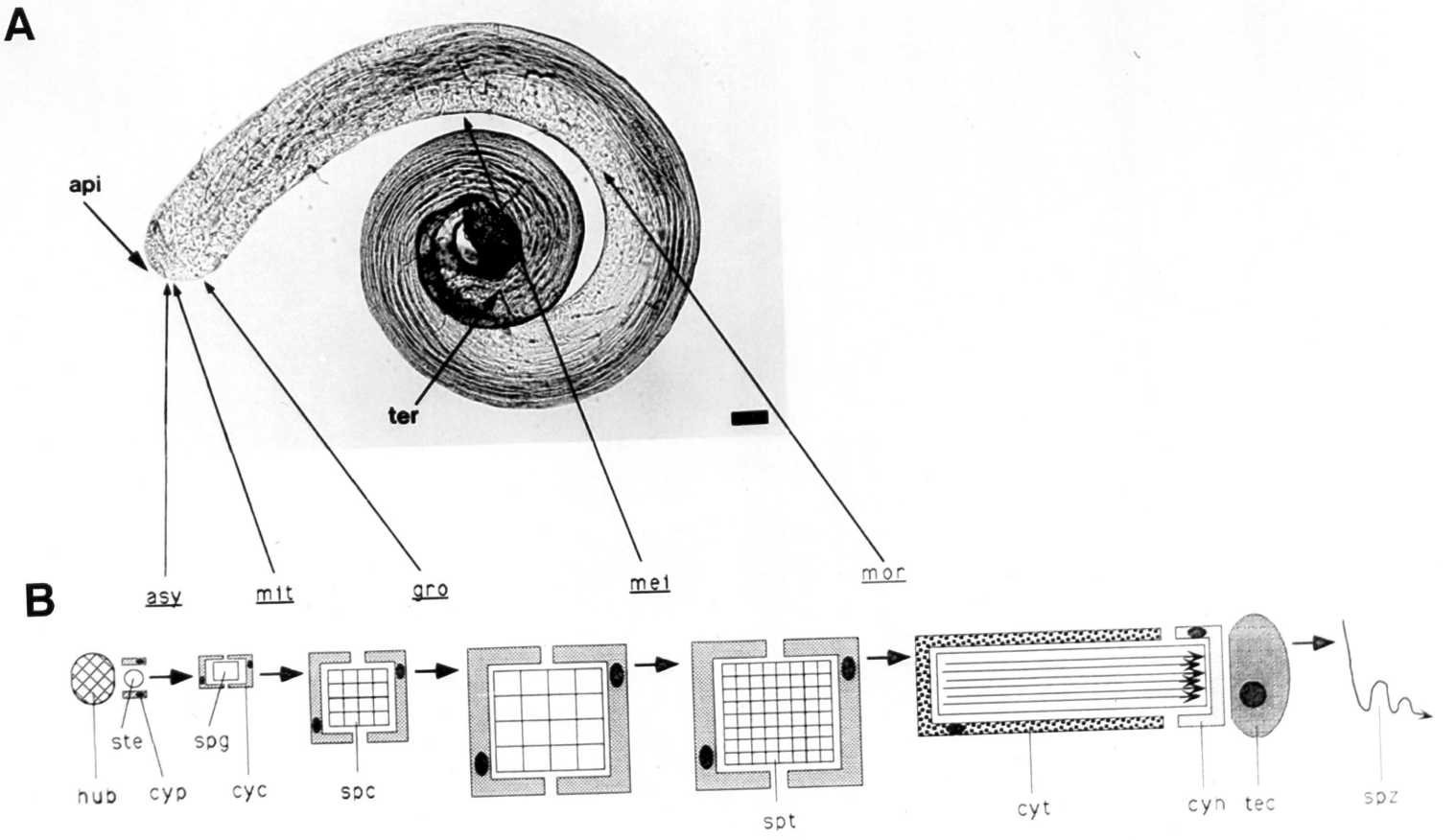
A
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Fig. 2 The beginning of spermatogenesis at the tip of the testis. **(A)** A marker line (842, see Chapter 2) staining the nuclei of hub cells (arrowhead), as well as those of cyst progenitor cells (thin arrowheads; these two nuclei are weakly stained and partially out of focus) and early cyst cells (not indicated, but see stronger staining towards the right). This panel illustrates the spatial relationship between cells at the tip of the testis; germ line stem cells (arrow) are not stained, but are clearly recognizable by their size and radial positioning around the hub. Immunoperoxidase reaction revealing anti-β–galactosidase antibody stain. The field is about 40 μm in width. **(B)** Schematized illustration of one germ line stem cell and two neigbouring cyst progenitor cells at three successive moments of their concerted division cycle. (1) The germ line stem cell (arrow) is in contact with the basal lamina surrounding the hub (arrowhead), and so are the two surrounding cyst progenitor cells (thin arrowheads). (2) The three cells divide in concert, and are in the process of completing cytokinesis; cytokinesis is delayed, as serial reconstructions have revealed that most germ line stem cells in the testis tip are still connected by a thin intercellular bridge to their daughter (Hardy el al., 1979). (3) The pairs of daughters from these three divisions have distinct fates. While the daughters still in contact with the hub remain self-renewing stem cells, and will subsequently undergo more rounds of the same division cycle, the three detached daughters embark on the following steps of the developmental program. The daughter of the germ line stem cell is called a primary gonial cell, and it is surrounded by the daughters of the two somatic cyst progenitor cells, which are called cyst cells. The two cyst cells surround the primary gonial cell, thus forming a cyst, the fundamental unit of spermatogenesis. See Figure 1 for details on subsequent stages.

Figure 2

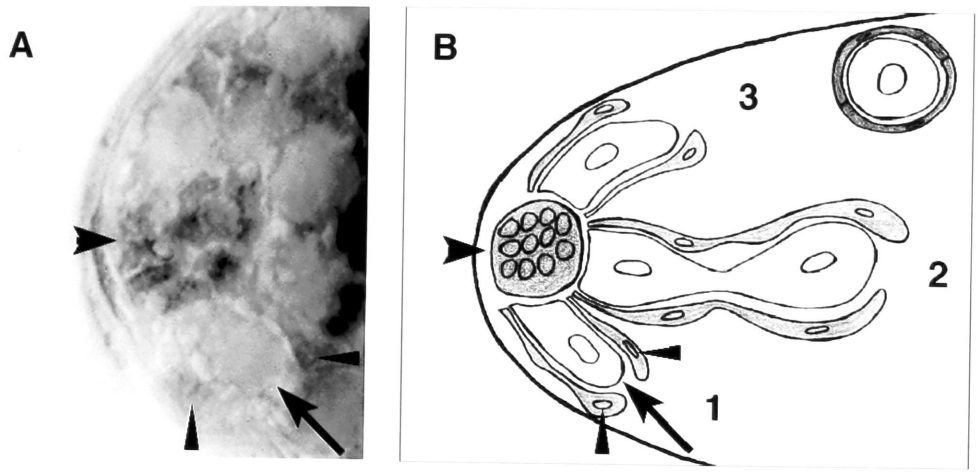


Fig. 3 Broad germ cell marker lines. All figures in Chapter 2 show X-gal staining unless otherwise noted. **(A)** Testis, w^-. No X-gal staining is visible, except in degenerating cysts in the proliferation region (arrow) and waste bags (arrowhead) **(B, C, D, E)** (817) A germ cell marker line initiating expression early in the growth phase. **(B)** Testis. **(C)** Apical region, showing perinuclear staining in growing spermatocytes, recognizable by their large nucleus and prominent nucleolus (arrrow); note the strong staining in sperm tails (arrowhead) **(D)** Cyst of 16 germ cells (arrow) in telophase of the first meiotic division (note that the staining is present in the whole cell) and cyst of 64 young post-meiotic spermatids (arrowhead), some of which are out of focus. **(E)** Two cysts of spermatids at different stages of post-meiotic differentiation, as judged by sperm head morphology; note the more mature rod-shaped sperm heads (arrow). **(F, G)** (I-58) A germ cell marker line initiating expression late in the growth phase. **(F)** Testis. **(G)** Apical region, showing the lack of staining in growing spermatocytes (arrow); note the strong staining in sperm tails (arrowhead). **(H, I)** (ms1090/TM3) A marker line expressing lacZ in germ cells of all stages. **(H)** Testis. **(I)** Apical region, showing staining in all early germ cells, including stem cells (arrow). Bars=50 μm.

Figure 3

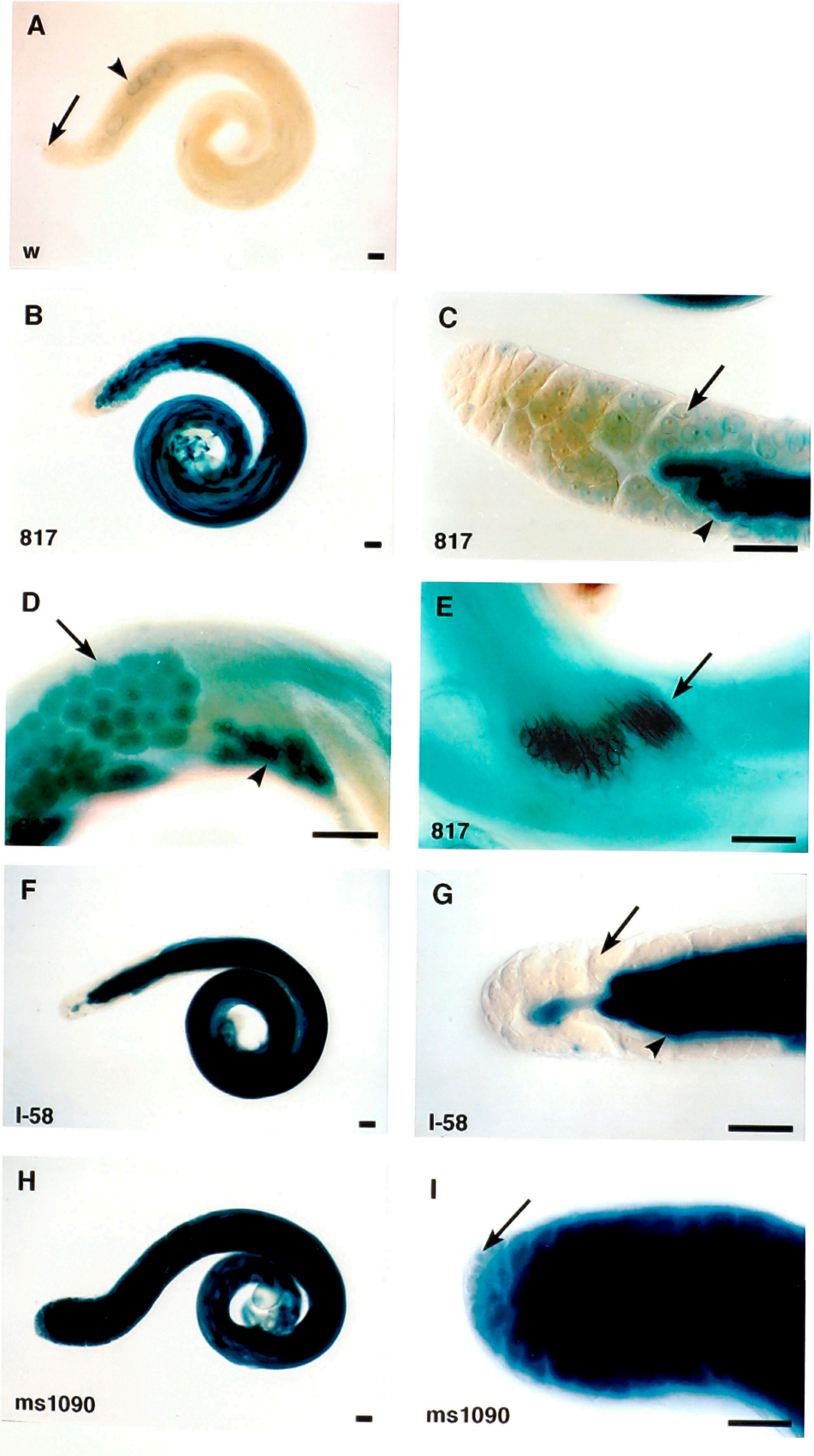


Fig. 4 Early germ cell marker lines. Apical region of testes. **(A)** (M34a) Labeling is restricted to germ line stem cells (arrow) and their immediate daughter; note that the apical region is enlarged in this strain. **(B)** (M5-4/CyO) LacZ expression is in the germ line stem cells (arrow) and their immediate daugther, as well as in the somatic cells of the apical hub (arrowhead). **(C)** (606) Labeling is in germ line stem cells and germ cells in the proliferation region (arrow), but not in later stages (arrowhead). **(D)** (ms987/TM3) LacZ expression in germ cells is in a "stripe" at the border between the proliferation and the growth phase regions (arrow); note that earlier (arrowhead) or later (thin arrowhead) germ cells do not stain . Bar=50 μm; all panels are at the same magnification.

Figure 4

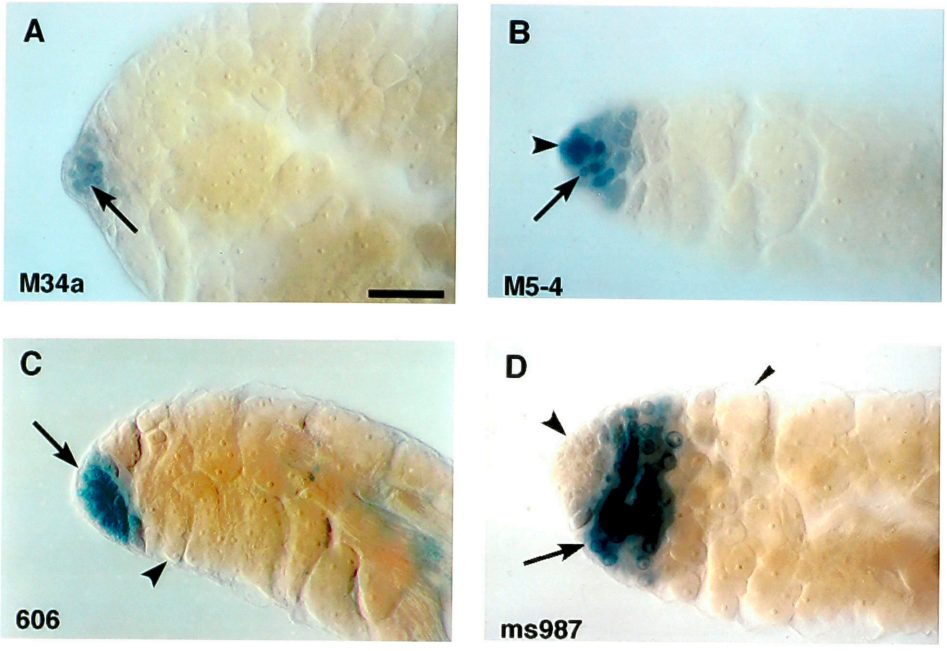


Fig. 5 Early markers of male germ cell identity. Testes (first column), wild-type ovaries (second column), *snf* ^{1621} mutant ovaries (third column), *Sxl*^{f4} mutant ovaries (fourth column). **(A, B, C, D)** M5-4/CyO. **(A, B)** M5-4 expresses lacZ in the hub (A, arrowhead), as well as in germ line stem cells and their immediate daughter in testes (A, arrow), but not in the corresponding stages in ovaries (B, arrowhead). **(C, D)** LacZ expression is turned on in small clusters of germ cells (C and D, arrows) in ovaries homozygous for a mutation in *snf* or for a germline-specific mutation in *Sxl*. **(E, F, G, H)** 606. **(E, F)** 606 expresses lacZ in early germ cells in testes (E, arrow), but not in ovaries (F, arrowhead); note that the somatic stalk cell that link egg chambers are labeled (F, thin arrowhead). **(G)** Most cells express lacZ (arrows) in ovaries homozygous for a mutation in *snf*. **(H)** Small clusters of germ cells (arrow) express lacZ in ovaries homozygous for a germline-specific mutation in *Sxl*. **(I, J, K, L)** Q13/CyO. **(I, J)** Q13 does not express lacZ in testes (arrowhead), while it stains early germ cells in ovaries (arrow points to a germarium). **(K, L)** LacZ is still broadly expressed (K and L, arrows) in ovaries homozygous for a mutation in *snf* or for a germline-specific mutation in *Sxl*. Note that more cells are staining than in wild-type ovaries, probably because germ cells are blocked at an early stage in the developmental program. Bar=50 μm; all panels are at the same magnification.

Figure 5

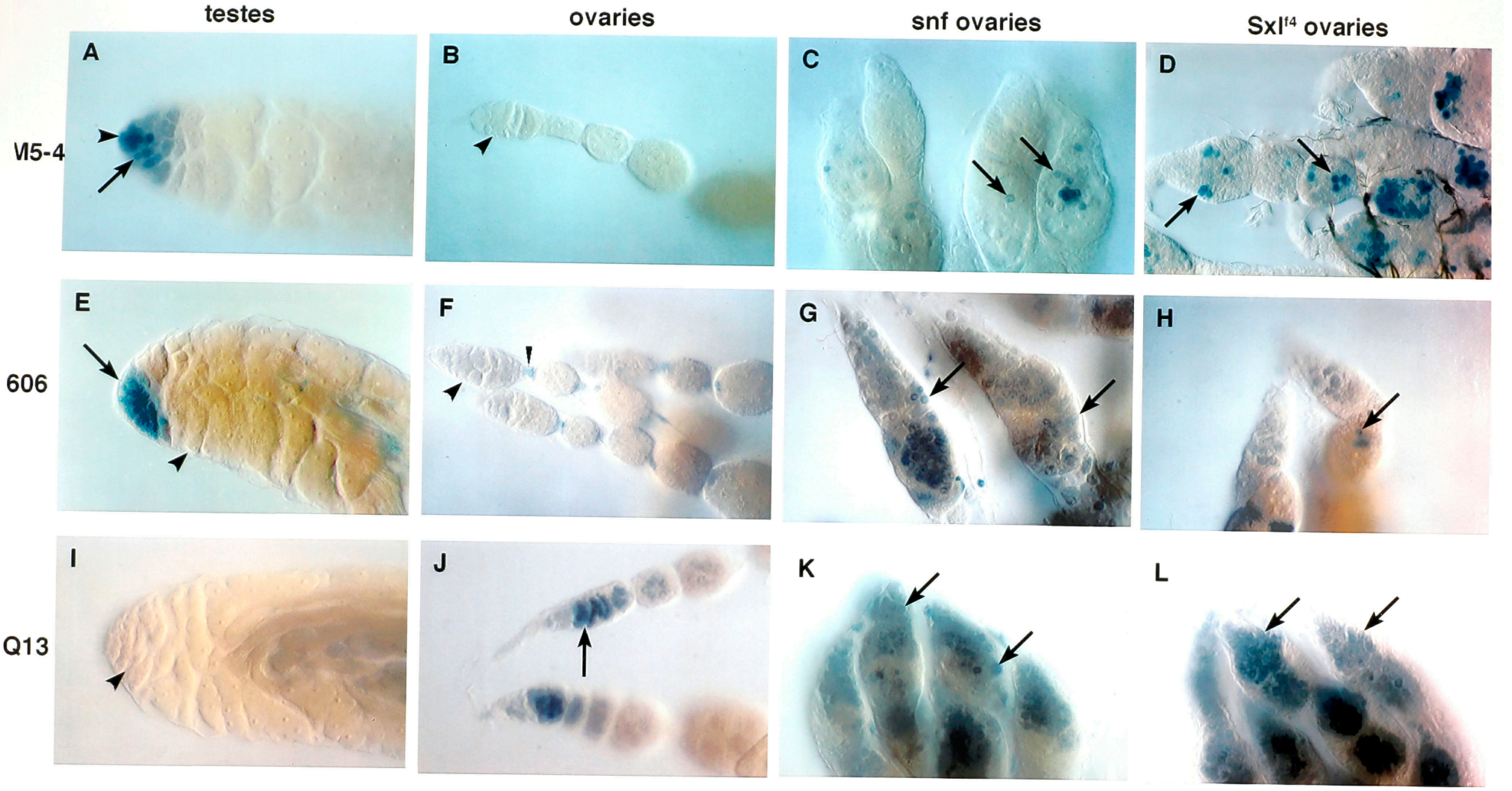

testes
ovaries
snf ovaries
Sxl[f4] ovaries
M5-4
606
Q13
A
B
C
D
E
F
G
H
I
J
K
L

Fig. 6 Marker lines labeling cyst cells. **(A, B, C, D)** (600) A marker line labeling cyst cells starting in the growth phase. **(A)** Testis; note that this line also labels the hub (arrow) and the terminal epithelial cells (arrowhead), as well as the pigment cells of the sheath (not easily distinguishable in this focal plane). **(B)** Apical region, showing two labeled nuclei (arrows) nested at the periphery of each growing cyst. **(C, D)** Localization of ß-galactosidase signal to cyst cells. **(C)** Third instar larval gonad labeled with anti-ß-galactosidase antibody visualized by indirect immunofluorescence. Three positive nuclei are in the plane of focus (two of them highlighted by arrows). Larval gonads contain the same cell types and stages as those found in adult testes for the period prior to the meiotic divisions, including cyst cells. **(D)** Same gonad as in (C) labeled with anti-ß3-tubulin antibody visualized by indirect immunofluorescense. The signal reveals cyst cell cytoplasm, which surrounds the developing germ cells (not labeled). Note that ß-galactosidase positive nuclei are located within ß3-tubulin positive cytoplasm (compare arrows in C and D). **(E, F, G, H)** Particular marker lines label cyst cells in a stage-specific manner **(E)** (842) Apical region, showing labeling of early cyst cells, including cyst progenitor cells (arrows). Note a neighbouring unlabeled germ line stem cell (thin arrowhead) and the labeled apical cells of the hub (arrowhead). This line also labels pigment cells, terminal epithelial cells and head-cyst cells (not shown here). **(F)** (901) Apical region, showing transient labeling of cyst cells in the late proliferative and early growth phase regions (arrowhead and arrow point at the youngest and oldest labeled cyst cells, respectively). **(G)** (ms-985/TM3) Testis showing labeling of cyst cells post-meiotically only (arrows). Head-cyst cells are indicated by an arrowhead. This line also labels germ line stem cells (not visible at this magnification) and terminal epithelial cells (thin arrowhead). **(H)** (57) Head-cyst cells. Immunoperoxidase staining of the basal region of a testis with anti-ß-galactosidase antibody. Hoechst counterstain shows the association of rod-shaped sperm head nuclei (arrows) with the ß-galactosidase positive head-cyst cell nuclei (arrowheads) (this line displayed unusually high levels of endogenous ß-galactosidase activity which necessitated analysis using the antibody directed against bacterial ß-galactosidase; this line also labels tail-cyst cells (not shown). **(I, J)** (498) Tail-cyst cells. **(I)** Testis, showing labeling of tail-cyst cells only (arrows). **(J)** Tail-cyst cell nucleus (arrow) between two elongated sperm tail bundles. Bars=50 µm.

Figure 6

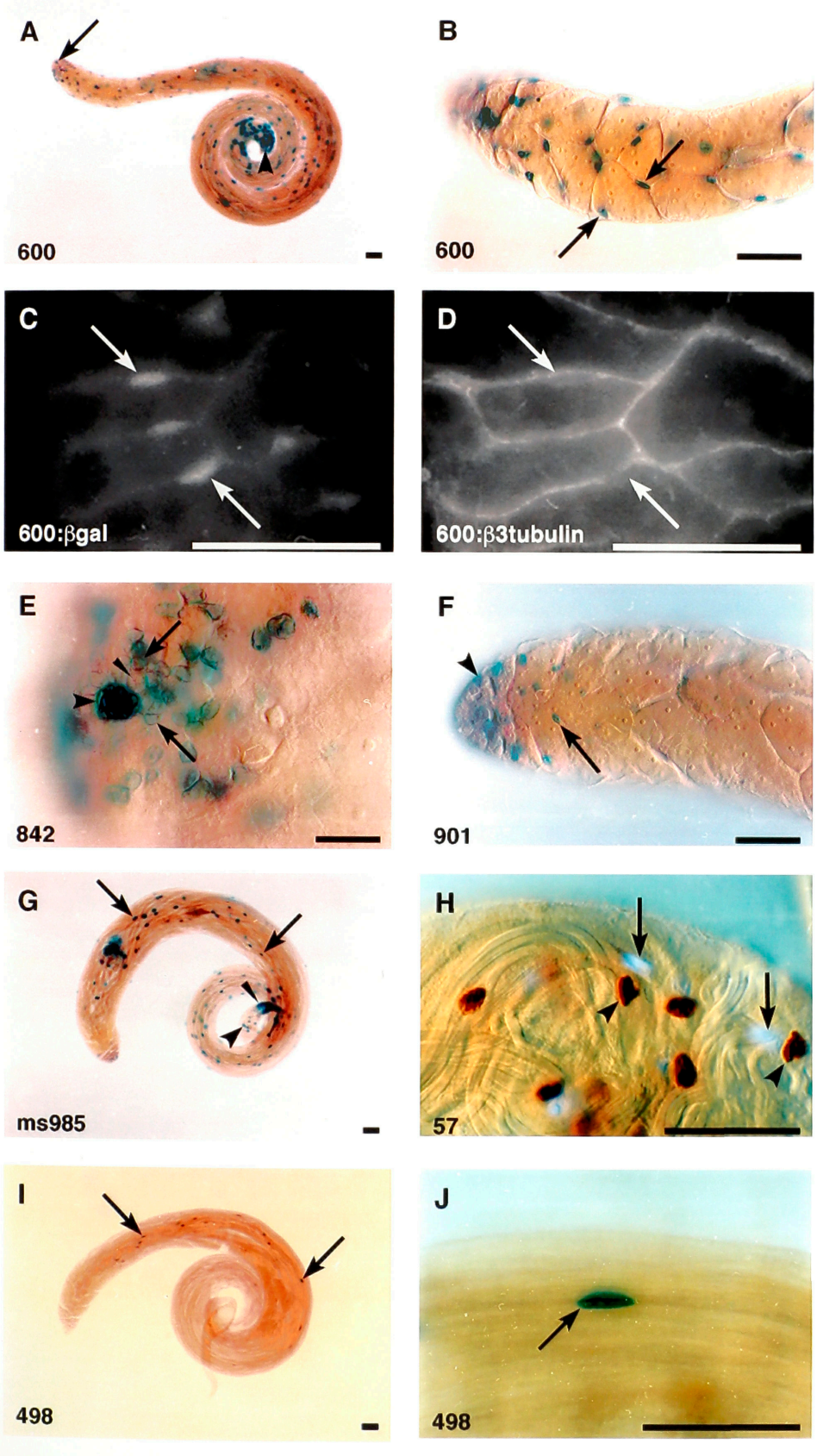

A
600
B
600
C
600:βgal
D
600:β3tubulin
E
842
F
901
G
ms985
H
57
I
498
J
498

Fig. 7 Marker line labeling the apical cells of the hub. (254/CyO) **(A)** Testis, showing labeling of the hub located at the apical tip of the testis (arrow). **(B)** Apical-most portion, showing labeling of the clustered somatic cells of the hub (arrow) and unlabeled germ line stem cells (arrowheads) radially anchored around the hub. **(C, D, E)** Identification of the ß-galactosidase positive cells as the apical cells of the hub. **(C)** Hoechst counterstain revealing all nuclei present at the apical tip of the testis, viewed from above. **(D)** Same apical tip as in (C) labeled with ß-galactosidase antibody visualized by indirect immunofluorescence; note the clustering of labeled cells (arrow). **(E)** Same apical tip as in C and D labeled with fasciclin III antibody visualized by indirect immunofluorescence; note that the labeled cells (arrow) are the same as those positive for ß-galactosidase (compare arrows in D and E). **(F)** Immunoperoxidase staining with anti-ß-galactosidase antibody of a 13-15 hour embryonic gonad. The gonad was dissected away from the embryo to highlight the labeling observed in the apical portion of the gonad (arrow); note the prominent pole cells (arrowhead). Other cell types also stain during embryogenesis. Bars=50 µm.

Figure 7

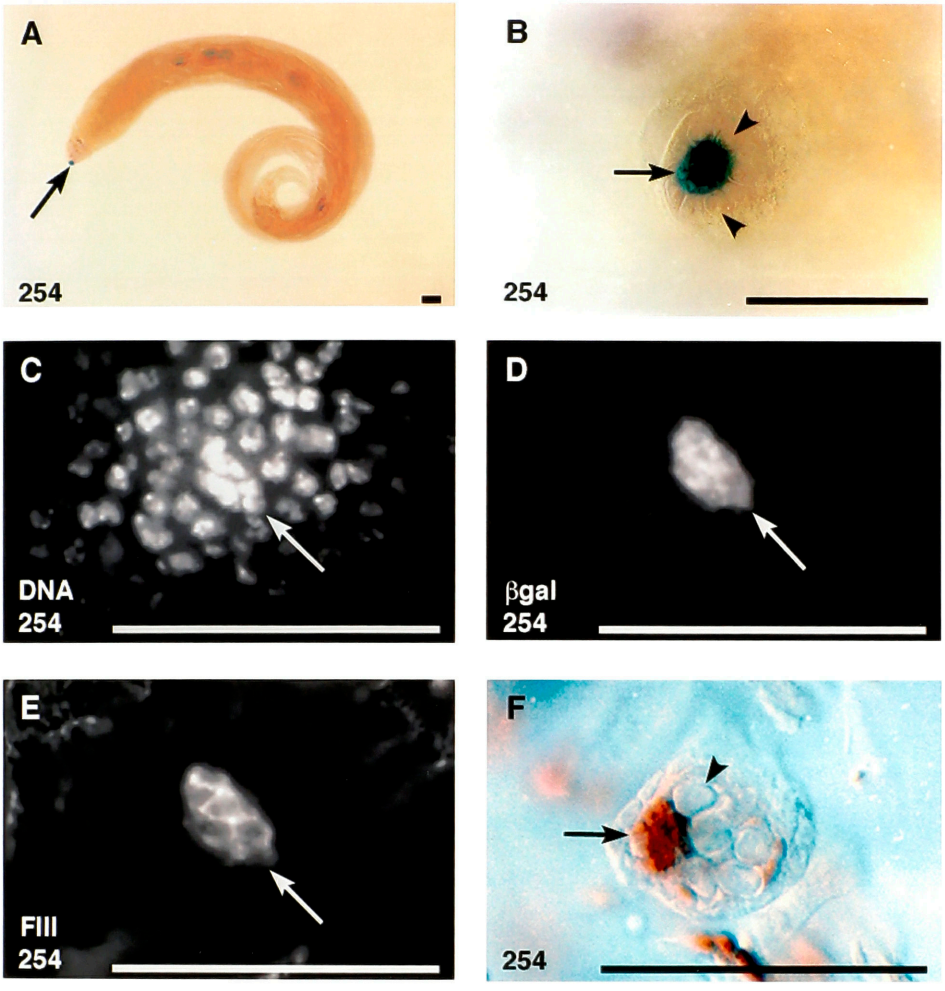


Fig. 8 Marker lines labeling other somatic cells types. **(A,B)** (34) Marker line staining all terminal epithelial cells. **(A)** Testis; staining begins in a region where coiling of sperm bundles occurs (arrow) and extends down to the junction with the seminal vesicle (arrowhead). This line also weakly labels cyst cells in the late proliferative and early growth phase regions (not visible in this focal plane) **(B)** Terminal region, showing that the labeled cells (arrows) are part of the epithelium of the testis rather than the lumen (arrowhead). **(C)** (429) Terminal region, showing that only a subset of epithelial cells are labeled in this line (arrows), probably those that entrap the head-cyst cells. Note the lack of staining in the more terminal epithelial cells (arrowheads), near the junction with the seminal vesicle (thin arrowhead); note also the presence of coiling sperm bundles in the area where the labeled cells are located. **(D)** (D-66) Terminal region, showing labeling in the reciprocal subset of epithelial cells (arrow), those closest to the seminal vesicle. Note the lack of staining in the more apical epithelial cells (arrowhead), those furthest away from the seminal vesicle. **(E, F)** (857) Marker line labeling the pigment cells. **(E)** Testis; pigment cell nuclei (arrow) are sparse. This line also stains the terminal epithelium (arrowhead). **(F)** (side and top views) The pigment cell nuclei are located in the sheath of the testis (arrow, top panel) and are very round (arrow, bottom panel). **(G, H)** (L44a) Marker line labeling the muscle cells. The muscle cell nuclei are smaller and also located in the sheath of the testis (arrows). Bars=50 μm.

Figure 8

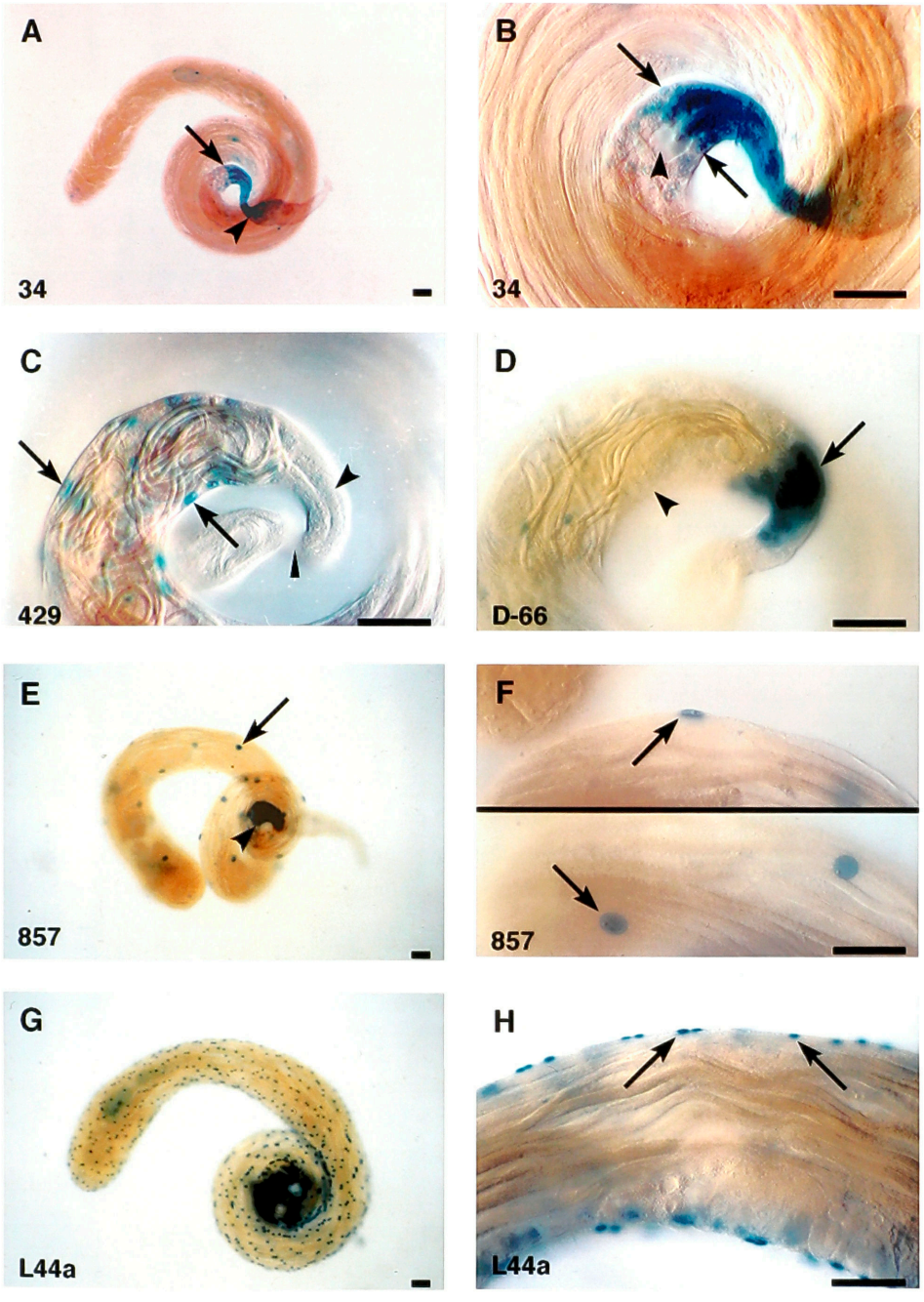
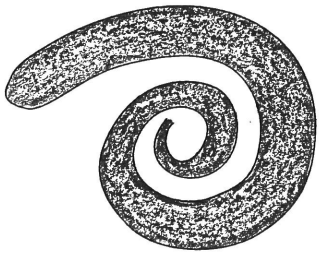


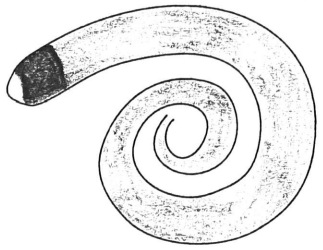
Table 1

Marker lines described in Chapter 2 and other marker lines kept in our laboratory

Marker lines are listed once and arranged according to their most notable expression pattern during spermatogenesis, which is summarized visually for each category. Levels of staining may vary between different lines. For each strain, the following information is given in the third column: name (e.g. ms1090/TM3), chromosomal and cytological location (e.g. III; 83B), origin (1a: first set, Jans' lab; 1b: first set, Fuller and Scott labs; 1c: first set, Kassis lab; 1b: first set, Fasano; 1e: first set, stock center; 2: second set, UCLA labs; 3: third set, DiNardo lab, see Material and Methods for details). Strains that are illustrated in figures of this Chapter are indicated. Additional domains of expression in testes are indicated for individual strains, when applicable {G: germ cells; C: cyst cells; H: cells of the hub; E: epithelial cells; P: pigment cells; M: muscle cells; s: stem cell region; p: proliferation region; g: growth phase region; m: meiotic region; pm: post-meiotic region; a: apical-most; d: distal-most; e: early; l: late; h: head; t: tail; w-: weak, and s-: strong levels of expresion, respectively; -: no expression}. Expression in testes but not ovaries {Mspecific} or at least in the male but not the female germ line {Mglspecific} is indicated. ND: not determined.

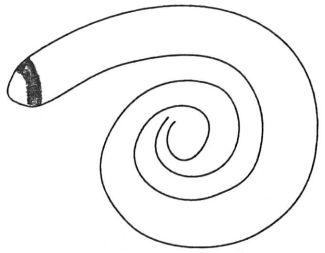
Staining pattern in testes	Visual summary	Strains
Germ line		
All stages, starting in stem cells.		ms1090/TM3 (III;83B) [2] {E} **Fig.3H,I** 21 other male-sterile lines 704 (ND) [1b] {H;E:d} 6P8 (X) [3] {C:g/p} K-24 (X) [3] {C:g,pm;E;P;M}

All stages; stronger in early germ cells, including stem cells, weaker thereafter.



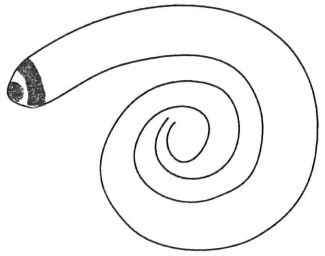
25 (ND) [1a] {E}
67 (III;97F/98A) [1a] {G:g:E}
733 (III;100D/E) [1a] {G:g;E}
D-61 (II) [3] {C:g;E:d;P}
E-90 (X) [3] {E}
K-39 (X) [3] {Mglspecific;C:g,pm;E;P}
S2-26 (X) [3] {Mspecific}

Stem cells (and their progeny)



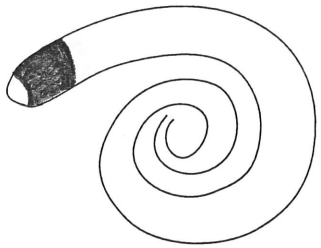
M-34a (III;70D/F) [1d] {enlarged tip} **Fig.4A**
S1-33 (ND) [3] {Mspecific}
S3-40 (II) [3] {Mspecific;C:p,g;P}
S3-46 (X) [3] {E}

Stem cells (and their progeny) and cells of the hub



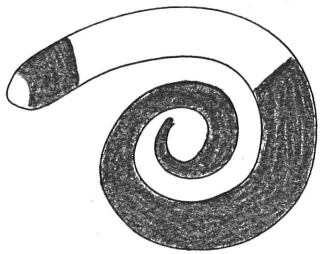
M5-4/CyO (II;35C/*esg*) [3] {Mspecific} **Fig.4B,5A**
M1-83/CyO; M5-3/CyO; M5-23/CyO; M5-64/CyO; M5-75/CyO (all II;35C/*esg*) [3] {Mspecific}
542 (II;35C/*esg*) [1a] {Mspecific;w-adults, s-larvae}
S3-58 (ND)[3] {w-G:p}
S3-61 (ND) [3] {w-H}
S3-74 (ND) [3] {E:d}

Stem cells and proliferation region



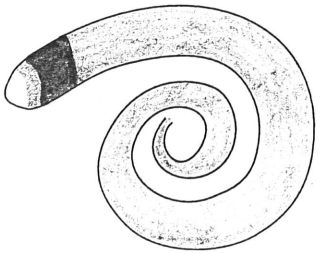
606 (II;28C) [1a] {C:t;E;Mspecific}
Fig.4C,5E
590 (II;28C) [1a] {Mglspecificw-G:g,pm;C:t;E:d}
743 (III;66A/B) [1b] {Mspecific}
6-23A (X) [1a]
D-44 (X) [3] {Mglspecific;E:d}
E-92 (X) [3] {w-G:g,pm;C:g,pm;E}
S3-45 (ND) [3] {w-G:pm;C:g,pm}
895 (ND)[1b] {only in larvae}

Stem cells and proliferation region; re-expression from late growth phase onwards



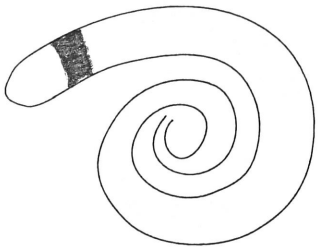
245 (II;27E) [1c]

Weak in stem cells, stronger in proliferation region, weak thereafter



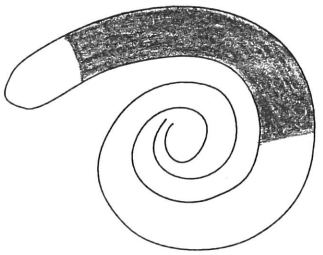
G-14 (X) [3] {Mglspecific;C:g,pm}

"Stripe" of cells in late proliferation/early growth phase



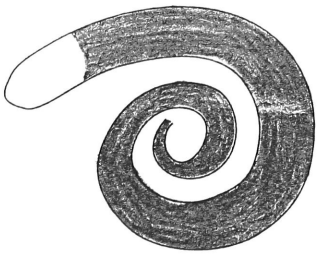
ms987/TM3 (III;85D) [2] {G:m; C:pm}
Fig.4D

Expression in growth phase, stopping shortly after meioisis; not much seen in tails



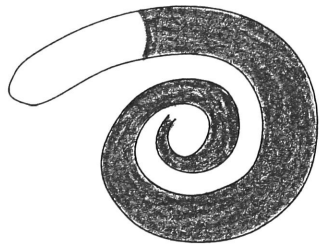
526 (III;82E) [1a]

Growth phase onwards



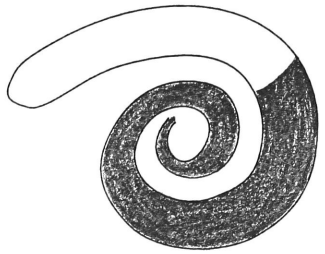
817 (II) [1b] **Fig.3B,C,D,E**
436 (ND) [1a]
712 (III;83C) [1b] {E:d}
859 (ND) [1b]

Mid-growth phase onwards



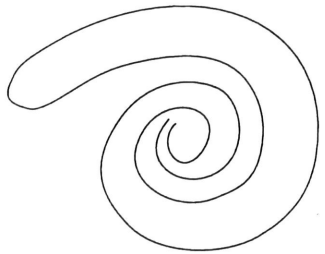
61 (III;66B/C) [1a]

Late-growth phase onwards



I-58 (X) [3] **Fig.3F,G**

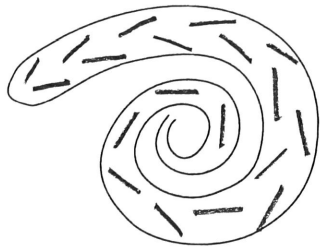
No staining in male germ line



Q13/CyO (II;51C/D) {Female-specific; weak expression in male growth phase upon longer incubations or when homozygous; E:subset} **Fig.5I,J**

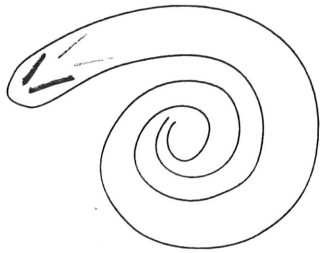
Cyst cells

All cyst cells



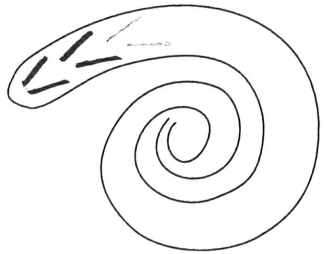
M12-41 (X) [3] {H;E}

Cyst progenitor cells (and their progeny)



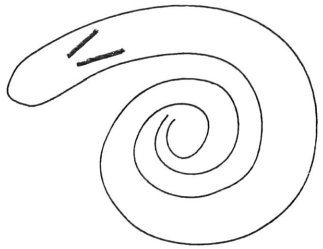
K59a2 (II;57B) [1d] {E:a}
L18a (II;24F/25A) [1d] {G:g,pm}

Early cyst cells, including cyst progenitor cells



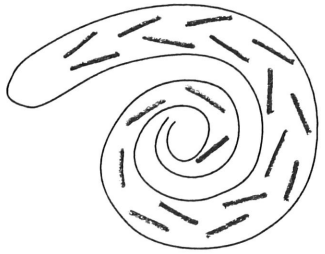
842 (II;57F) [1b] {C:h;H;E;P} **Fig.6E**
26 (II;57F) [1a] {C:h;H}
51 (III;68F/69A) [1a] {H}
517 (III;61D) [1a] {H;E}
S1-77 (X) [3] {ND}

Cyst cells at the border between proliferation and growth phase regions



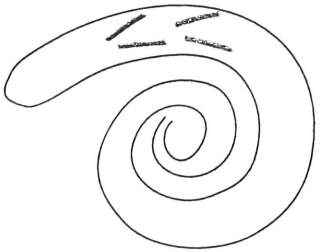
901 (III;92B/C) [1b] **Fig.6F**
831 (ND) [1b] {w-H}
K-49 (X) [3] {C:h,t}

Cyst cells starting in the growth phase region and onwards



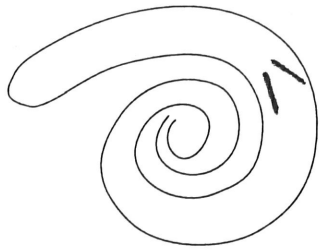
600 (II;21F/22A) [1a] {H;E;P}
Fig.6A,B,C,D
473 (II;53F) [1a] {w-G:g,pm}
P573 (II) [1e] {w-G:g,pm;E:d)
C-84-CyO (II) [3] {w-G:g,pm}
I-93 (X) [3] {E:a}

Cyst cells transiently in the growth phase region



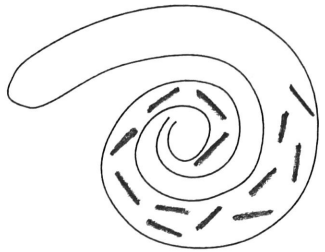
37 (II;28D) [1a]
484 (II;25F/26A) [1a] {E:d}
C1-14 (X) [3] {P}

Cyst cells very transiently in the region of the meiotic divisions



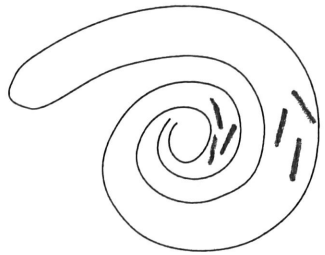
97 (III;62B) [1a]

Cyst cells in postmeiotic aspects



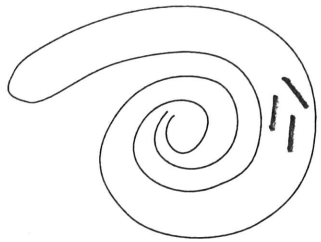
ms985/CyO (II;28C) [2] {G:s;E} **Fig 6G**
873 (III;100E) [1b] {E:d;P}

Head- and tail-cyst cells



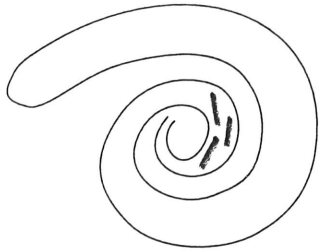
57 (III;82C) [1a] **Fig.6H**
858 (ND) [1b] {weaker in C:t}
J-87 (ND) [3]

Tail-cyst cells



498 (II;24A) [1a] **Fig.6I,J**
R21b (ND) [1d]

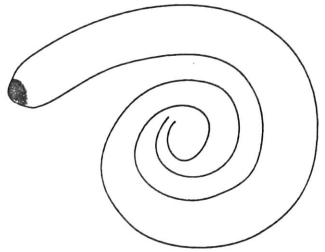
Head-cyst cells



234 (III;82C/D) [1c]

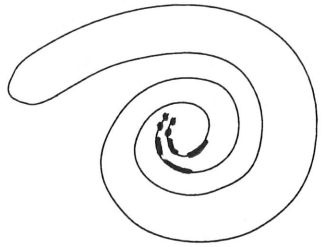
Other somatic cells

Cells of the hub



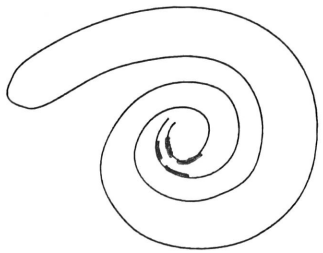
254 (II;35C/*esg*) [1c]
Fig.7A,B,C,D,E,F
C1-21 (X) [3] {w-G:s,p}
C6-28 (III) [3] {w-G:s;C:g;w-H}
M2-39 (X) [3] {w-G:s,p;E}
M2-14 (ND) [3] {w-G:s}
S1-19 (X) [3] {G:s,p;C:g,pm;E;P}
S2-11 (X) [3] {w-C:p,g}

Terminal epithelial cells



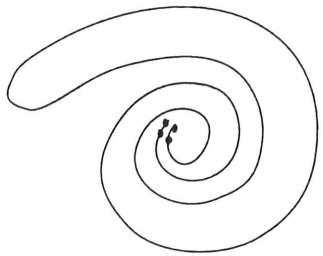
34 (III;62A/B) [1a] {C:p,g} **Fig.8A,B**
717 (II;26A) [1b] {C:p;w-H}
25-13 (ND) [3]
A-6 (X) [3] {C:t}
K-78 (II) [3] {w-H}

Subset of terminal epithelial cells (larger, apical most)



429 (III;88E) [1a] {C:t} **Fig.8C**

Subset of terminal epithelial cells (smaller, closer to the seminal vesicle)

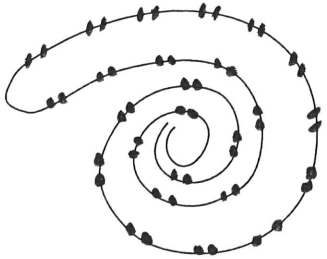


D-66 (ND) [3] **Fig.8D**

Pigment cells

857 (ND) [1b] {E} **Fig.8E,F**
365 (ND) [1a] {E}

Muscle cells



L44a (II;57A/C) [1d] **Fig.8G,H**

DISCUSSION

Little is known about the genes governing the differentiation of germ line and somatic cells during spermatogenesis in *Drosophila.* As a first step towards a molecular genetic dissection of this developmental program, we have characterized enhancer trap marker lines that label specific cell types and stages during spermatogenesis. We have examined over 4200 enhancer trap lines and describe lacZ staining patterns for the different cell types present in testes, defining in some cases stages that had not been recognized previously by morphological criteria. The array of staining patterns observed in the collection of 89 marker lines that we retained for further studies is given in Table 1.

Expression in the germ line

By close examination of a subset of 120 viable and fertile lines, we observed that ß-galactosidase activity is found in the male germ line in 68 % of the lines, a figure that is more than double that reported in two similar studies conducted with oogenesis (Fasano and Kerridge, 1988; Grossniklaus et al., 1989). This could simply be indicative of a larger number of cis-acting elements active during male *versus* female gametogenesis. More probably, this could be due to the fact that the lines were generated by a transposition event occuring in the male germ line. This view is consistent with the observation that transposition events occurring in the male germ line lead to a higher frequency of lacZ expression in testes than in ovaries, the reverse being the case when the transposition event occurs in the female germ line (Bownes, 1990).

Most lines that stain germ cells start expressing lacZ in the growth phase. This is consistent with uridine incorporation experiments which showed that the bulk of transcription in the germ line occurs during this phase (Olivieri and Olivieri, 1965; Gould-Somero and Holland, 1974). Those studies also led to the postulate that transcription ceases prior to the meiotic divisions during *Drosophila* spermatogenesis.

Accordingly, we have not identified any line in which lacZ expression occurs strictly post-meiotically in the germ line. The latest onset of lacZ expression observed is late in the growth phase, just prior to the meiotic divisions (Fig. 3F and 3G). Strict post-meiotic transcription units may yet exist in *Drosophila*, but be refractory to P-element insertion and thus be under-represented among enhancer trap lines.

A few lines have an expression pattern that is quite restricted to germ line stem cells (Fig. 4A). However, such lines also have weaker staining in the immediate daughter of the stem cells. This is probably due to the intercellular bridge that connects the stem cell to its daughter during most of the cell cycle following the initial stem cell division (Hardy et al., 1979). Such a persisting bridge likely prevents complete segregation of lacZ mRNA or ß-galactosidase protein exclusively produced in stem cells. Similar bridges persist between cyst progenitor cells and their daughter (Hardy et al., 1979), and probably also explain why we did not observe any strain with exclusive cyst progenitor cell labeling.

Male germ cell identity

Some marker lines express lacZ in the proliferative phase of male but not female gametogenesis, characterizing them as markers of early male germ cell identity. Work has begun to unravel the sex-determination pathway in the germ line (reviewed by Pauli and Mahowald, 1990; Steinmann-Zwicky, 1992). *snf* and *Sxl* are two genes that are part of this pathway, and which are required for proper female differentiation of XX germ cells (Schüpbach, 1985; Perrimon et al., 1986; Oliver et al., 1988; Steinmann-Zwicky, 1988; Steinmann-Zwicky, 1989; Salz, 1992). In snf^{1621} or Sxl^{f4} mutants, ovaries are filled with small cells that morphologically resemble germ cells embarked on a male differentiation program. Moreover, *snf* XX germ cells splice the male-specific variant of Sxl (Bopp et al., 1993; Oliver et al., 1993), and both *snf* and Sxl^{f4} express Stellate and mst-325 mRNA's, which are usually normally only transcribed in testes (Wei et al., 1994).

Accordingly, in both *snf* and Sxl^{f4} mutant ovaries, germ cells express the two male-specific markers that we have tested, M5-4 and 606 (Fig. 5). Using our strains 590 and 606 (see Table 1), Wei and coworkers (1994) similarly reported lacZ expression in *snf* and Sxl^{f4} ovaries. Together, these results concur to demonstrate that these marker lines can serve to probe early male germ cell identity.

While 606-lacZ expression is present in most *snf* XX germ cells (Fig. 5G), M5-4-lacZ expression is limited to small clusters of cells in both *snf* and Sxl^{f4} mutant ovaries (Fig. 5C, 5D), as is 606-lacZ expression in Sxl^{f4} (Fig. 5H). The limited expression could reflect the fact that most transformed XX germ cells are past the stage in the developmental program at which they would express the marker. This may be especially true for M5-4, whose expression in the male germ line is restricted to stem cells and their daughters. This view is consistent with the more extensive 606-lacZ expression, at least in *snf*, as 606 expression in testes persists until slightly later stages than M5-4. Curiously, M5-4 X-gal positive cells are often found away from the mutant germaria, where stem cells and their daughters are located in the wild-type.

Limited expression could also be due to incomplete sexual transformation of XX germ cells. The occasional presence of pseudonurse cells in Sxl^{f4} ovaries (data not shown) is compatible with this hypothesis. Moreover, snf^{1621} is a somewhat temperature-sensitive allele, as more tumourous germ cells accumulate at 29 °C than at lower temperatures (Gollin and King, 1981). We conducted our experiments at 25 °C, and it is likely that male-specific expression would have been more extensive in snf^{1621} mutant ovaries at 29 °C.

Interestingly, the male-specific and female-specific forms of Sxl mRNA are concomitently observed in *snf* mutant ovaries (Bopp et al., 1993; Oliver et al., 1993). As those experiments were conducted with total ovary RNA, it remained possible that individual mutant cells were expressing both forms at once. Our single-cell resolution

analysis instead suggests that sexual transformation in *snf* and Sxl^{f4} mutant ovaries occurs in some germ cells, but not others.

Cyst cell differentiation

We have shown that most lines that stain cyst cells label only a subset of those present during the course of spermatogenesis. For the period prior to the meiotic divisions, we find at least four distinct types of staining patterns in cyst cells. The first labels early cyst cells, including cyst progenitor cells and shows little or no expression in later stages of cyst cell differentiation (Fig. 6E). The second type labels cyst cells exclusively at the border between the proliferation and growth phase regions (Fig. 6F). The third type labels cyst cells only transiently in the growth phase (data not shown). Finally, the fourth and most common type labels cyst cells starting in the growth phase region and most or all cyst cells in the period after the meiotic divisions (Fig. 6A, 6B). These distinct types of staining patterns suggest that there are different stages in cyst cell differentiation in the period prior to the meiotic divisions. Such distinctions had not been detected by morphological criteria.

After the meiotic divisions take place in germ cells, the two cyst cells become structurally distinct, one being associated with the sperm heads, and the other with the elongating sperm tails. Accordingly, we found lines that label either only tail-cyst cells (Fig. 6I, J), only head-cyst cells (data not shown) or both tail- and head-cyst cells (Fig. 6H). Thus, cyst cell differentiation for the period both before and after the meiotic divisions can be broken down into a succession of stages revealed by the marker lines.

Gonadogenesis

At about 12 hours during development, pole cells and somatic gonadal mesodermal cells coalesce to form the embryonic gonad (Sonnenblick, 1941; Aboïm, 1945; Mahowald, 1962; Hay et al., 1988; Brookman et al., 1992; Boyle and DiNardo, 1995). All

morphologically distinct somatic cells of the adult testis derive from the somatic mesodermal precursor cells. Marker lines that label specific somatic cells early in development are instrumental in addressing when these different cell types assume their fate. We have shown that a marker line labeling the apical cells of the hub in the adult testis represents an early molecular marker for the presumptive apical cells of the embryonic gonadal mesoderm (Fig. 7G). Several marker lines that identify other somatic cell types in testes also label presumptive cells in the embryonic gonad (M. Boyle, P.G., S. Viswanathan, S. DiNardo, unpublished observations). Studies using such marker lines and the genes identified by them can unravel the origin and lineage of the different somatic components of male gonads (Boyle and DiNardo, 1995).

Marker lines identify genes expressed during spermatogenesis

By revealing the expression pattern of neighbouring genes expressed in similar domains, this collection of marker lines should allow the characterization of genes expressed at specific times and places during spermatogenesis. For instance, a marker line expressing lacZ in the cells of the hub (Fig. 7A) bears a P-element inserted in the gene *esg* (Whiteley et al., 1992). By *in situ* hybridization, esg mRNA is indeed found in the cells of the hub, as well as in germ line stem cells (C. Bromleigh, P.G. and S. DiNardo, unpublished observations). The expression in germ line stem cells was unexpected, as it had not been predicted by the lacZ expression pattern. However, additional enhancer trap lines inserted at *esg* express lacZ not only in the cells of the hub, but also in germ line stem cells, in a male-specific manner (Figs. 4B, 5A, 5B and Table 1). Accordingly, Northern blot analysis reveals that esg mRNA is not present in wild-type ovaries, but accumulates in *snf* mutant ovaries (C. Bromleigh, P.G. and S. DiNardo, unpublished observations).

esg is an essential gene which encodes a transcriptional repressor required to prevent polyploidisation in the abdominal histoblasts (Hayashi et al., 1993; Fuse et al.,

1994). Null mutants in *esg* die as first instar larva, making it difficult to assess whether *esg* is required for spermatogenesis, which is just begining at this stage. One could envisage transplanting a mutant first instar larval gonad into a wild-type host to ask whether it would mature as a control wild-type transplant (see Gloor, 1943; Rungger-Brändle, 1976), but we have not yet attempted this experiment. To test a potential role of *esg* specifically in germ line stem cells, we have recently removed *esg* function in the germ line by FLP-mediated recombination (see Chapter 7 for a description of this system). We failed to observe any defect during spermatogenesis, as wild-type looking cysts of homozygous mutant germ cells were found in the proliferation region, growth phase and post-meiotically (data not shown). Thus, *esg* does not appear to be required in the germ line for the proper execution of spermatogenesis.

We have cytologically mapped 42/89 and assigned to a chromosome 70/89 of the marker lines reported in Table 1, thus facilitating the future characterization of neighbouring genes, as well as the use of marker lines in genetic crosses. This collection of marker lines is instrumental in investigating the fate of specific cells in various mutants and experimental situations (see Chapters 3 and 4, for instance) and thus forms an important foundation for the molecular genetic dissection of spermatogenesis.

Note: Parts of this Chapter have been published in:

P. Gönczy, S. Viswanathan and S. DiNardo (1992). Probing spermatogenesis in *Drosophila* with P-element enhancer detectors. Development *114*: 89-98.

CHAPTER 3: THE AGAMETIC TESTIS AS A MODEL SYSTEM TO PROBE THE INFLUENCE OF GERM CELLS ON SOMATIC CELL FATES

INTRODUCTION

In many animals, including insects and vertebrates, there is an early separation between the germ line and the soma, and only later during embryogenesis do cells from these two lineages come together to form a gonad (reviewed by Dixon, 1994). Once in their proper somatic environment, germ cells undergo a stereotyped sequence of events which eventually leads to the production of mature gametes. Intercellular signalling events control a variety of developmental processes in metazoans, and the influence that the somatic and germ line components of gonads have on each other has been a subject of long-standing interest.

The influence of somatic cells on germ cell differentiation has been studied in many organisms, including mammals, worms and flies. In mammals, the somatic Sertoli cells appear to play some role in several aspects of testicular development and function (reviewed by Russell, 1980; Pelliniemi et al., 1993). In *C. elegans*, a specialized somatic cell at the tip of the gonad, the distal tip cell, produces a signal that is required by germ cells for continued mitotic proliferation (Kimble and White, 1981). lag-2 has been recently identified as the likely signal emanating from the distal tip cell, while glp-1 is the probable receptor of that signal on germ cells (reviewed by Kimble and Ward, 1988; Clifford et al., 1994). glp-1 is a member of the Notch/lin12 family of transmembrane cell surface receptors, while lag-2 is homologous to Delta, a ligand of the Notch receptor in *Drosophila* (Austin and Kimble, 1989; Yochem et al., 1989; Fehon et al., 1990; Heitzler and Simpson, 1991; Henderson et al., 1994; Tax et al., 1994). During the early stages of

Drosophila oogenesis, a group of somatic cells also regulates neighbouring germ cells, in this case by inhibiting the division rate of stem cells (Lin and Spradling, 1993). The nature of the signaling system has not yet been identified in this situation. Later during *Drosophila* oogenesis, a bidirectional interaction between the soma and the germ line is key in establishing the dorso-ventral polarity of the oocyte (reviewed by Schüpbach et al., 1991; Chasan and Anderson, 1993). Thus, specific signals coming from somatic cells are crucial in many organisms for the orderly progression of germ cell differentiation.

The potential influence of the germ line on the differentiation and function of the somatic cells of the gonad has received comparatively little attention. An early experiment demonstrated that gonads would not form in chick embryos in which the germinal precursors had been eliminated (Dantschakoff, 1941). Thus, in birds, the germ line has an influence on the capacity of the somatic cells to organize into a gonad. This, however, turned out not to be the case in other species where the question has been examined. For instance, gonads form normally in mice bearing certain alleles at the *atrichosis* (*at*) or the *White* (*W*) locus, despite the fact that the genital ridges fail to become adequately populated with primordial germ cells (Mintz and Russel, 1957; Hummel, 1966). Interestingly, the resulting testes have essentially normal structural and temporal maturation of Sertoli cells (Handel and Eppig, 1979). Thus, these somatic cells seem able to differentiate appropriately in the absence of a germ line.

Geigy and Aboïm addressed the same question in *Drosophila* over 50 years ago (Geigy, 1931; Aboïm, 1945). By UV-irradiating the posterior cap of 2 hours 30 min embryos, they selectively destroyed pole cells, the presumptive germ cells, hours before they would come together with somatic mesoderm to form a gonad. The resulting adults had ovaries or testes extremely reduced in size, owing to the absence of germ cells, but their architecture appeared relatively normal (Geigy, 1931). Aboïm extended Geigy's study by following the morphology of the somatic cell types in these "agametic" gonads throughout development. Both in males and females, the somatic cell types recognizable

in the wild-type appeared to be present and differentiated on schedule in agametic gonads. In testes, the apical cells of the hub, the cyst cells -called "interstitial" at the time-, the terminal epithelial cells -called "of the canal"- as well as both layers of the sheath were present. Although the overall organization of the cells differed somewhat from the wild-type, Aboïm concluded that the germ line exerted little or no influence on the differentiation of the somatic cells of *Drosophila* gonads.

We wanted to reinvestigate this question by using the marker lines that we had developed, in order to follow specific somatic cell fates at the gene expression level, rather than by morphological criteria alone. Instead of ablating pole cells by UV-irradiation, we made use of a temperature sensitive allele of the maternal effect gene *oskar* (*osk*). Whereas embryos derived from mothers null for *osk* function die with posterior defects, embryos derived from mothers carrying the temperature-sensitive allele osk^{301} survive at the permissive temperature, but still lack the determinants required for pole cell formation (Lehmann and Nüsslein-Volhard, 1986). By crossing males from marker lines labeling specific somatic cells to osk^{301} females, we obtained agametic progeny that carry a copy of the enhancer trap.

We confirm Aboïm's results and report that the cells of the hub, cyst cells, terminal epithelial cells and cells of both layers of the sheath are present in most agametic testes. We expand his observations by showing that cyst cells begin their differentiation program in the absence of germ cells. Unexpectedly, we find that 20 % of agametic testes do not contain a hub or cyst cells. Thus, germ cells are required for the proper organization of somatic cells in some cases. Moreover, expression of fasciclin III, a cell adhesion molecule expressed in the cells of the hub, is altered in agametic testes. We conclude that germ cells are required to maintain proper fasciclin III expression restricted to the hub. Finally, we show that, on average, twice as many cyst cells undergo S phase in the agametic testis than in wild-type. Consequently, we suggest that germ cells may play a role in inhibiting the proliferation of neighbouring cyst cells.

RESULTS

Somatic cell fates in agametic testes

We analysed agametic testes in the progeny of crosses between *osk*^{301} females and males of specific enhancer trap marker lines. We first examined strains that express lacZ in the apical cells of the hub. As expected from Aboïm's work, these lines allowed us to locate the hub in the agametic testis (Fig. 9A and 9B, arrows). Although the hub was present in most cases (but not all, see below), it was often larger and located some distance away from the tip compared to the wild-type (Fig. 9A, arrowhead). The number of cells making up the hub was somewhat variable, going from about 10 to over 30 in some cases (data not shown). Although there is some fluctuation in this number among wild-type testes as well (Hardy et al., 1979), the variability is smaller than that observed among agametic testes. Moreover, the cells of the agametic hub tended to be larger than those in the wild-type (data not shown). Taken together, our data confirm that the apical cells can organize into a hub in the absence of a germ line. However, they also suggest that germ cells play a role, direct or indirect, in refining the size of the somatic hub and its position in the testis.

We next examined strains expressing lacZ in cyst cells. We looked at lines that label all cyst cells in the testis (Fig. 9C). In the agametic situation, these markers revealed that the mass of cells usually located beneath the hub is comprised of cyst cells (Fig. 9D and 9E, arrows). To investigate whether some of these cyst cells had begun their differentiation program, we analyzed marker lines whose expression in cyst cells normally begins only in the growth phase region (Fig. 9F, arrow). In the agametic testis, such marker lines labeled cyst cells some distance away from the hub (Fig. 9I and 9J, arrows), but failed to label cyst cells just beneath the hub (Fig. 9H, arrowhead). Therefore, cyst cells are present in agametic testes and are able to progress through some aspects of their differentiation program in the absence of germ cells.

As shown in Fig 9A, the hub is displaced from the very tip of the agametic testis. By using a marker line, we determined that the cells occupying the apical-most position in the agametic testis were muscle cells of the sheath (Fig. 9J and 9K, arrows). Aboïm similarly reported a thickening of the inner-most part of the sheath in agametic testes. He also discussed a comparable thickening of the outer-most layer of the sheath, that comprised of pigment cells. We made use of a marker line labeling the pigment cells to confirm his observation. As a consequence of the thickening of this cellular layer, the pigment cell nuclei were closer to each other in the agametic testis than in the wild-type (compare Fig. 9L and 9M, pairs of arrows).

From this series of experiments, we derive an anatomy of the canonical agametic testis very similar to that described by Aboïm, with the hub, cyst cells and cells of the terminal epithelium (data not shown), within a somewhat thickened sheath (Fig. 9N). However, we observed quite some variability among agametic testes in terms of their size and shape (compare various agametic panels of Fig. 9), as well as in terms of the number of hub and cyst cells. Unexpectedly, we even found that about 20 % of agametic testes (7/30 and 6/38 in two independent experiments) entirely lacked the hub and cyst cells, while retaining the components of the sheath (data not shown). Such a class of agametic testes had not been reported by Aboïm. From this observation, we infer that the germ line is required to allow for the proper organization of the hub and cyst cells in all cases. The low penetrance of the phenotype may indicate that the germ line requirement is partially redundant with a strictly somatic function.

Armadillo and fasciclin III expression

We next investigated whether two proteins known to be present in the cells of the hub were still expressed in agametic testes. We first looked at the expression of armadillo (arm). arm is a *Drosophila* ß-catenin homologue that plays an essential role in cell adhesion and wingless-dependent signal transduction (reviewed by Peifer et al., 1993a).

In testes, arm protein was particularly abundant in the cells of the hub (Fig. 10A and 10B, arrows) and was also present at lower levels in cyst cells (Fig. 10A and 10B, arrowheads; Peifer et al., 1993b). In the agametic case, arm protein was still expressed in the cells of the hub (Fig. 10C, arrow) and in cyst cells (Fig. 10C, arrowhead). We have not yet examined enough agametic testes to ascertain whether the levels of arm protein are always as in the wild-type. However, we reach the preliminary conclusion that arm expression in the somatic hub and cyst cells does not depend on the presence of germ cells.

Another molecule expressed in the cells of the hub is fasciclin III (Brower et al., 1981). Fasciclin III was identified as a cell surface glycoprotein abundant on a subset of axon bundles during embryogenesis (Patel et al., 1987). In testes, whereas fasciclin III was always strongly expressed in the wild-type hub (Fig. 10D and 10E, arrows) and terminal epithelium (Fig. 10D, arrowhead), its expression at the hub was variable in agametic testes. For instance, in a double-labeling experiment in which fasciclin III expression was examined along with the fate of the cells of the hub and the cyst cells (see legend to Fig. 10), agametic testes (n=30) could be divided into the four following categories. First, 7/30 testes had no fasciclin III expression besides that in the terminal epithelium, and they also had no hub or cyst cells. These represent the 20 % unusual agametic testes discussed above. Second, 8/30 testes had no fasciclin III expression at the hub (just like the testis in Fig. 10F), although they all had cyst cells and 6/8 were positive for the hub marker. Third, 6/30 testes had only weak fasciclin III expression at the hub (just like the testis in Fig. 10G, arrow), and all had a hub and cyst cells. Finally, 9/30 testes had levels of fasciclin III expression comparable to the wild-type in the proximity of the hub. Interestingly, 8 of these 9 agametic testes had fasciclin III expression extending beyond the hub (Fig. 10I and 10J, arrowheads), into the cyst cell territory (Fig. 10J and 10I, arrows). Usually, the cells of the hub proper had much weaker fasciclin III expression (Fig. 10J, arrowhead).

Despite the variability observed among agametic testes, our observations suggest that the germ line may play two distinct roles in regulating fasciclin III expression in neighbouring somatic cells. First, germ cells seems required for the correct initiation or maintenance of fasciclin III expression in the cells of the hub. Second, germ cells appear to prevent fasciclin III expression in neighbouring cyst cells.

Cyst cells in agametic testes undergo more S phases

Squeezed between germ line stem cells, the somatic cyst-progenitor cells are also arranged around the hub (Hardy et al., 1979). Two cyst-progenitor cells are thought to divide in concert with one neighbouring germ line stem to give rise to two cyst cells surrounding one daughter gonial cell (see Fig. 2). While this gonial germ cell will undergo four mitoses, the two cyst cells no longer divide during the remainder of spermatogenesis. We wanted to investigate whether germ cells may play a role in regulating the proliferation of the cyst cell lineage.

To begin addressing this question, we performed BrdU incorporation experiments, comparing the number of cyst cells undergoing S phase in a 30 minute labeling period in the wild-type and agametic testes. For the wild-type, we utilized a marker line expressing lacZ in early cyst cells and the hub. An immunofluorescence double-labeling experiment was carried out such that both ß-galactosidase and BrdU signals could be followed; the data was analyzed at the confocal microscope (see Materials and Methods). Doubly-labeled cells (Fig. 11A, arrows) were scored as cyst cells that were in S phase during the labeling period. We analyzed 21 such testes, and plotted the distribution of the number of cyst cells labeled in each testis (Fig. 11B). We observed a mean of 6.1 cyst cells doubly-labeled per wild-type testis, as well as a wide spread around that mean. Curiously, some of the doubly-labelled cells were some distance away from the hub (data not shown), suggesting that they were not cyst-progenitor cells.

For the agametic testis, we determined the number of BrdU positive cells within the area defined by the marker lines as containing cyst cells (see Fig. 9E). We scored 47 testes, and observed a mean of 12.9 cells labeled per agametic testis, more than twice that seen in the wild-type (Fig. 11B). The spread around this mean was even more accentuated than that observed in the wild-type. Most cells undergoing S phase in the agametic testis were located in the vicinity of the hub (Fig. 11C, arrows), but some were located further away (Fig. 11C, thin arrowhead).

Our results suggest that the germ line could be required to control the proliferation of somatic cyst cells.

Fig. 9: Marker lines reveal the architecture of the agametic testis. Apical regions of control testes or entire agametic testes in sons of *osk*^{301} mothers; both control and agametic testes carry one copy of the P-element; immunoperoxidase staining with anti β–galactosidase antibody. The third column schematizes the data for the agametic testis. **(A, B)** A marker line (254) specific for hub cells in the wild-type (arrowhead) locates the hub in the agametic testis (A and B, arrows); note that the agametic hub is slighlty larger and does not occupy the apical-most position as in the wild-type. **(C, D, E)** A marker line (D-39) labeling all cyst cells (C, arrow) and the hub (C, arrowhead) identifies the cells beneath the hub in the agametic testis as cyst cells (D and E, arrows); the arrowhead in (D) points to the likely position of the hub. **(F, G, H)** A marker line (P-573) staining cyst cells beginning in the growth phase region in the wild-type (F, arrow) labels the cyst cells furthest away from the hub in the agametic testis (G and H, arrows); note that early cyst cells are not labeled in either wild-type or agametic testis (F, G and H, arrowheads). **(I, J, K)** A marker line (L44a) specific for the muscle cells (I, arrow) demonstrates the thickening of this layer of the sheath in agametic testes; muscle cells thus make up most of the tissue located between the hub and the very tip of the agametic testis (J and K, arrows). **(L, M, N)** A marker line (857) labeling the pigment cells demonstrates the thickening of the sheath in agametic testes; note how nuclei are much closer to each other in the agametic testis (M, arrows) than in the wild-type (L, arrows); the very apex of the control testis (panel L) is not shown. Bar=50 μm; all panels are at the same magnification.

Figure 9

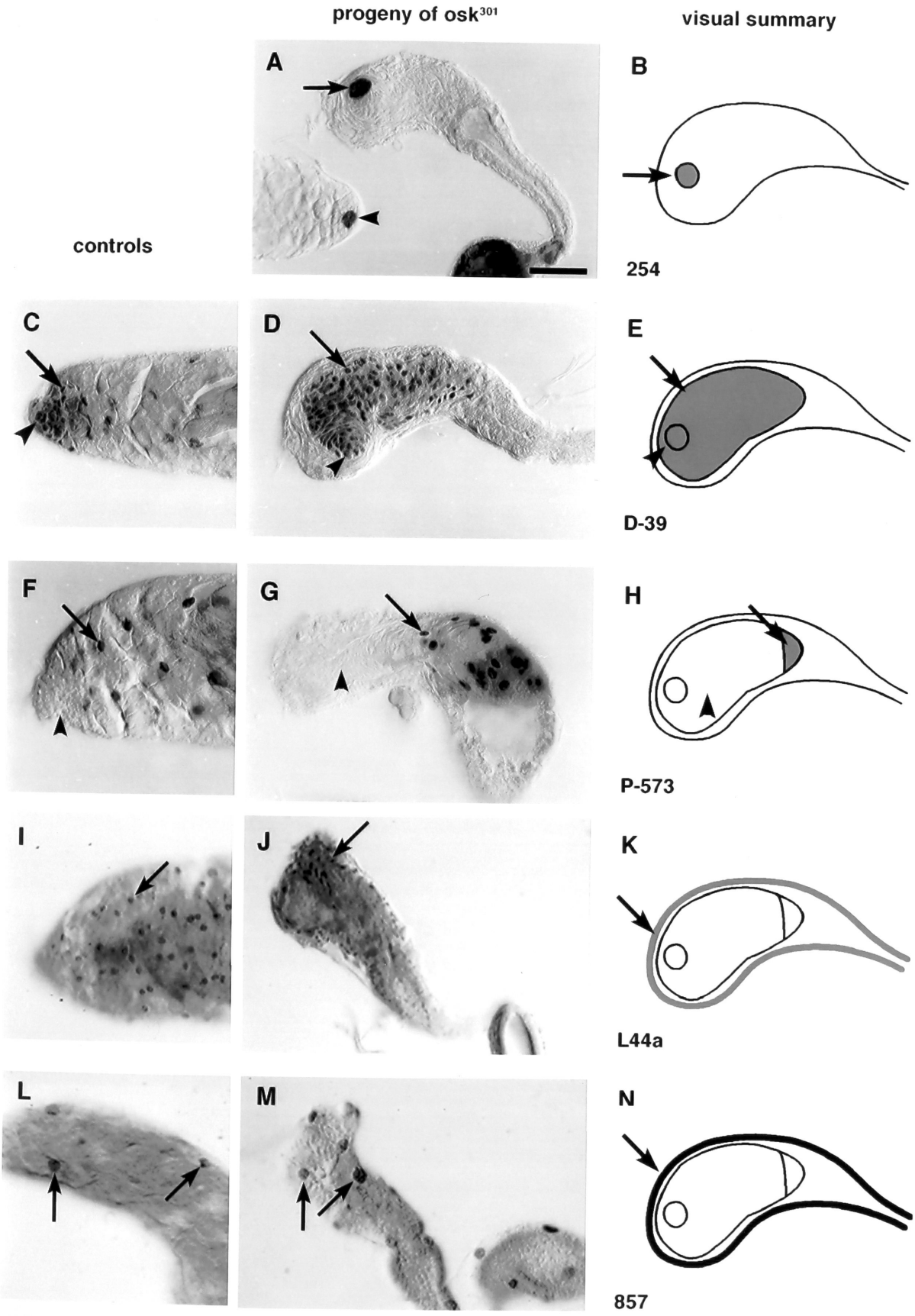

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Fig. 10 Armadillo and fasciclin III expression in agametic testes. **(A, B, C)** Immunoperoxidase staining with anti-armadillo antibody. **(A)** Wild-type testis and **(B)** apical region of wild-type testis. arm is expressed at high levels in the cells of the hub (A and B, arrows), and at lower levels in early cyst cells (A and B, arrowheads), recognizable by their thin cytoplasm surrounding developing germ cells. **(C)** Agametic testis; arm is still expressed in the hub (arrow) and in cyst cells (arrowhead). **(D, E, F, G)** Immunoperoxidase staining with anti-fasciclin III antibody. **(D)** Wild-type testis and **(E)** apical region of wild-type testis. Fasciclin III is expressed in the cells of the hub (D and E, arrows), and in the terminal epithelium joining the base of the testis to the seminal vesicle (D, arrowhead). **(F, G)** Representative examples of fasciclin III expression in two classes of agametic testes (see Text). **(F)** In one class of agametic testes, fasciclin III expression at the hub is absent altogether, although expression in the cells of the terminal epithelium is unaffected (arrowhead) **(G)** In another class of agametic testes, fasciclin III expression in the hub is much weaker than for coprocessed wild-type testes (G, arrow, compare with E, arrow). **(H, I, J)** A class of agametic testes ectopically expresses fasciclin III in cyst cells. Double indirect immunofluorescence of an agametic testis carrying the marker S2-11, which labels the cells of the hub and cyst cells at the border between the proliferation and growth phase regions; genotype of parents: ( ) S2-11/S2-11; osk^{301}/osk^{301} x ( ) w^{-}/Y. Anti β-galactosidase and anti-fasciclin III antibodies visualized by indirect immunofluorescence with rhodamine and fluorescein conjugated secondary antibodies, respectively; cyst cells beneath the hub are followed by Hoechst staining **(H)** Hoechst staining labels the DNA of cells present in the agametic testis; the arrow and arrowhead are in the same position as in panels I and J. **(I)** β-galactosidase staining reveals the cells of the hub (arrowhead), as well as maturing cyst cells (arrow). **(J)** Fasciclin III is ectopically expressed in maturing cyst cells (arrow); note the much weaker fasciclin III expression in the cells of the hub (arrowhead). The thin arrowhead points to the cells of the terminal epitelium. Bars=50 μm.

Figure 10

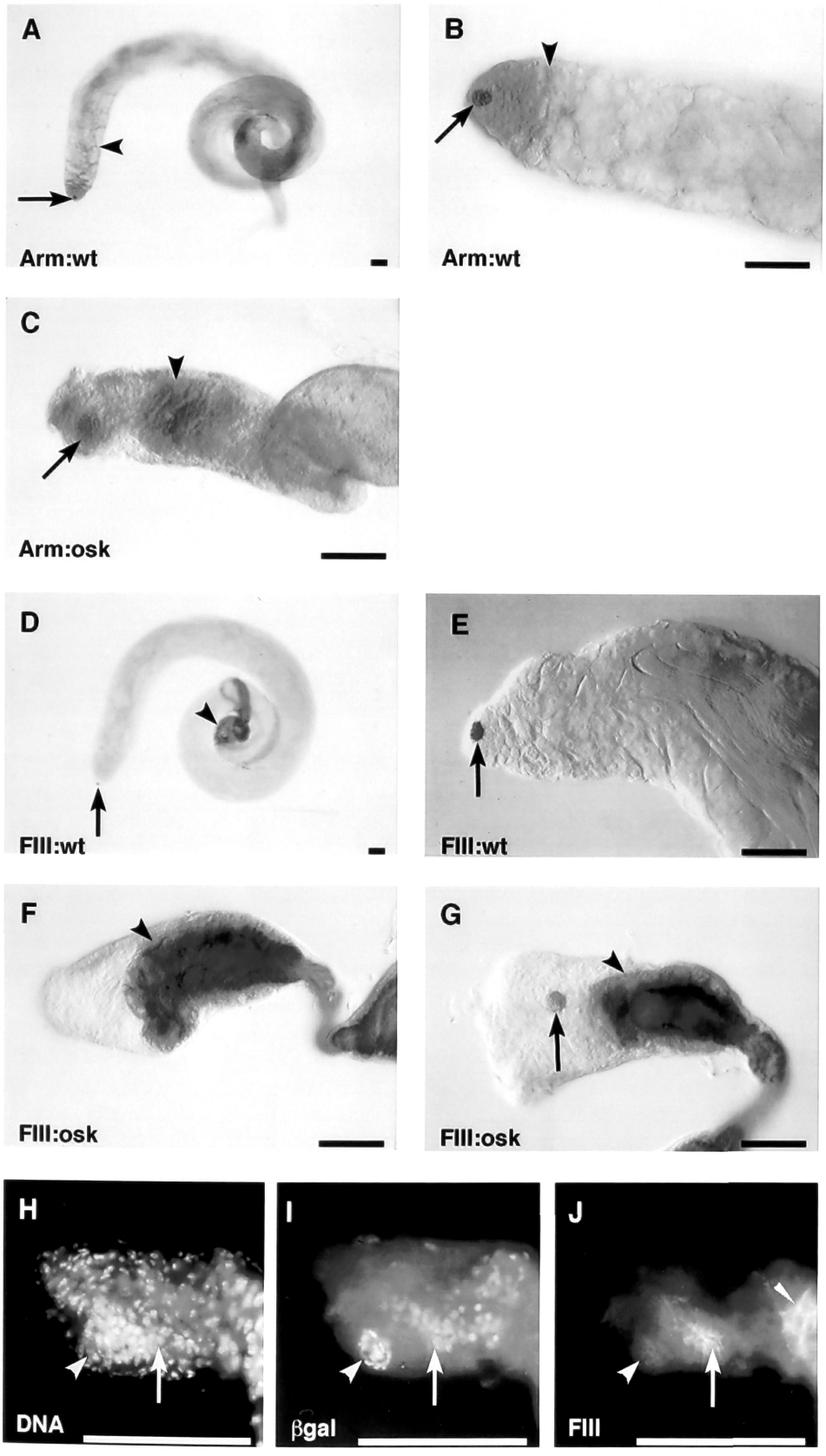
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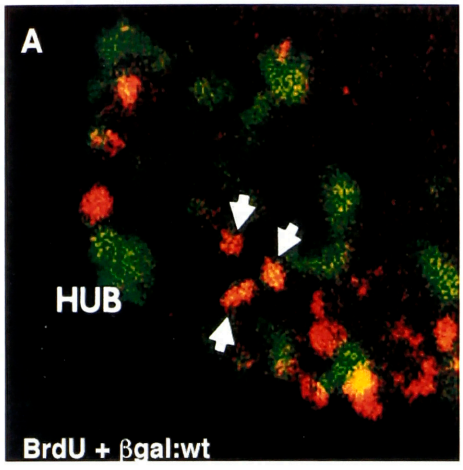
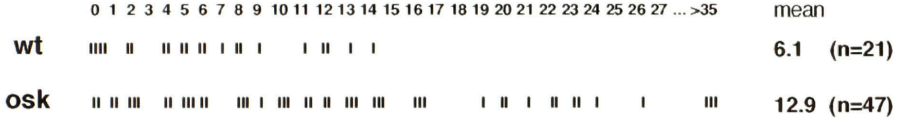
Fig. 11 More cyst cells undergo S phase in agametic testes. **(A)** Testis tip in a marker line (842) labeling early cyst cells and hub cells, and which underwent BrdU incorporation during a 30 minute labeling period. Anti β–galactosidase and anti-BrdU antibodies visualized by indirect immunofluorescence with fluorescein and rhodamine conjugated secondary antibodies, respectively. β–galactosidase expression is seen in green, BrdU incorporation in red. Orange nuclei (resulting from a spatially coincident strong red signal and weaker green signal) identify cyst cells that incorporated BrdU during the labeling period; 3 such nuclei are visible (arrows) in this 10 μm thick stack of sections gathered at the confocal microscope. The bigger yellow patch towards the bottom right of the panel most likely results from the superimposition, in the 10 μm stack, of a maturing cyst cell with one or several closely underlying germ cells undergoing S phase. **(B)** Distribution of the number of cyst cells in S phase in the wild-type and agametic testes. Each vertical bar represents one testis; the mean number of cyst cells undergoing S phase is given; note the wide spread around the mean in both cases. 21 wild-type testes were analyzed at the confocal microscope (see above), while 47 agametic testes were examined by regular fluorescent microscopy for the presence of BrdU positive nuclei within the area containing cyst cells. Agametic testes without a hub or cyst cells were probably (my notes are fuzzy) not considered in this experiment. **(C)** Agametic testis carrying a marker line (M5-4) labeling the cells of the hub, and which underwent BrdU incorporation during a 30 minute period. Anti β–galactosidase and anti-BrdU antibodies were revealed by successive rounds of immunoperoxidase staining (see Material and Methods), first to reveal the BrdU signal (brown) and, second, β–galactosidase expression (purple). Over 20 cyst cells in this agametic testis underwent S phase during the labeling period; note that while most labeled nuclei (arrows) are next to the hub (arrowhead), others are some distance away from it (thin arrowhead). Bar=50 μm.

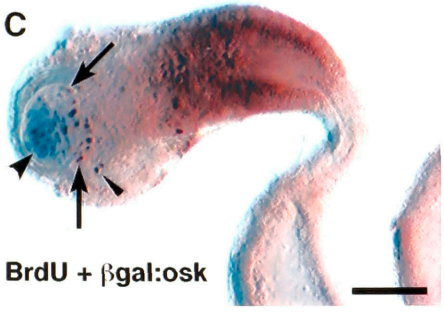
Figure 11



B

Number of cyst cells in S phase





DISCUSSION

We investigated whether germ cells influence neighbouring somatic cells during the early steps of spermatogenesis. We confirm the findings of Aboïm, as all somatic cell types seem present in most agametic testes. We extend his work by showing that cyst cells progress in their differentiation program in the absence of germ cells. Importantly, we find in addition that germ cells play a role in at least three aspects of somatic cell function: proper organization of hub and cyst cells in all cases, regulation of fasciclin III expression at the hub, and proliferation of cyst cells.

Some agametic testes lack a hub

20 % of agametic testes do not contain a hub or cyst cells beneath it. While describing variations in the appearance of the hub and cyst cells during pupation, Aboïm failed to mention agametic testes entirely lacking these somatic cells (Aboïm, 1945). The discrepancy with our data might be due to the method utilized to obtain agametic animals. Whereas we eliminated germ line determinants genetically, presumably preventing the formation of pole cells, Aboïm ablated already formed pole cells by UV-irradiation. Pole cell debris that resulted from the operation were often observed as late as 12 hours into development, appropriately positioned within gonadal mesoderm (Aboïm, 1945). Such germ line debris may have retained some signalling capability toward the somatic component of the gonad. If this explanation were correct, it would suggest that germ cells play a role during embryogenesis to organize somatic cells in all gonads.

Agametic testes lacking cyst cells always lack the hub. We do not know whether this correlation reflects a simultaneous requirement for germ cells for both somatic cell types or, alternatively, a primary requirement for one of them, followed by a secondary signalling event between the hub and cyst cells. Close examination of these cell types in

genetically ablated agametic gonads throughout development may allow to discriminate between these alternatives.

The hub does not maintain fasciclin III expression in the absence of germ cells

While the cells of the hub are present in most agametic testes, they fail to properly express the cell adhesion molecule fasciclin III. Fasciclin III expression is thus often lacking or reduced at the hub in the absence of germ cells. Similarly, in mammals, while morphologically normal Sertoli cells are found in mutant mice genetically deficient in germ cells (Handel and Eppig, 1979), Sertoli cells nevertheless require the presence of the germ line for proper function (reviewed by McGuinness and Griswold, 1994). For instance, the removal of germ cells from cultured Sertoli cells results in a decreased secretion of androgen-binding protein and transferin by these somatic cells (Welsh et al., 1985; LeMagueresse et al., 1988). Loss of germ cells in vivo or in vitro leads to an increased secretion of testins, a family of structurally related Sertoli cell proteins (Cheng et al., 1989). Thus, in mammals as in *Drosophila*, while the germ line seems mostly dispensible for proper morphological differentiation of somatic cell types, it still is required for the appropriate expression of specific proteins by the somatic cells of the gonad.

We believe that germ cells play a role in maintaining, rather than initiating, fasciclin III expression in the cells of the hub. We favor this hypothesis as fasciclin III expression is usually either reduced in level or absent, possibly representing two subsequent stages in maintenance failure. Evidence for this view could come from an examination of fasciclin III expression in agametic gonads earlier during development, where it should be stronger, as well as in older adults, where fasciclin III expression should be weaker.

We crossed 12 marker lines that label the cells of the hub into an agametic background in the hope of identifying genes whose expression would strictly depend on

the germ line. While 3 lines appeared to be expressed at lower levels, possibly identifying additional genes whose maintenance requires the germ line, we failed to identify any line in which ß-galactosidase was absent altogether. We infer that such genes, if they exist, are likely to be rare.

Cyst cells ectopically express fasciclin III in agametic testes

Close to 40 % of agametic testes with a hub display ectopic fasciclin III expression in cyst cells beneath it. Interestingly, the cells of the hub and those of the cyst cell lineage are closely related, as almost every enhancer trap line that labels cyst progenitor cells also labels the cells of the hub (see Table 1 and data not shown). Therefore, cyst cells may be near to having the potential to express some hub-specific genes.

As a working model, we propose that germ cells could have two distinct roles on fasciclin III expression in neighbouring somatic cells. First, they could be required for the maintenance of fasciclin III expression in the cells of the hub, as judged by the diminished or inexistant expression in most agametic testes. Second, germ cells could also play a role in repressing fasciclin III expression in neighbouring cyst cells, as judged by the ectopic expression observed in some agametic testes.

The germ line negatively regulates cyst cell proliferation

On average, about twice as many cyst cell nuclei undergo S phase in the agametic testis than in the wild-type, although there is a wide spread around the mean in both cases. This observation raises the possibility that germ cells play a role in restricting the proliferation of neighbouring cyst progenitor cells. However, we need to determine at least two facts before reaching this conclusion. First, we need to ascertain whether these extra S phases are followed by cell division, or whether they simply represent endoreduplication cycles. Preliminary observations indicate that the number of cyst cells does increase with age, in at least some agametic testes, suggesting that S phases are

followed by cell divisions. Second, we need to determine whether the cells undergoing extra S phases are cyst progenitor cells, or their daughter cyst cells. In the latter case, germ cells would play a role in inhibiting proliferation in the daughter cyst cells, rather than in cyst progenitor cells per se. This will be difficult to evaluate as we do not have marker lines that only label cyst progenitor cells, and not their immediate progeny.

Cell-cell interations are crucial for cell-cycle control in several systems. For instance, in *S. cerevisiae*, pheromones secreted by cells of the opposite mating type induce cell cycle arrest of target cells (reviewed by Marsh et al., 1991; Reed, 1991). The mechanisms by which the pheromone signal is transduced from the surface of the target cell to inhibiting the cdk2 kinase essential for the G1 to S transition are being elucidated in this system (see for instance Peter et al., 1993). Many vertebrate growth factors can also cause cells to either proliferate or become quiescent (reviewed by Pardee, 1989). In this case as well, work has begun to link the signal transduction cascade to the modulation of basic components of the cell-cycle machinery (see for instance Ewen et al., 1993). We can only hope that our understanding of the regulation of cyst cell proliferation by germ cells will some day be as detailed.

Is there a future for agametic testes ?

In a remarkable piece of work published 50 years ago, Aboïm described the morphology of somatic cells in agametic gonads of both sexes throughout development. He concluded that the germ line plays little role, if any, in directing somatic cell fates. By reinvestigating this question using molecular markers, we modify Aboïm's conclusions and show that germ cells play a role, direct or indirect, in three aspects of somatic cell fates: presence of a hub and cyst cells, regulation of fasciclin III expression in the hub and neighbouring cyst cells, and proliferation of early cyst cells.

We do not know when during development germ cells are needed for sending the appropriate signals to somatic cells, nor do we know anything about the nature of these

signals. The agametic testis may not be well suited to investigate these questions further. Indeed, one would probably have to follow the fate of specific somatic cells as agametic gonads develop, which is a difficult task owing to their small size. Moreover, the variability observed in all three aspects of germ line requirement renders the agametic testis a less than ideal experimental situation. As an alternative, one might gain further insight into these questions by selectively eliminating germ cells in the adult testis, for instance by using a GAL4 system driving a toxin (Bellen et al., 1992; Brand and Perrimon, 1993), or by identifying loci involved in these signaling events.

CHAPTER 4: FURTHER CHARACTERIZATION OF *bam* AND *bgcn*, TWO OVERPROLIFERATION MUTANTS OF THE MALE AND FEMALE GERM LINE

INTRODUCTION

Germ line stem cells divide asymmetrically, giving rise to two daughters with distinct fates. Whereas one daughter remains a self-renewing stem cell, the other becomes a gonial cell that undergoes a limited number of mitoses, before switching to the meiotic cell cycle, and then completing gametogenesis. A defect in three early fate decisions may be expected to result in a phenotype of overproliferating germ cells. First, if the gonial cell were unable to adopt a fate different from its parental stem cell, it would still behave as a stem cell and thus indefinitely proliferate. Second, if the mitotically dividing germ cells were unable to limit their number of mitoses, or, third, were unable to enter the meiotic cell cycle, they may also keep proliferating. Thus, the analysis of mutants with overproliferating germ cells should provide important information about the regulation of early fate decisions during gametogenesis.

In *Drosophila* females, overproliferating germ cells can also result from inappropriate germ line sex determination (reviewed by Pauli and Mahowald, 1990; Steinmann-Zwicky, 1992; Spradling, 1993; Gateff, 1994). Sex determination in the germ line is in part different from sex determination in somatic cells. First, whereas sex-determination in the soma is cell autonomous, XX germ cells determine their sex by combining a cell autonomous mechanism with an inductive signal from the soma (Steinmann-Zwicky et al., 1989). Second, many of the genes required for somatic sex determination are dispensible in the germ line (Marsh and Wieschaus, 1978; Schüpbach,

1982). However, *Sxl*, the master regulator of the somatic sex determination cascade, is also essential for germ line sex determination. XX germ cells lacking *Sxl* function and transplanted into wild-type XX host ovaries form aberrant multicellular cysts, demonstrating that *Sxl* is required in germ cells for proper differentiation along the female pathway (Schüpbach, 1985). Such transplanted *Sxl*$^-$ germ cells differentiate morphologically as early spermatocytes, confirming the central role of *Sxl* in germ line sex determination (Steinmann-Zwicky et al., 1989). Moreover, *Sxl* alleles specifically affecting germ line sex determination have ovaries similarly filled with multicellular cysts (Salz et al., 1987; Lindsley and Zimm, 1992; Bopp et al., 1993).

A group of female-sterile loci, including *ovarian tumor* (*otu*), *ovo* and *sans-fille* (*snf*; also known as *fs(1)1621*), display a similar overproliferation mutant phenotype during oogenesis (Gollin and King, 1981; King and Riley, 1982; Oliver et al., 1990). They have thus been proposed to also affect sex determination in the germ line (reviewed by Pauli and Mahowald, 1990; Spradling, 1993). Morphological, molecular and genetic data confirm that these three loci play a role in germ line sex determination. First, mutant XX germ cells resemble spermatocytes and express enhancer trap lines or mRNA's normally restricted to the male germ line (Pauli et al., 1993; Wei et al., 1994). Moreover, they also express the male-specific form of Sxl mRNA (Bopp et al., 1993; Oliver et al., 1993; Pauli et al., 1993). Finally, mutations in *otu*, *ovo* and *snf* can be suppressed by constitutive alleles of *Sxl*, indicating that these loci are required upstream of *Sxl* during germ line sex determination (Steinmann-Zwicky, 1988; Salz, 1992; Oliver et al., 1993; Pauli et al., 1993).

Two other loci, *bag-of-marbles* (*bam*) and *benign gonial cell neoplasm* (*bgcn*) (Gateff, 1982; McKearin and Spradling, 1990), have a similar overproliferating mutant phenotype during oogenesis, and have thus been postulated to be also involved in germ line sex determination (Mahowald and Wei, 1994; Wei et al., 1994). However, both *bam* and *bgcn* mutations also accumulate undifferentiated germ cells in testes (Gateff, 1982;

McKearin and Spradling, 1990), whereas *otu*, *ovo* and *snf* have no effect on spermatogenesis. Therefore, rather than being needed for germ line sex determination, *bam* and *bgcn* could instead be required in both sexes for one of the fate decisions regulating proliferation of early germ cells (Gateff, 1982; McKearin and Spradling, 1990).

We investigated the *bam* and *bgcn* phenotypes during spermatogenesis to help elucidate the function of these two loci. We present evidence against a sexual transformation of mutant germ cells, and show that *bam* and *bgcn* overproliferating cells retain some characteristics of stem cells. Moreover, we report that *bam* and *bgcn* germ cells overproliferate in synchrony, within two cyst cells that appear to be also blocked in their differentiation.

RESULTS

bam and *bgcn* mutant testes were essentially identical by all criteria examined. Therefore, the data in this Chapter are only illustrated for one mutant, while minor differences are pointed out in the legends to the figures.

In the wild-type, the gonial daughter of the germ line stem cell undergoes four mitotic divisions to form a cyst of 16 germ cells, contained within two somatic cyst cells. The 16 germ cells then enter a long growth phase leading to the meiotic divisions. Cysts of 16 germ cells at different stages of the growth phase can be easily observed in live preparations of wild-type testes (Fig. 12A, arrows). In contrast, cysts of 16 germ cells were rarely found in *bam* and *bgcn* testes. Such rare cysts contained small germ cells (Fig. 12B, arrowhead, compare with Fig. 12A, arrows), indicating that they had not entered the growth phase. As previously reported, *bam* and *bgcn* testes mostly contained giant cysts, filled with often over one hundred small germ cells (Fig. 12B, arrow; Gateff, 1982; McKearin and Spradling, 1990). Older giant cysts contained refractile germ cells that seemed to be dying (data not shown, but see McKearin and Spradling, 1990, as well as the Discussion of this Chapter, and Chapter 8).

Thus, *bam* and *bgcn* germ cells undergo four mitotic divisions to form cysts of 16 germ cells, but then abberantly undergo additional rounds of divisions instead of exiting the proliferation phase and progressing into the growth phase.

bam and *bgcn* germ cells within a cyst overproliferate in synchrony

We next examined the BrdU incorporation pattern of wild-type and mutant testes to begin to characterize the cell cycling properties of *bam* and *bgcn* germ cells.

Wild-type testes that incorporated BrdU during a 30 minute labeling period had BrdU-positive nuclei only at their very tip, in the proliferation region (Fig. 12C, arrow). No incorporation was observed further down, in the growth phase region (Fig. 12C,

arrowhead). All germ cells within a cyst underwent S phase in near synchrony (Fig. 12E). Germ cells in a given cyst similarly undergo M phases in near synchrony (data not shown). The cyst furthest from the hub (Fig. 12E, arrowhead) which incorporated BrdU contained 16 cell germ cells, presumably in premeiotic S (Fig. 12E, arrow). Such labeled cysts were always located still close to the tip, indicating that premeiotic S closely follows the last amplifying mitotic division. No incorporation was observed beyond that point, as germ cells entered the extended G2 period leading to the meiotic divisions.

In contrast, *bam* and *bgcn* testes not only had BrdU positive nuclei at their tip, but also further down (Fig. 12D, arrows), past the region to which proliferation is normally restricted. Mutant germ cells within a cyst still underwent S phases in synchony (Fig. 12F). As expected, most labeled cysts contained well over 16 germ cells (Fig. 12F, arrows).

We conclude that S phases are synchronized among germ cells within a cyst in the wild-type, and that *bam* and *bgcn* germ cells retain a similar synchrony while overproliferating. An examination of mitotic figures in mutant testes yielded identical conclusions for M phases (data not shown).

bam and _bgcn_ germ cells are surrounded by two developmentally arrested cyst cells

Overproliferating germ cells seemed to be contained within a cyst, as judged both by morphological criteria (see Fig. 12B) and by their synchronous proliferation (see Fig. 12F). Nevertheless, we wanted to verify that the correct number of cyst cells were surrounding the overproliferating *bam* and *bgcn* germ cells. To this end, we crossed both mutants to a marker line labeling cyst cells starting in the early growth phase region. Just like in the wild-type (Fig. 12G, arrows), *bam* and *bgcn* overproliferating germ cells were also surrounded by two cyst cells (Fig. 12H, arrows). Therefore, *bam* and *bgcn* do not affect the proliferation of the somatic cyst cells that surround the developing germ cells.

We then addressed whether these two cyst cells differentiate appropriately in *bam* and *bgcn* mutant testes, despite the germ cells not progressing beyond the early stages of spermatogenesis. We utilized a marker line that only labels early cyst cells in the wild-type (Fig. 12I, arrow). In mutant testes, cyst cells maintained lacZ expression (Fig. 12J, arrowheads) past the region to which it is normally restricted (Fig. 12J, arrow). Although we have tested only one marker line for each *bam* and *bgcn* so far, we tentatively conclude that, just like germ cells, cyst cells are blocked in their differentiation program.

bam and *bgcn* germ cells are not sexually transformed

We next investigated whether the overproliferating germ cells were sexually transformed. In testes, *bam* and *bgcn* mutant germ cells still expressed a male-specific marker (Fig. 13A and 13B), which is off in wild-type XX germ cells, but whose expression is turned on in sexually transformed *snf* and Sxl^{f4} mutant ovaries (see Fig. 5F, 5G and 5H). In testes, *bam* and *bgcn* mutant germ cells did not express a marker which is off in wild-type testes (Fig. 13C and 13D), but on in wild-type ovaries (see Fig. 5J). Thus, sexual transformation is unlikely to be reponsible for the *bam* and *bgcn* spermatogenesis phenotype.

bam and *bgcn* overproliferating germ cells have a mixed early identity

We then analyzed how far *bam* and *bgcn* germ cells progress along spermatogenesis.

Mutant germ cells did not express a marker labeling germ cells starting in the growth phase in the wild-type (Fig. 13E and 13F), confirming that mutant cells are blocked at a prior stage of the developmental program. Overproliferating *bam* and *bgcn* mutant germ cells maintained the expression of a marker labeling wild-type germ cells in a "stripe" at the border between the proliferation and the growth phase regions (Fig. 13G and 13H). Accordingly, mutant germ cells also failed to maintain expression of some

stem cell specific markers (Fig. 13I and 13J). Thus, overproliferating *bam* and *bgcn* germ cells have some characteristics of germ cells in the mitotic amplificatory stage of spermatogenesis. However, overproliferating germ cells did maintain the expression of some other stem cell speficic markers (Fig. 13K and 13L).

Taken together, these observations indicate that overproliferating *bam* and *bgcn* germ cells are blocked early in spermatogenesis with a mixed identity, retaining some characteristics of stem cells while at the same time expressing features of slightly more mature proliferating germ cells.

Fig. 12 *bam* and *bgcn* mutant germ cells overproliferate in synchrony within two cyst cells. *bgcn*/+=*bgcn*^{QS2}/CyO; *bgcn*/*bgcn*=*bgcn*^{QS2}/*bgcn*^{QS2}; *bam*/+=*bam*$^{\Delta 86}$/+; bam/bam=*bam*$^{\Delta 86}$/*bam*$^{\Delta 86}$. **(A, B)** Live testis squash **(A)** *bgcn*/+ germ cells, organized in cysts of 16 cells (arrows); note that the size of germ cells increases in more mature cysts (right-most arrow); the refractile cyst cell cytoplasm can be seen in intact cysts (thin arrowhead). **(B)** *bgcn* mutant; while rare cysts contain 16 small germ cells (arrowhead), most contain many more, up to over one hundred (arrow). **(C, D)** 30 minute BrdU incorporation experiment; immunoperoxidase reaction with anti-BrdU antibody. **(C)** Wild-type testis. BrdU incorporation occurs at the tip (arrow), in the region where stem cells and mitotically dividing cells are located; no S phases occur further down, either in the growth phase (arrowhead) or during the remainder of spermatogenesis. **(D)** *bgcn* mutant testis. BrdU incorporation occurs not only at the tip, but also further down (arrows), in a region where no S phases are observed in the wild-type; note that the *bgcn* testis is shorter. **(E, F)** 30 minute BrdU incorporation experiment; immunoperoxidase reaction with anti-BrdU and anti-fasciclin III antibodies. **(E)** Apical region of a wild-type testis. Some nuclei in the vicinity of the hub (arrowhead) have undergone S phase; slightly away from the hub, cysts of germ cells at the 4-, 8- and 16- (arrow) cell stage have incorporated BrdU; note that germ cells within a cyst are synchronously undergoing S phase. **(F)** Apical region of a *bgcn* testis. Some nuclei in the vicinity of the hub (arrowhead) have undergone S phase; cysts that contain up to over one hundred synchronously replicating nuclei are found further down (arrows), in an area normally devoid of proliferating cells. **(G, H)** X-gal staining of a marker line (473) labeling cyst cells starting in the early growth phase, demonstrating the presence of two cyst cell nuclei (G and H, arrows) per cyst of germ cells, both in the wild-type and *bgcn*. **(I, J)** X-gal staining of a marker line (K59a) labeling very early cyst cells (I and J, arrows), demonstrating that cyst cells are arrested in their differentiation program in *bam* mutant testes, as they maintain lacZ expression (J, arrowheads). A marker line (L18a) with a similarly restricted expression in cyst cells gave the same result in *bgcn* testes. Bar=50 μm.

Figure 12

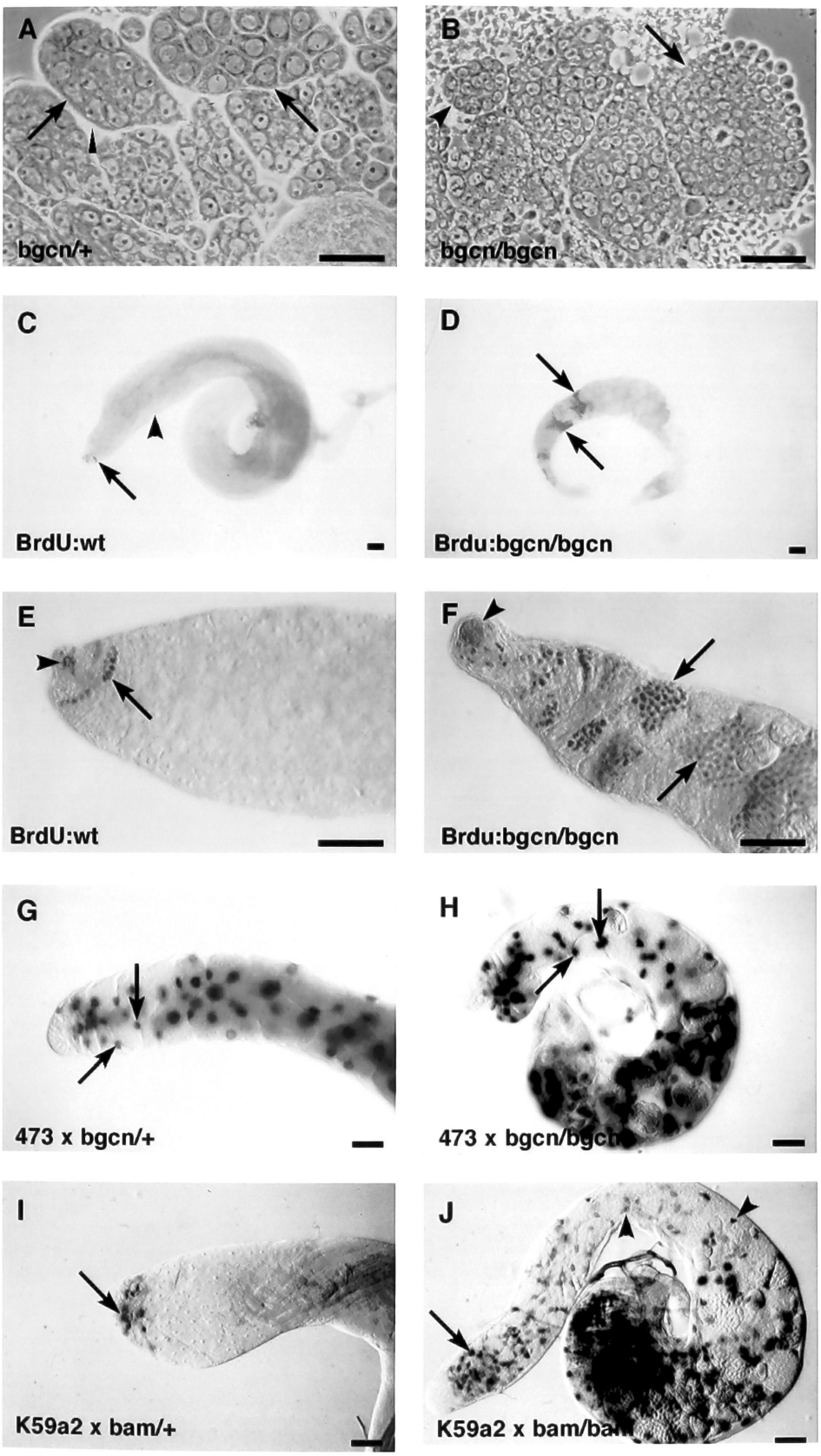
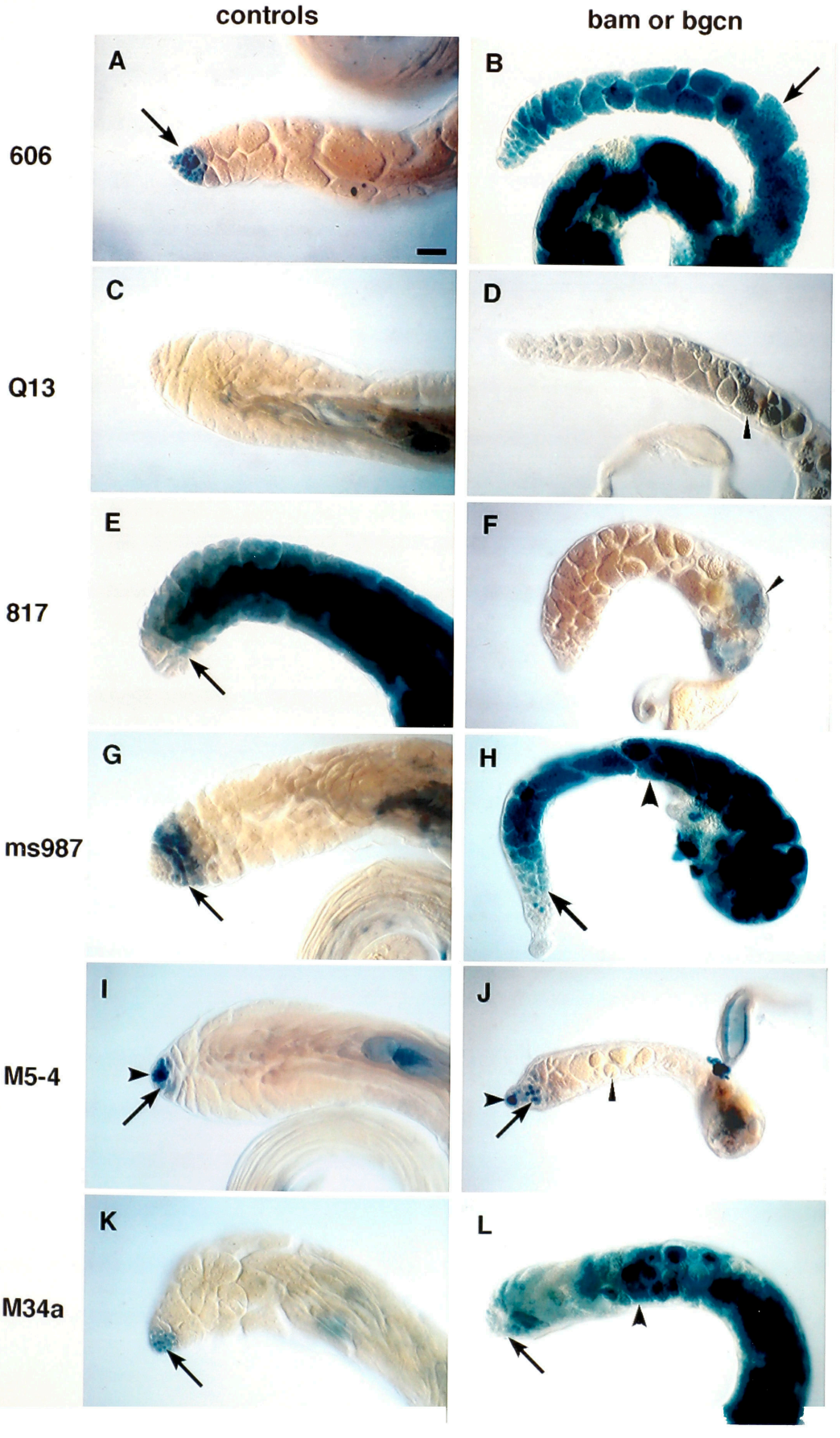

A
bgcn/+
B
bgcn/bgcn
C
BrdU:wt
D
Brdu:bgcn/bgcn
E
BrdU:wt
F
Brdu:bgcn/bgcn
G
473 x bgcn/+
H
473 x bgcn/bgcn
I
K59a2 x bam/+
J
K59a2 x bam/bam

Fig. 13 *bam* and *bgcn* mutant germ cells have an early male fate. X-gal staining. **(A, B)** A marker line (606) that labels early germ cells in the male (A, arrow) but not the female (see Fig. 5B) expresses lacZ in *bgcn* mutant testes (B, arrow). Genotypes: 606/606; $bam^{\Delta 86}$/TM3Sb (control); 606-$bgcn^{QS2}$/606-$bgcn^{QS2}$. An identical result is obtained with *bam* mutant testes. **(C, D)** A marker line (Q13) that labels early germ cells in the female only (see Fig. 5J) fails to substantially express lacZ in *bam* mutant testes; traces of expression are occasionally seen towards the base of the testis (thin arrowhead). Genotypes: Q13/CyO; $bam^{\Delta 86}$/TM3; (control); Q13/CyO; $bam^{\Delta 86}$/$bam^{\Delta 86}$. An identical result is obtained with *bgcn* mutant testes. **(E, F)** A marker line (817) staining germ cells beginning in the growth phase (E, arrow), fails to substantially express lacZ in *bam* mutant testes; traces of expression are sometimes seen towards the base of the testis (not shown). Note also the background staining towards the base of the mutant testis (F, thin arrowhead), probably resulting from dying germ cells; identical staining is observed in mutant testes not carrying a marker line, demonstrating that it does not result from lacZ expression. Genotypes: 817/817; $bam^{\Delta 86}$/TM3 (control); 817/817; $bam^{\Delta 86}$/$bam^{\Delta 86}$. An identical result is obtained with *bgcn* mutant testes. **(G, H)** A marker line (ms 987) labeling germ cells in a "stripe" at the border between the proliferation and the growth phase regions (G and H, arrows) is maintained in overproliferating cysts in a *bam* mutant testis (H, arrowhead). Genotypes: ms987-$bam^{\Delta 86}$/TM3 (control); ms987-$bam^{\Delta 86}$/$bam^{\Delta 86}$. An identical result is obtained with *bgcn* mutant testes. **(I, J)** A marker line (M5-4) labeling germ line stem cells (I, arrow) and their immediate daughters, as well as the hub (I, arrowhead), is not maintained in a *bam* mutant testis: whereas both the hub (J, arrowhead) and very early germ cells (J, arrow) are labeled, older cysts are not (J, thin arrowhead). Genotypes: M5-4/CyO; $bam^{\Delta 86}$/TM3Sb (control); M5-4/CyO; $bam^{\Delta 86}$/$bam^{\Delta 86}$. Similar results are obtained for *bam* with marker lines S1-33 and S3-45 and for *bgcn* with marker lines M5-23 and S1-33. **(K, L)** A marker line (M34a) labeling germ line stem cells (K, arrow) and their immediate daughters, is expressed both in young (L, arrow) and older (L, arrowhead) *bgcn* mutant germ cells. Genotypes: *bgcn*/CyO; M34a/M34a; $bgcn^{QS2}$/$bgcn^{QS2}$; M34a/M34a. A similar result is obtained in *bam* mutant testes, although maintenance is less pronounced than in *bgcn*. Similar results are obtained for *bam* with marker lines M10-39, S3-40 and S3-46. Bar=50 μm; all panels are at the same magnification.

Figure 13


controls
bam or bgcn
606
Q13
817
ms987
M5-4
M34a
A
B
C
D
E
F
G
H
I
J
K
L

DISCUSSION

bam and *bgcn* are the only known mutations that result in an overproliferation of germ cells in both sexes. *bam* and *bgcn* could control stem cell fate, the counting of mitotic divisions, or entry into meiosis. Germ cell overproliferation represents the likely null phenotype for both loci (McKearin and Christerson, 1994; Wei et al., 1994). Therefore, *bam* and *bgcn* can be considered as germ line specific tumor suppressor loci (Weinberg, 1991; Gateff, 1994). The *bgcn* gene extends over a 13 kb genomic region encoding 6 transcripts (Gateff, 1994), but it is not yet clear which of these may provide *bgcn* function. *bam* encodes a protein of unknown function, which shares 20 % sequence similarity with the product of the *otu* gene (McKearin and Spradling, 1990). We began the phenotypic characterization of these mutants during spermatogenesis to better understand how *bam* and *bgcn* function may regulate the proliferation of early germ cells.

Male germ cells overproliferate in *bam* and *bgcn* testes

bam and *bgcn* have been suggested to interfere with germ line sex determination, as they have a phenotype during oogenesis similar to that of loci involved in that process (Mahowald and Wei, 1994; Wei et al., 1994). We show that the spermatogenesis phenotype is not likely due to a defect in germ line sex determination, as mutant germ cells still express male-specific markers, but not markers characteristic of female germ cells.

Nevertheless, it is possible that defects in germ line sex determination play a role in the *bgcn* oogenesis phenotype, as XX mutant germ cells expressed two male-specific markers (606 and M5-23), at least to some extent (data not shown). Additional experiments will be needed to ascertain whether *bgcn* is a *bona fide* component of the germ line sex determination pathway. In contrast to *bgcn*, *bam* mutant ovaries failed to express a male-specific marker (M5-4) (data not shown). In apparent contradiction with

this last observation, others have reported traces of male-specific lacZ expression (marker lines 606 and 590) in *bam* mutant ovaries (Wei et al., 1994). Trace amounts of the male-specific Sxl mRNA are also detected in XX *bam* germ cells (Bopp et al., 1993; Oliver et al., 1993). However, the vast majority of Sxl RNA is still spliced in the female mode. Both male-specific lacZ expression and splicing occur at much lower levels in *bam* than in *otu* or *snf* mutant ovaries (Bopp et al., 1993; Oliver et al., 1993; Wei et al., 1994). Thus, probably only a few cells may adopt a male fate in *bam* ovaries, possibly as a secondary consequence of not appropriately progressing through the female differentiation program. Moreover, the *bam* mutant phenotype can not be suppressed by a constitutive *Sxl* allele (McKearin and Christerson, 1994). While this result is also compatible with *bam* functioning downstream of *Sxl*, together with the other observations, it suggests that *bam* does not play a role in determining the sex of XX germ cells.

A very early defect in gametogenesis

bam and *bgcn* germ cells do not progress beyond the early stages of spermatogenesis. We show that overproliferating germ cells maintain the expression of some stem cell markers, but not others. It is possible that *bam* and *bcgn* germ cells are truly overproliferatimg as stem cells, but that some stem cell markers may never express lacZ in ectopically located germ cells, if for instance their expression depended on the presence of the neighbouring hub. Alternatively, *bam* and *bgcn* mutant germ cells may truly have a mixed fate of stem cells and sligthly later germ cells. The fact that they also express a marker specific for the border between the proliferation and growth phase regions is consistent with this view. Such mixed characteristics are common in transformed cell lines and tumors (reviewed by Heppner, 1984; Leith and Dexter, 1986).

During oogenesis, *bam* germ cells appear to similarly fail to progress beyond the very early stages. While Sxl protein is cytoplasmic only up to the 2- or 4-cell stage during

wild-type oogenesis, it is found in the cytoplasm of all *bam* mutant germ cells (Bopp et al., 1993). In addition, *bam* germ cells usually form pairs of cells connected by a single intercellular bridge, consistent with them being unable to progress past the 2-cell stage (McKearin and Christerson, 1994).

Arrested cyst cell differentiation

Just as *bam* and *bgcn* germ cells do not progress beyond the early stages of spermatogenesis, the surrounding two cyst cells do not seem to mature past the corresponding early stages of cyst cell differentiation. This could be due to a simultaneous requirement for *bam* and *bgcn* function in both the germ line and the somatic cyst cells. The requirement for *bgcn* during oogenesis has been addressed by reciprocal pole cell transplants, which demonstrated that the *bgcn* overproliferation phenotype is autonomous to the germ line (Wei et al., 1994). Unexpectedly, these experiments also revealed a distinct role for *bgcn* in the soma to support oogenesis (Wei et al., 1994). A potential somatic requirement for *bgcn* during spermatogenesis has not been addressed. Similar experiments have not been conducted in the case of *bam*, althouth *bam* is probably only required in the germ line, as both *bam* mRNA and protein are exclusively detected in germ cells (McKearin and Spradling, 1990; McKearin and Christerson, 1994). If *bam* and *bgcn* were truly only required in germ cells during spermatogenesis, it is possible that cyst cells in mutant testes arrest their differentiation because they lack some signal from adequately maturing germ cells.

Synchronous overproliferation within a cyst

In testes, overproliferating *bam* and *bgcn* germ cells undergo S and M phases in synchrony. In ovaries, since *bam* germ cells form pairs of interconnected cells (McKearin and Christerson, 1994), the hundreds of overproliferating germ cells within an egg chamber are not in a functional synticium. Therefore, mutant germ cells in ovaries should

not necessarely undergo S phases in synchrony, and our preliminary observations confirm that expectation. Why, then, are *bam* and *bgcn* germ cells synchronized within a cyst during spermatogenesis ? One possibility is that, in contrast to the situation in ovaries, they are actually part of an enormous syncitium of partially interconnected cells. Alternatively, the surrounding cyst cells may play some role in synchronizing their proliferation.

Overproliferation, differentiation and death

Cells must exit the cell cycle in order to progress through a differentiation program, and this dependency is being elucidated at a molecular levels in several cases. During myogenesis for example, while the phosphorylated form of the retinoblastoma gene product (Rb) leads to continued cell-cycling, the unphosphorylated form of Rb forms a complex with MyoD to promote myogenic differentiation (Gu, et al., 1993). Inactivation of the unphosphorylated form of Rb inhibits myogenesis and allows terminally differentiated cells to reenter the cell cycle (Gu, et al., 1993).

Cells that simultaneously receive conflicting stimuli, to both progress through the cell cycle and begin differentiation, undergo programmed cell death. For instance, cells expressing high levels of proliferation promoting oncogenic proteins like c-myc or E1A, while being at the same time deprived of serum, undergo massive apoptosis (Evan et al., 1992; Lowe and Ruley, 1993; Lowe et al., 1994). During spermatogenesis, germ cells cease proliferating as they enter the meiotic cell cycle and embark on a terminal differentiation program. Intringuingly, *bam* and *bgcn* germ cells overproliferate at first, but die after undergoing a few extra rounds of division. While this may be due to a variety of reasons, one can speculate that death, in this case also, may result from the reception of conflicting stimuli instructing mutant germ cells to both proliferate and begin the differentiation program.

CHAPTER 5: IDENTIFICATION OF MALE-STERILE MUTANTS GENERATED BY SINGLE P-ELEMENT MUTAGENESIS

INTRODUCTION

Despite a detailed understanding of spermatogenesis at the morphological level, relatively little is known about the genes controlling this developmental program. Exceptions include the testis-specific ß2-tubulin, which is required for microtubule-based processes during meiosis and post-meiotic differentiation, and the *cdc25* homolog *twine*, wich controls entry into meiosis I (Kemphues et al., 1982; Alphey et al., 1992; Courtot et al., 1992). Both ß2-tubulin and *twine* were identified as homologues of gene products studied in other organisms.

Extensive female-sterile screens have identified a few loci that can simultaneously mutate to a female- and male-sterile phenotype (see Schüpbach and Wieschaus, 1991). For instance, both *bam* and *bgcn*, which play a crucial role in regulating proliferating of early germ cells have been identified on the basis of their female sterile phenotype (Gateff, 1982; McKearin and Spradling, 1990; Schüpbach and Wieschaus, 1991). Screens directed specifically at identifying loci required for male fertility have also been conducted, mostly with chemical mutagens (reviewed by Lifschytz, 1987; Fuller, 1993). However, most of the mutants generated in this manner have not been mapped, and the majority are no longer available.

We have chosen a different approach, that of single P-element insertional mutagenesis (Cooley et al., 1988), to conduct a genetic analysis of spermatogenesis in *D. melanogaster*. With this method, each mutation is genetically tagged by an eye color marker and can be readily located on the physical map by *in situ* hybridization to polytene

chromosomes. Furthermore, new mutations, including null alleles, can be efficiently generated by remobilization of the P-element with a transposase source. Lastly, DNA flanking an insertion site can be readily cloned by bacterial transformation or by PCR, greatly facilitating molecular analysis of the locus.

In this Chapter, we report the isolation of 111 male-sterile mutations generated by P-element mutagenesis. We then present the genetic and phenotypic characterization of 83 of them, which was conducted in collaboration with the Wasserman lab. The vast majority of male-sterile mutations affect spermatogenesis, with defects ranging from non proliferation of germ cells to the production of defective sperm. For the subset of 83 strains, the results of complementation tests with available chromosomal deficiencies, together with reversion analyses, indicate that the P-element insertions are responsible for the observed male-sterile phenotypes. This collection of mutants therefore provides a starting point for the molecular dissection of mechanisms controlling the course of spermatogenesis.

RESULTS

We describe to varying degrees three sets of P-element induced male-sterile mutations.

We generated a first set of male-sterile mutations on the X chromosome. The mutagenesis scheme was modified from autosomal P-element insertional mutageneses (Bier et al., 1989; Wilson et al., 1989; Karpen and Spradling, 1992), in order to recover male-sterile and lethal mutations on the X chromosome (Fig. 14). We obtained 14 male-sterile and semi-sterile mutations on the X chromosome (Table 2). One mutation from this set (*roughex*^{9}) had a unique phenotype during spermatogenesis and is described in detail in Chapter 8. The others have not yet been further characterized and are not discussed in the text.

We identified a second set of 28 autosomal male-sterile mutations among single P-element insertion lines provided to us by the fly groups at UCLA, as well as among lines we had screened for lacZ expression (see Materials and Methods). We carried out a preliminary phenotypic analysis on this second set to identify those male-sterile mutations affecting the regulation of the early stages of spermatogenesis. From this initial analysis, we retained two male-sterile mutations, *chickadee*^{11} and *ms(2)916*, which are discussed in more detail in Chapter 6. We localized the P-element on the polytene map in all 28 lines, and placed them in *trans* to a cytologically defined deficiency spanning the insertion site, when available (Table 3). In most cases where a deficiency was not available, we addressed whether the resident P-element was responsible for the mutant phenotype by looking for reversion to fertility among flies whose parents had been provided with a transposase source to induce remobilization of the P-element. From the complementation data with the available deficiencies and the results from the reversion analyses, we determined that 14 male-sterile mutations from this second set were tagged

by the P-element (Table 3). Except for *ms(2)916* (see Chapter 6), the remaining 14 are no longer considered here.

An expanded collection of male-sterile mutations

The 14 tagged male-sterile mutations from the second set were merged with 69 male-sterile mutations identified by the Wasserman lab, to form the third set, a collection of 83 male-sterile mutations. Complementation tests and phenotypic analyses were performed in a collaborative effort between the two labs on this expanded collection.

We begin by presenting the results from an initial screen carried out by the Wasserman lab so that the frequencies of different classes of mutants (lethal, female-sterile, male-sterile, male- and female-sterile) could be tabulated. They screened a subset of 1919 P[Z] element insertion lines (see Materials and Methods) for male-sterility with no preselection other than for some degree of homozygous viability (Table 4A). 28 (1.5%) caused partial or complete male sterility; 4 of these 28 also affected female-fertility. An additional 48 inserts (2.5%) were strictly female-sterile. Viability was good (>70% expected) for 26 of the 28 male-sterile lines. 24 mutations affected spermatogenesis; the remaining 4 led to anatomical or behavioral defects. Of the spermatogenesis mutants, the majority affected post-meiotic differentiation; only 5 of the 24 disrupted earlier stages (Table 4B).

Since the remaining 55 male-sterile mutations (41 from the Wasserman lab and 14 from us) were identified among lines which no longer included all viable mutants generated, we did not measure the frequency of male-sterile mutations in these cases.

Mapping and complementation experiments indicated that the 83 male-sterile or semi-sterile mutations affected 63 loci on the second and third chromosomes. For about three-fourths of these mutations (61/83), we have used excision of the P-element or complementation tests with deficiencies or other mapped insertion alleles to confirm that the insertion is responsible for the mutant phenotype (Tables 5 and 6).

The mutants have been placed into seven phenotypic classes, five of which involve defects at successive stages of spermatogenesis (proliferation phase, growth phase, entry, into the meiotic divisions, meiotic divisions, post-meiotic differentiation) and two of which affect mating or sperm transfer.

In this Chapter, we chose to only briefly describe the mutant phenotypes in Table 5. Figures and additional details on the phenotypic characterization of the expanded collection can be found in the Genetics paper mentioned at the end of this Chapter.

Fig. 14 Scheme of a P-element mediated mutagenesis aimed at recovering male-sterile mutations on the X chromosome. **(a)** The yw X chromosome was isogenized prior to the mutagenesis; we used two different launching P[lacW] elements (Bier et al., 1989), located in 24A and 35C, respectively. **(b)** Δ2-3 (Robertson et al., 1988), was used as the source of transposase. **(c)** A lethal derivative of the Binsinscy balancer, termed Binsinscy-l(1)A, was generated following standard EMS mutagenesis procedures (Grigliatti, 1986). **(d)** Dysgenic male; the * indicates all the chromosomes that potentially have a novel P[lacW] insertion. About 4000 vials were set up at this stage. **(e)** Only females come out of this cross, as both $runt^{LB5}/Y$ (Gergen and Wieschaus, 1986) and Binsinscy-l(1)A males die. Whenever possible, one exceptional female with an eye color was picked per vial; such females must carry a novel insertion on one of the chromosomes marked with an *, which they inherited from their father. About 3500 independent novel events were selected in this manner (occasionally, nonCyO exceptional females were picked, provided they had a clearly different eye color from that of the launching element). **(f)** At this step, all flies were transfered to fresh vials, without examination on the pad. The males have an X chromosome which potentially carries a novel P-element insertion; if this event is lethal, no males will be present and the vial will not take; if the event is male-sterile, males will be present but fail to give rise to progeny and the vial will not take either. Thus, we were able to rapidly select candidate vials. From the candidate vials, females carrying FM7c, but not Binsinscy-l(1)A, were mated to FM7c males to establish stocks, and lethality or male-sterility was subsequently verified. Examination of the females carrying FM7c, but not Binsinscy-l(1)A, indicated the chromosome on which the novel P-element was likely located. Thus, the P was assigned to the X chromosome if all females had a similar dark eye color, to the CyO balancer if only curly females had darker eyes, and to the third (or possibly fourth) chromosome if half of the females had darker eyes, irrespective of them being curly. In some cases, the location could not be determined owing to a very pale eye color or some book keeping error/historical incident. Using an identical procedure, we determined the frequencies of events on the X chromosome among 100 random lines: 29 of them had a P-element on the X chromosome. Therefore, we estimate that about 1015 lines altogether (29% of 3500) had an insertion event on the X chromosome. We recovered about 100 lethals, a few semi-lethals, as well as 14 male-sterile and semi-sterile strains. The frequencies of these categories of mutants is comparable to those found in P-element mediated autosomal screens (see for instance Table 4). In some cases, the resident P-element was clearly not responsible for the mutation on the X chromosome, as the eye color marker segregated with an autosome (see Table 2).

Figure 14

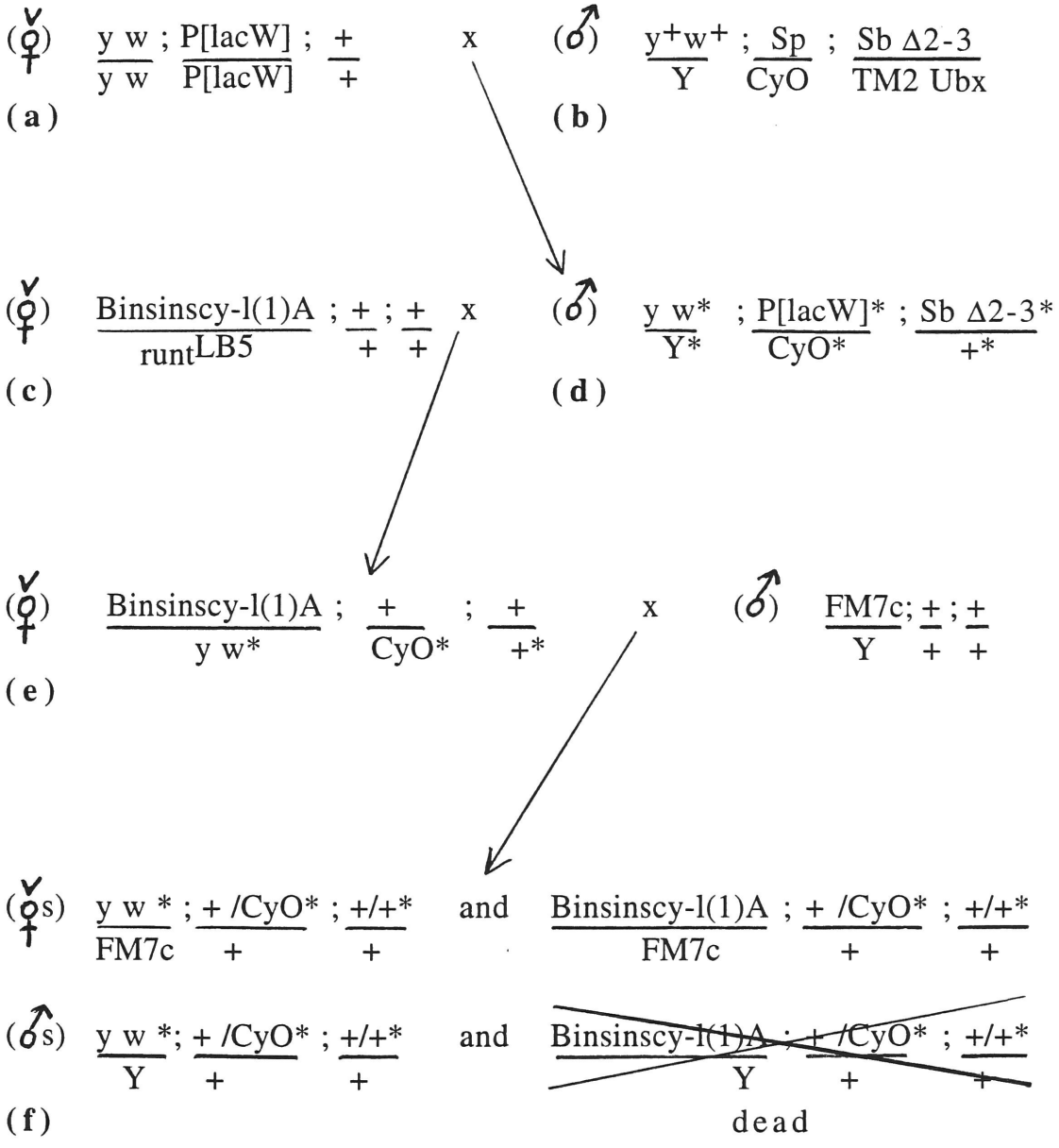

(♀) y w / y w ; P[lacW] / P[lacW] ; + / +
x
(♂) y+w+ / Y ; Sp / CyO ; Sb Δ2-3 / TM2 Ubx
(a)
(b)
(♀) Binsinscy-l(1)A / runtLB5 ; + / + ; + / +
x
(♂) y w* / Y* ; P[lacW]* / CyO* ; Sb Δ2-3* / +*
(c)
(d)
(♀) Binsinscy-l(1)A / y w* ; + / CyO* ; + / +*
x
(♂) FM7c / Y ; + / + ; + / +
(e)
(♀s) y w * / FM7c ; + /CyO* / + ; +/+* / +
and
Binsinscy-l(1)A / FM7c ; + /CyO* / + ; +/+* / +
(♂s) y w *; + /CyO* ; +/+*
Y + +
and
Binsinscy-l(1)A ; + /CyO* ; +/+*
Y + +
dead
(f)

Table 2

Identification of 14 P-element induced male-sterile mutations on the X chromosome

Isolation#/name	Phenotypic description	P-element[a]
ms(1)9C01	Meiotic division defect; spermatids contain 4 nuclei associated with a large nebenkern	X; 11C/D; tagged
ms(1)9C02	Motile sperm in seminal vesicle; semi-lethal.	X
ms(1)9C03	Semi-sterile	Not on X
ms(1)9C04	Semi-sterile	III
ms(1)9S05	Non-motile sperm in seminal vesicle	Not on X
ms(1)9C06	Some sperm in seminal vesicle	X
ms(1)9S07	Growth phase defect; testes are short and contain cyst of germ cells which degenerate prior to the completion of the growth phase	X; 13E/F; tagged
ms(1)5C47	Somewhat thin testes; spermatid nuclei fail to elongate	Not on X

ms(1)5C48	Additional M phase after meiosis II	X; 5D (*roughex*9); tagged
ms(1)SRT13	Semi-sterile; sperm in seminal vesicle	X
sl(1)5C20	Semi-lethal; thin testes; variable sizes of both nuclei and nebenkern at onion stage	X
sl(1)9S30	Semi-lethal; semi-sterile; growth phase defect: a dark dot is seen in late spermatocytes and during meiosis I	X[b]
sl(1)9S48	Semi-lethal; wing phenoyype in escapers	X[b]
sl(1)CRT7	Semi-lethal	X[b]

[a] Chromosomal location of the P-element could usually be determined when establishing the stock (see legend to Figure 14). The cytological position was only determined in three cases. Reversion analysis was also performed in those three cases by remobilizing the P-element in 10-15 individual derivatives and asking whether reversion to fertility was observed in a few derivatives; in such an event, the mutation was deemed to be tagged by the P-element.
[b] Seems to have a second P-element on an autosome.

Table 3

Identification of 28 autosomal P-element induced male-sterile mutations

Cytology	Isolation#/name	Deficiency tested[a]	U[b]	R[c]	3rd[d]
26A	*ms(2)1052/l(2)26Ab*	Df(2L)GpdhA [25D7-E1;26A8-9]	+	+	+
26A	*ms(2)1112(1)/chickadee11*	Df(2L)GpdhA [25D7-E1;26A8-9]	+	+	+
28C	*ms(2)985(2)*	Df(2L)spdX4 [27E;28C]	-	-	-
30D	*ms(2)1083(3)*	–	NA	ND	-
35B/C	*ms(2)1006*	Df(2L)osp29 [35B1-3;35E6]	-	ND	-
39A	*ms(2)913*	Df(2L)TW84 [37F5-38A1;39D3-E1]	-	ND	-
43F	*ms(2)426(2,4,5)*	Df(2R)cn9 [42E;44C]	-	+	-
43F+56F	*ms(2)776(4,6)*	–	NA	ND	-
46C	*ms(2)990(2,7)/dispersed*	Df(2R)eve1.27 [46C3-4;46C9-11]	-	+	+
46F	*ms(2)938/mulet*	–	NA	+	+
49B	*ms(2)911*	Df(2R)vg135 [48D-E;49D-E]	-	ND	-
50D	*ms(2)1059/peanuts*	–	NA	+	+
51E/F	*ms(2)916(2)*	Df(2R)XTE18 [51E3;52C9-D1]	-	-	-
53E	*ms(2)904*	–	NA	ND	-

55A	*ms(2)1058$^{(8)}$/halley*	Df(2R)Pc4 [55A;55F] Df(2R)Pc17B [54E8-F1;55B9-C1]	+ +	+	+
55C	*ms(2)109$^{(2,4,9)}$*	Df(2R)Pc4 [55A;55F]	+	+	-
56F	*ms(2)1028$^{(10)}$*	Df(2R)AA21 [56F9-17;57D11-12]	-	-	-
56F	*ms(2)356$^{(4)}$*	Df(2R)AA21 [56F9-17;57D11-12]	-	ND	-
61B	*ms(3)1024$^{(2)}$/pointless*	_	NA	+	+
64B+97D	*ms(3)993$^{(6,11,12)}$*	Df(3L)GN24 [63F4-7;64C13-15] Df(3R)Tl-P [97A;98A1-2]	+ +	ND	-
68C	*ms(3)973$^{(2)}$/pistacchio*	Df(3L)vin2 [67F2-3;68D6]	+	ND	+
83B	*ms(3)1090$^{(2)}$/gorp*	_	NA	+	+
85D	*ms(3)987$^{(2)}$*	Df(3R)by10 [85D8-12;85E7-F1]	-	ND	-
85D	*ms(3)1073$^{(11)}$*	Df(3R)by10 [85D8-12;85E7-F1]	-	ND	-
88B	*ms(3)1104/cashews*	Df(3R)red1 [88B1;88D3-4]	+	ND	+
88D	*ms(3)975/effete^{6}*	Df(3R)red1[88B1;88D3-4]	+	ND	+
	ms(3)1011/effete^{7}	Df(3R)red1 [88B1;88D3-4]	+	ND	+
97E/F	*ms(3)969/goulash*	Df(3R)Tl-P [97A;98A1-2]	+	ND	+
98D	*ms(3)1066$^{(11)}$/gruyère*	_	NA	+	+

[a] Deficiencies are referenced by Lindsley and Zimm (1992); the left and right breakpoints are indicated.

[b] Indicates whether the deficiency uncovers the P-element, as judged by the sterility, or sometimes lethality, of trans-heterozygotes.

[c] Reversion analysis performed by trying to remobilize the P-element in 10-15 individual derivatives; +=reversion to fertility was observed in a few derivatives; -=reversion was never observed; ND=not done

[d] Only male-sterile mutations that appeared to be tagged by the P-element on the basis of failure to complement with the deficiency and/or the reversion data were transfered to the third set for further analysis (see Text). Additional information about lines that were kept in the third set is found in Table 5.

(1) Female-sterile as well

(2) Semi-sterile

(3) *ms(2)1083* has a potentially interesting phenotype, since testes are typically small and contain apparently early germ cells; flies also have a bristle phenotype. *ms(2)1083* was, however, not pursued further, as the spermatogenesis phenotype was variable and dependent on genetic background; this also rendered reversion analysis impossible.

(4) Mutation was induced with the P[lacW] element by Bier et al. (1989); all other mutations listed in Table 3 were induced by the fly labs at UCLA with P[lac,ry+] (O'Kane and Gehring, 1987) (see Materials and Methods).

(5) Results from the reversion analysis and deficiency mapping are in contradiction; reversion is possibly because the phenotype, which is semi-sterile to start with, is ameliorated by the new genetic background. This line was not considered to be tagged.

(6) Two P-elements were found.

(7) The small deficiency should not have necessarely uncovered the locus; reversion analysis indicates tagging.

(8) Lethal over both deficiencies.

(9) The mutation was not sterile enough to properly interpret the results of the complementation and reversion analyses.

(10) May revert.

(11) Semi-lethal.

(12) Both deficiencies uncovered a mutation: male-sterility associated at 64B, semi-lethality at 97D; was not kept for the third set because of the two P-elements.

Table 4

Screen for male-sterile mutations among a set of random single P-element insertions on the second and third chromosomes (Wasserman lab)

	n	% Total Hits
A. Total insert lines	1919	100
Homozygous lethal insertions	262	14
Female-sterile (male-fertile)[a]	48	2.5
Male-sterile (female-fertile)[a]	24	1.3
Male- and female-sterile[a]	4	0.2
B. Phenotypes of male sterile insertions[a, b]		
	n	% Male Steriles
I. Defects in spermatogenesis		
Proliferation defect	2	7
Growth phase defect	1	4
Entry into the meiotic divisions defect	0	-
Meiotic division defect	2	7
Post-meiotic differentiation defect	19	68
	24	
II. Defects in mating		
Anatomical	1	4
Behavioral	3	11
	4	

[a] Sterile classes include a few semi-sterile lines which produce a limited number of progeny, no more than 10% the number seen with wild-type flies (see Table 5).

[b] Includes all male-sterile lines, both female-fertile and female-sterile.

Table 5

Expanded collection: loci on the second and third chromosomes mutating to male-sterility

Locus/Cytology	Allele numbers	Phenotypic description[a]
Proliferation defect [2 genes]		
chickadee/26A	*chic*^{10}, *chic*^{11}(b), *chic*^{12}, *chic*^{13}, *chic*^{14}, *chic*^{15}	Testes have reduced germinal content. Alleles *chic*^{10} and *chic*^{14} are male semi-sterile. Allele *chic*^{13} is female semi-sterile. Allele *chic*^{11} is female-sterile. In *trans* to a deficiency, inserts are male- and female-sterile and phenotypes are more severe. Locus was discovered by Schüpbach and Wieschaus (1991). Encodes profilin (Cooley et al., 1992).
diaphanous/38E	*dia*^{1}	Late-stage cysts are present at eclosion. Testes are empty in five-day old males. Insert is male-sterile and female semi-sterile in *trans* to deficiency. Ovaries contain few egg chambers. Null alleles are lethal (Castrillon and Wasserman, 1994).
Growth phase defect [5 genes]		
bocce/51D	*boc*^{1}	Nuclear size and number is variable in cysts of spermatocytes. Infrequent cysts of spermatids are aberrant. Testes are small. Females are semi-sterile; few eggs are laid. Phenotype is more severe in *trans* to deficiency: no spermatids are found in males; females lay no eggs.
cueball/62A	*cue*^{2}	Spermatocytes contain cytoplasmic abnormalities. Rare post-meiotic cysts are defective. Testis is short with defective sheath. Females are semi-sterile; ovaries are small and misshapen. Phenotypes are more severe in *trans*

		to a deficiency. Locus was discovered by P. Wilson and M. Fuller (personal communication).
fumble/77B	*fbl*^{1}, *fbl*^{2}	Testes from *fbl/fbl* males contain spermatids with large nebenkerne and micronuclei at onion stage. Testes from *fbl/Df* males contain degenerating spermatocytes. Classification is tentative. Phenotype is more severe in *trans* to deficiency and earlier stages are affected (see Appendix in Materials and Methods).
scratch/63AB	*stc*^{1}	Needle-shaped crystals accumulate throughout developing germline. Spermatids contain nuclei and nebenkerne of variable size. Phenotype resembles that of XO males.
l(2)26Ab	*l(2)26Ab*$^{4(b)}$, *l(2)26Ab*^{5}	Testes are short and filled with cysts of 16-cell spermatocytes, which degenerate prior to completion of the growth phase. Mutation is allelic to *l(2)gdh-2* (Kotarski, et al., 1983).
Entry into the meiotic divisions defect [2 genes]		
boule/66F	*bol*^{1}	Some 16-cell cysts resemble those seen in *pelota* (see below); others contain multiple nuclei in addition to the abnormally large nebenkerne.
pelota/30C	*pelo*^{1}	Meiosis does not occur, but abnormally large nebenkerne form in late spermatocytes. Mutation is cold-sensitive, female-sterile, and semi-lethal.
Meiotic division defect [3 genes]		
bobble/82D	*bob*^{1}	A few spermatids per cyst have nuclei or nebenkern of abnormal size. Mutation is semi-lethal.
doublefault/32A	*dbf*^{1}	Size varies for both nuclei and nebenkern at onion stage. Spermatid nuclei fail to change shape.

shank /82C	*shk*^{1}	Spermatids contain two or four nuclei associated with a single large nebenkern. Females are semi-sterile. Phenotype is less severe in *trans* to deficiencies.
Postmeiotic differentiation defect [46 genes]		
arrest/33CD	*aret*^{9}	Mutation is semi-lethal in *trans* to deficiency, but male-sterile phenotype is no more severe. Locus was previously identified as male- and female-sterile (Schüpbach and Wieschaus, 1991).
bellwether/ 58F	*blw*^{1}	Mutation is allelic to *ms(2)Pry58F* (Cooley et al., 1988).
betel/78D	*bet*^{1}	
blanks/36AB	*bln*^{1}	
capon/85A	*cap*^{1}	Mutation is allelic to *ms(3)Pneo85A* (Cooley et al.,1988).
cashews/88B	*cas*^{1}(b)	
dispersed/46C	*disd*^{1}(b)	Elongated spermatid nuclei are dispersed. Males are semi-sterile.
effete/88D	*eff*^{6}(b), *eff*^{7}(b,c), *eff*^{8}, *eff*^{9}(c), *eff*^{10}, *eff*^{11}, *eff*^{12}, *eff*^{13}	Mutations are allelic to *ms(3)88D* (Berg and Spradling 1991)[c].
emmenthal/56E	*emm*^{1}	Nebenkerne are vacuolated.
gelded/28D	*gel*^{1}	
gorp/83B	*gor*^{1}(b)	Males are semi-sterile.
goulash/97EF	*goul*^{1}(b)	

gruyère/98D	gru^1(b)	Nebenkerne are vacuolated. Mutation is female-sterile and semi-lethal.
halley/55A	hal^1(b)	Spermatid nuclei fail to elongate; mutation is lethal over deficiency.
hephaestus/100E	$heph^1$, $heph^2$	Tip of testis is dilated to approximately twice wild-type circumference.
jaguar/95F	jar^1, jar^2	Males are semi-sterile.
mulet/46F	mlt^1(b)	Mutation is allelic to *ms(2)ry-3* (Berg and Spradling 1991).
oxen/49C	ox^1	
peanuts/50D	pea^1(b)	
pistachio/68C	pto^1(b)	Males are semi-sterile.
purity of essence /28E	poe^1, poe^2	
Rb97D	$Rb97D^1$	Mutation is insertion in RNA binding protein gene (Karsch-Mizrachi and Haynes, 1993)
seedless/84F	sdl^1, sdl^2	
scattered/30B	$scat^1$	Elongated spermatid nuclei are dispersed.
thousand points of light /86E	tho^1	Elongated spermatid nuclei are dispersed.
trail mix/57E	tmx^1	Males are semi-sterile.
ms(2)21D		

ms(2)27C	
ms(2)27CD	Mutation is semi-lethal.
ms(2)29F	
ms(2)30C	Males are semi-sterile; elongated spermatid nuclei are dispersed.
ms(2)42A	Spermatid nuclei are not clustered.
ms(2)42D	Males are semi-sterile.
ms(2)43C	Spermatids occasionally contain variably sized nuclei and nebenkern. Mutation is semi-lethal and female semi-sterile.
ms(2)46BC	
ms(2)46C	Elongated spermatid nuclei are dispersed.
ms(3)61CD	
ms(3)65E	
ms(3)72D	Post-meiotic nuclei have sickled appearance.
ms(3)73D	
ms(3)80	
ms(3)85D	
ms(3)89B	
ms(3)90E	Mutation is semi-lethal.
ms(3)98B	Males are semi-sterile.
ms(3)100EF	Males are semi-sterile.

Behavioral Defect [2 genes]		
cuckold//28A	*cuc*^{1}, *cuc*^{2}	Males are semi-sterile due to failure to court and mate females. Longevity of both males and females is decreased.
fruitless/91B	*fru*^{3}, *fru*^{4}	Males court females and males, but fail to mate. Male-specific abdominal muscle is reduced. Locus was previously described by Gill (1963) and by Gailey and Hall (1989).
Sperm Transfer Defect [3 genes]		
ken and barbie/60A	*ken*^{1}	External genital structures are absent in some males and females. Aristae are sparse and unpigmented. Mutation is semi-lethal and both male and female semi-sterile.
pointless/61B	*ptl*^{1}(b)	Males are semi-sterile, with wild-type levels of motile sperm in seminal vesicle. Little or no sperm is transferred to females.
twig/89E	*twig*^{1}	Anal-genital plate is twisted in both sexes. Females are sterile.

[a]Mutations are male-sterile, female-fertile, and have good viability, unless otherwise noted. Phenotype of mutations over deficiency, where available, is similar unless noted. [b]Mutation was induced with the P[lac, ry+]A element. All other mutations were induced with the P[Z] element. [c]Alleles *eff*^{7} and *eff*^{9}, as well as two preexisting P-insertions in *effete*, affect viability and female fertility, but heteroallelic combinations are viable and female-fertile.

Table 6

Expanded collection: male-sterile loci by cytology

Cytology	Locus	Deficiency For Locus	Deficiency Breakpoints Left	Right	R[a]
21D	*ms(2)21D*				
26A	*chickadee*	Df(2L)GpdhA	25D7-E1	26A8-9	√
26A	*l(2)26Ab*	Df(2L)GpdhA	25D7-E1	26A8-9	√
27C	*ms(2)27C*				
27CD	*ms(2)27CD*				
28A	*cuckold*				
28D	*gelded*				√
28E	*purity of essence*				
29F	*ms(2)29F*				
30B	*scattered*	Df(2L)30A; C	30A	30C	
30C	*pelota*	Df(2L)30A; C	30A	30C	√
30C	*ms(2)30C*				
32A	*doublefault*	Df(2L)J2	31B	32A	
33CD	*arrest*	Df(2L)escP3-0	33A1-2	33E	
36AB	*blanks*	Df(2L)H20	36A8-9	36E3-4	
38E	*diaphanous*	Df(2L)TW84	37F5-38A1	39D3-E1	√
42A	*ms(2)42A*				√
42D	*ms(2)42D*	Df(2R)pk78s	42C1-7	43F5-8	
43C	*ms(2)43C*				
46AB	*ms(2)46AB*				

46C	*ms(2)46C*				
46C	*dispersed*				√
46F	*mulet*				
49C	*oxen*	Df(2R)vg-C	49B2-3	49E7-F1	
50D	*peanuts*				√
51D	*bocce*	Df(2R)JP1 (1)	51C3	52F5-9	√
55A	*halley*	Df(2R)Pc4	55A	55F	
56E	*emmenthal*	Df(2R)G100-L141 (2)	56D	56F	
57E	*trail mix*	Df(2R)PuD17	57B4	58B	
58F	*bellwether*				
60A	*ken and barbie*	Df(2R)bwS46	59D8-11	60A7	
61B	*pointless*				√
61CD	*ms(3)61CD*				
62A	*cueball*	Df(3L)Ac14-8 (3)	61C3,4	62A8	√
63AB	*scratch*				√
65E	*ms(3)65E*				
66F	*boule*	Df(3L)29A6	66F3	67B1	√
68C	*pistachio*	Df(3L)vin2	67F2-3	68D6	
72D	*ms(3)72D*				
73D	*ms(3)73D*				
77B	*fumble*	Df(3L)rdgC (4)	77A1	77D1	
78D	*betel*	Df(3L)Pc-MK	78A3	79E1-2	
80	*ms(3)80*				
82C	*shank*	Df(3R)Z-1 (5)	82A5-6	82E4	
82D	*bobble*	Df(3R)6-7 (6)	82D3-8	82F3-6	
83B	*gorp*				√

84F	*seedless*				
85A	*capon*	Df(3R)p13 (7)	84F2	85B	
85D	*ms(3)ry85D*				
86E	*thousand points of light*	Df(3R)TE32 (8)	86E2-4	87C6-7	√
88B	*cashews*	Df(3R)red1	88B1	88D3-4	
88D	*effete*				√
89B	*ms(3)89B*				
89E	*twig*	Df(3R)bxd100	89B5-6	89E2-3	
90E	*ms(3)90E*				
91B	*fruitless*	Df(3R)ChaM5	91B3	91D1	√
95F	*jaguar*	Df(3R)crbS87-5	95F7	96A17-18	√
97D	*Rb97D*	Df(3R)Tl-I	97B	97E	
97EF	*goulash*	Df(3R)Tl-P	97A	98A1,2	
98B	*ms(3)98B*				
98D	*gruyère*				√
100E	*hephaestus*				
100EF	*ms(3)100EF*				

All deficiencies are referenced in Lindsley and Zimm 1992 except as noted: (1) Saxton et al., 1991; (2) Lindsley et al., 1972; (3) J. Posakony, personal communication; (4) Steele and O'Tousa, 1990; (5) Letsou et al., 1991; (6) S. Alexander and S. Wasserman, unpublished results; (7) K. Kemphues, personal communication; (8) Gausz et al., 1991. [a] A checkmark under R (revertability) indicates a locus for which transposase-induced excision of the P-element was carried out and male-fertile revertants were obtained; absence of a checkmark merely indicates that reversion was not attempted.

DISCUSSION

From a genetic standpoint, spermatogenesis is readily tractable, since sterile mutations can be easily generated, identified, and investigated. The presence or absence of a germ line, the number and quality of mitotic and meiotic divisions, and the production of motile sperm can all be scored in the light microscope. Mutations provide the key to initiating molecular biological studies, a transition greatly facilitated by the use of mutations induced with single P-elements.

Mutation frequencies

Male-sterile mutations were identified in our screen about one-tenth as frequently as lethal mutations. Given that the number of loci in *Drosophila melanogaster* that can mutate to lethality is about half the number of polytene bands (Perrimon et al., 1989), or approximately 2500, it can be calculated that at least 250 genes should mutate to male-sterility. In fact, as discussed by others (Lindsley and Tokuyasu, 1980; Perrimon et al., 1986; Schüpbach and Wieschaus, 1989; Schüpbach and Wieschaus, 1991), the number of genes required for fertility in either sex is likely to be much greater. Germ line clones for a large fraction of zygotic lethal mutations on the X chromosome are sterile in females (Perrimon et al., 1989). Moreover, flies carrying temperature-sensitive lethal mutations often become sterile when shifted to non-permissive conditions as adults (Shellenbarger and Cross, 1979). Thus many loci required for fertility may mutate only rarely, or not at all, to give viable, sterile phenotypes. Consideration of allele frequencies indicates that our collection does not represent the majority of loci mutating to a male-sterile phenotype. Only two loci, *chickadee* and *effete*, are represented by more than two alleles; both represent previously identified hotspots for P-element insertion (Berg and Spradling, 1991; Cooley et al., 1992). Of the remaining loci, 53 out of 61 are represented by only a single allele. It is not surprising, therefore, that we did not obtain mutations in a number

of autosomal loci known to mutate to male sterility, such as *bag-of-marbles, benign gonial cell neoplasm, twine,* or *ß2-tubulin*, particularly since some loci may not be mutable with P-elements. Nevertheless, we did isolate mutations in previously characterized male-sterile loci, including *arrest*, *chickadee*, and *fruitless*. We also identified a mutation in a locus for an RNA binding protein (*Rb97D*), for which there were no known mutations (Karsch-Mizrachi and Haynes, 1993), as well as a male-sterile allele of a lethal complementation group (*l(2)26Ab*).

Classification of male-sterile mutations

The range of male-sterile phenotypes we observed is similar to that seen in screens with chemical mutagens (Lifschytz, 1987; Hackstein et al., 1990; Hackstein, 1991). As in these previous screens, mutations affecting germ line proliferation, spermatocyte growth and meiosis were relatively rare, while mutations affecting post-meiotic differentiation were the most common.

We classified male-sterile phenotypes with regard to the distinct stages that characterize spermatogenesis and grouped the mutations based on the earliest phenotypic deviation from wild-type observable in the light microscope. These classes are broad and surely encompass a number of cellular processes. Nevertheless, we believe that the current classification of mutants represents a good starting point. By focusing on loci in which mutations affect a given developmental stage, it should be feasible to dissect particular processes in spermatogenesis, despite the large number of genes required overall.

Two lines of evidence indicate that the earliest spermatogenesis phenotype detected in our mutants is a property of the locus rather than of particular mutant alleles. First, in the eight cases for which multiple alleles of a locus were isolated, all alleles belong to the same phenotypic category. Second, with one exception, when a mutation was placed in *trans* to a deficiency, the resulting defects in spermatogenesis fell into the same

phenotypic class as observed for the homozygous P-element. This was true even in those cases where the insertion was homozygous viable and female fertile, but became semi-lethal or female-sterile in *trans* to the deficiency.

Some mutations block the transition from one stage to the next. In such cases, the mutation may define a step in a dependent series along the spermatogenic pathway. For example, mutations in *l(2)26Ab* block development before the late spermatocyte stage. In the wild-type, therefore, subsequent processes depend on the execution of the step requiring *l(2)26Ab* function.

Spermatogenesis, is not, however, simply a linear series of dependent steps (discussed by Lifschytz, 1987; Fuller, 1993). For example, males lacking a Y chromosome exhibit defects in the spermatocyte growth phase, but still initiate meiosis and spermatid differentiation (Meyer, 1968). A number of mutations in our collection also affect a given stage without blocking further development. For example, the *scratch* mutation results in the formation of crystals apparent at the growth phase as well as additional defects at meiosis. These phenotypes may reflect a complete loss of function in one of two or more parallel processes in the spermatogenic pathway. It is also possible, however, that the allele is hypomorphic and that a null mutation would result in a complete developmental arrest.

Sterile mutations in essential genes

A number of our male-sterile mutations affecting early stages of spermatogenesis disrupt gamete development in both sexes (Table 5). This is not unexpected, since there are many similarities in the early steps of male and female gametogenesis and thus many opportunities for shared gene function (see Schüpbach and Wieschaus, 1991). Similarly, the fact that many of these mutations also affect viability presumably reflects the existence of functions common to cell growth or division in both the germ line and the

soma. Indeed, for females, at least, it appears that most genes which mutate to sterility are not exclusively required for gametogenesis (Perrimon et al., 1986).

Male-sterile or female-sterile alleles of essential loci offer the opportunity to study phenotypes that are frequently more readily interpretable than the lethality that characterizes null mutations. For example, in the case of the *Drosophila* homolog of the EGF receptor, a requirement for the locus in dorsoventral pattern formation was apparent from characterization of female-sterile *torpedo* alleles ($Egfr^{t}$), but not zygotic lethal *faint little ball* mutations ($Egfr^{f}$) in the same locus (Schüpbach, 1987; Price et al., 1989; Schejter and Shilo, 1989). Similarly, female-sterile alleles of *chickadee*, an essential locus (Verheyen and Cooley, 1994), revealed the participation of profilin, an actin binding protein, in the anchoring of nurse cell nuclei within the developing egg chamber (Cooley et al., 1992). Male-sterile *chickadee* alleles, which result in testes nearly devoid of germinal content, are likely to offer additional insight into profilin function (see Chapter 6).

Since P-elements frequently insert upstream of coding sequences in the 5' end of a gene, they may be particularly effective at inducing tissue or sex-specific alleles of essential loci. For example, many P-element insertions in *chickadee* that are viable but sterile lie within an alternative 5' exon (Cooley et al., 1992).

Proliferation phase

Mutations which disrupt male germ line proliferation provide an opportunity to dissect processes of inherent interest in spermatogenesis: the control of stem cell fate, the synchronization of germ line and somatic stem cell divisions, the counting of mitoses and entry into meiosis.

Germ line proliferation is affected by mutations in two of our male-sterile loci, *chickadee* and *diaphanous*. The phenotypes of these mutations suggest that these loci act prior to the growth phase. For *chic*, a proliferation defect has been demonstrated directly

by an examination of BrdU labeling in mutant and wild-type testes (see Chapter 6). For *dia*, null alleles are lethal at the pupal stage and produce aberrant mitotic figures in larval brains (Castrillon and Wasserman, 1994). Since P-element mutations in either gene lead to a loss of germ line cells in the testis, *chic* and *dia* are complementary in phenotype to *bgcn* and *bam,* mutations that cause an overproliferation of germ line cells (Gateff, 1982; McKearin and Spradling, 1990).

Entry into the meiotic divisions

Male-sterile mutations defective for entry into the meiotic divisions are also of experimental interest. Whereas the regulation of the meiotic cell cycle has been studied extensively in yeast (reviewed by Mitchell, 1988; Malone, 1990), much less is known about this process in multicellular organisms. Although gametogenesis in flies has been studied in females more than in males, spermatogenesis offers a significant advantage for the study of meiosis. The two meiotic divisions in spermatocytes occur in rapid succession in a discrete portion of the adult testis. In contrast, meiosis in females begins in the nuclei of pro-oocytes but is not completed until after fertilization, at least three days later.

The cysts found in testes from *pelota* males undergo incomplete chromosome condensation and thus have arrested at the earliest steps of meiosis I (Eberhardt and Wasserman, in preparation). Nevertheless, in *pelota* males, cells from these arrested cysts form a nebenkern, a mitochondrial form normally found only in post-meiotic spermatids. This uncoupling of meiosis and post-meiotic differentiation has also been observed for *twine*, as well as several other male-sterile mutations (Lifschytz and Hareven, 1977; Regan and Fuller, 1990; Alphey et al., 1992; Courtot et al., 1992).

Some mutations that we have placed into the growth phase class may also define genes required for entry into the meiotic divisions. For example, in *l(2)26Ab* mutant testes, maturing spermatocytes degenerate and no meiotic products are observed. The

possibility exists that germ cell degeneration is a secondary consequence of an alteration in cell fate due to the absence of a signal required for progress through meiosis.

Towards a molecular genetic analysis of spermatogenesis

We have reported the initial phenotypic characterization of a collection of 83 recessive autosomal male-sterile mutations, generated by single P-element mutagenesis. It is now possible to focus on select mutants that affect a given process during spermatogenesis, to identify the genes controling that aspect of the developmental program. This collection of male-sterile mutants thus provides the basis for a molecular genetic dissection of spermatogenesis.

Note: Part of this Chapter has been published in:
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CHAPTER 6: *chickadee* AND *ms(2)916* REGULATE GERM CELL PROLIFERATION

INTRODUCTION

Lineages in which differentiated cells are unable to divide and have a limited life span usually rely on the activity of stem cells for their renewal (reviewed by Hall and Watt, 1989; Evans and Potten, 1991). The proliferation of stem cells and their mitotically amplifying progeny must be carefully regulated to maintain an adequate number of differentiated cells. For instance, a normally quiescent population of myoblast precursors multiplies in response to tissue damage in verterbrate muscle fibers (reviewed by Rosenthal, 1989). The regulation of stem and progeny cell proliferation is likely achieved by the transduction of intrinsic and extrinsic signals to the basic components of the cell cycle machinery. A genetic analysis of these processes is thus expected to identify both specific signals that instruct the rate of proliferation, as well as more generally required cellular components which allow the execution of a given division rate.

Very little is known about the genes controling the proliferation of germ line stem cells and their mitotically amplifying progeny during *D. melanogaster* gametogenesis. In both females and males, the gonial daughter of the stem cell undergoes 4 mitotic divisions characterized by incomplete cytokinesis, and the 16 resulting germ cells are interconnected by intercellular bridges (reviewed by Fuller, 1993; Spradling, 1993). During each of the 4 divisions, residues from the mitotic spindle aggregate into an organelle called the fusome (Giardina, 1901; Maziarski, 1913), which links the 16 germ cells (Telfer, 1975; Lin et al., 1994).

Interestingly, while there are always 4 mitotic divisions during oogenesis in all

Drosophila species examined, this number is species-specific during spermatogenesis, being 4 in *D. melanogaster*, but 3 in *D. hydei* and 5 in *D. pseudoobscura* (Liebrich, 1984). In *D. melanogaster*, there is no need to undergo all 4 divisions to allow progress through subsequent stages of spermatogenesis. Indeed, germ cells carrying a temperature-sensitive mutation in a given essential locus undergo only 3 or 2 mitotic divisions at the semi-permissive temperature, but are still able to differentiate into post-meiotic spermatids (Lifschytz, 1978). It is not established, however, whether germ cells undergoing more than the usual 4 divisions would similarly progress through subsequent stages of the developmental program.

Mutations that affect the proliferation of germ cells are rarely recovered. For instance, less than 3% of male-sterile lines surveyed in an early study appeared to affect this step of spermatogenesis (Lifschytz, 1987). These lines were not analyzed in any detail and are no longer available. We have similarly found that only 3 of 111 P-element induced male-sterile mutations interfere with the proliferation of germ cells (see Chapter 5). Mutations affecting this step of spermatogenesis can be placed into two broad categories depending on whether they result in an underproliferation or overproliferation of germ cells.

In addition to loci required for the determination of the germ line in the embryo, an underproliferation mutation may identify at least three classes of loci regulating different aspects of stem cell biology. First, one class of genes could be required for the specification of stem cells. In mutants, pole cells would form and incorporate into the gonads, but never become functional stem cells. Second, some loci could be required for the maintenance of stem cell fate. In mutants of this class, stem cells would be initially present, but would undergo a symmetric division, such that both daughters embark on the differentiation program, thus depleting the testis of stem cells. A third class of genes could be required for the proper division of stem cells. In mutant testes, stem cells would

be present, but quiescent. In addition, an underproliferation phenotype may identify loci required for the survival or the amplification of the mitotically dividing germ cells.

Loci necessary for germ line proliferation have been characterized in *C. elegans* (reviewed in Clifford et al., 1994). *glp-1* is required autonomously in germ cells for their continued mitotic proliferation (Austin and Kimble, 1987). glp-1 likely receives a mitogenic signal sent by the neighbouring somatic distal tip cell (Kimble and White, 1981; Austin and Kimble, 1987). Similarly to *glp-1* mutants, *glp-4* mutants have only about 12 germ cells in adult gonads, instead of the usual 1000 (Beanan and Strome, 1992). In addition to *glp-1* and *glp-4*, a set of loci is required in the mother for proper germ line development in the progeny (Capowski et al., 1991). One of them, *mes-1*, is characterized by an abnormal division pattern in the early embryo, which results in larvae lacking the precursors of the germ line (Strome et al., 1994). *mes-1* is thus required for the determination of the germ line in the embryo, and not for a later step of germ cell development. The other *mes* genes, however, as well as *glp-4*, are required for post-embyonic proliferation of germ cells (Capowski et al., 1991; Strome et al., 1994).

As discussed in Chapter 4, mutants with the opposite phenotype, that of an overproliferation of germ cells, may identify loci required for the gonial daughter to adopt a fate different from its parental stem cell, for the restriction of the number of mitoses, or for entry into meiosis. *bam* and *bgcn* are *Drosophila* overproliferation mutants affecting both sexes (Gateff, 1982; McKearin and Spradling, 1990), and mutant germ cells appear to be blocked at an extremely early stage of gametogenesis (Bopp et al., 1993; Lin et al., 1994; McKearin and Christerson, 1994; see also Chapter 4). A conditional lethal mutation, *l(1)55*, has been reported to also have an overproliferation phenotype during spermatogenesis, but it is not clear whether a potential phenotype during oogenesis has been investigated (Lifschytz, 1978).

Mutants with overproliferating germ cells have also been characterized in *C. elegans*, as well as mammals. In worms, mutations in *gld-1* result in the overproliferation

of female germ cells only (Francis et al., 1995a,b). Direct observation of DNA condensation has revealed that *gld-1* mutant germ cells enter meiotic prophase before returning to indefinite mitotic proliferation (Francis et al., 1995a). Thus, *gld-1* is required for progress through an early stage of female meiosis. The *gld-1* phenotype is reminiscent of the capacity of yeast cells in the early stages of meiosis to resume the mitotic cycle if transfered to growth medium (reviewed by Esposito and Klapholz, 1981). Certain ovarian teratomas in mouse (Eppig et al., 1977) and human (Surti et al., 1990) may arise from germ cells similarly returning to a mitotic division program after having progressed through parts of the meiotic cell cycle. Other ovarian and testicular tumors probably arise as germ line stem cells or their mitotically amplifying progeny overproliferate before entry into the meiotic cell cycle (Mostofi and Sesterhenn, 1985).

To gain insight into the regulation of germ cell proliferation during *Drosophila* spermatogenesis, we focused on a subset of mutations derived from our collection of P-element induced male-sterile mutations. We first report that mutations affecting germ cell proliferation in either direction invariably result in testes that are smaller than wild-type. We then show that mutations in *chickadee*, which encodes *Drosophila* profilin, disrupt the proliferation of germ cells and alter the fate of neighbouring somatic cells. We also characterize a novel male-sterile mutation, *ms(2)916*, that results in an overproliferation of germ cells during spermatogenesis, but not during oogenesis. Finally, we show that germ cells undergoing 5 mitotic divisions instead of the usual 4 are still capable of differentiating into post-meiotic spermatids.

RESULTS

Mutants affecting the proliferation of germ cells have short testes

Only 3 of 111 P-element induced male-sterile mutations reported in Chapter 5 appeared to affect germ cell proliferation. Examination of the testis size and shape in these three mutants, *diaphanous*^{1} (*dia*^{1}), *chickadee*^{11} (*chic*^{11}) and *ms(2)916*, revealed that proliferation defects correlated with short and sometimes thin testes. This correlation is probably a consequence of the under-representation or absence of subsequent stages of spermatogenesis.

The phenotype of males mutant for *dia*^{1} was unusual in exhibiting a dramatic dependence on the age of the fly. At eclosion, *dia*^{1} testes were somewhat thinner and shorter than wild-type (Fig. 15B, compare with 15A). As a *dia*^{1} male aged, the early stages of spermatogenesis were not replenished, resulting in very thin testes, devoid of germinal contents (Fig. 15C). The *dia*^{1} mutation is thus likely to affect a process required for the continued proliferation of germ cells (see Discussion).

Alterations in testis morphology were also apparent in males homozygous for mutations in the *chic* locus. *chic*^{11} testes were reduced in size to a variable extent; an example of a mildly affected testis is shown in Figure 15D. The phenotype was more severe in stronger *chic* alleles, such as *chic*^{37}, in which the testis was thinner and smaller (Fig. 15E). The phenotype became worse over a deficiency, as *chic*^{11}*/Df* testes were even shorter (Fig. 15F). They thus resembled the agametic testes of the progeny of *osk*^{301} mothers (Fig. 15G).

Males mutant for *bam* also had smaller testes, which contain overproliferating germ cells (Fig. 15H, see Chapter 4). The small testis size in this case is likely due to the observed degeneration of overproliferating germ cells after a few extra divisions, and to the absence of subsequent stages of spermatogenesis. Males homozygote for the mutation *ms(2)916*, which also causes overproliferation of germ cells (see below), had smaller

testes as well; the size was somewhat variable in this case, and a typical testis is shown in Fig. 15I. Whereas some *ms(2)916* germ cells still progressed through spermatogenesis at 25 °C, this is was not the case at 18 °C, and the resulting testis was typically smaller (Fig. 15J).

Comparison of the testis phenotypes of *osk*, *chic*, *bam* and *dia* illustrates a relationship between the size of the testis and the extent of germ line division and growth. When the germ line is absent *ab initio*, as in the progeny of *osk* mothers, the testis is severely stunted (Fig. 15G). When germ line stem cells are present but do not proliferate appropriately, as in the case of $chic^{37}$ (see below), the testis is somewhat larger (compare Fig. 15G with 15E). A similar testis size is observed when germ cells initially overproliferate, but then degenerate, as in the case of *bam* (Fig. 15H). Lastly, when germ cells are present in young adults but are not replenished, as in *dia* males at day 5, the testis is somewhat larger and very thin (Fig. 15C).

***chickadee* is required for germ cell proliferation**

Although the smaller testis size and reduced germinal content in *chic* mutants was suggestive of a defect in germ line proliferation, the possibility remained that proliferation was unaffected, but that germ cells instead rapidly degenerated in *chic* mutant testes.

We performed BrdU incorporation experiments to directly assay the extent of germ cell proliferation in *chic* mutant testes. While in the wild-type, many germ cells underwent S phase during a 30 minute labeling period (Fig. 16A), most $chic^{11}$ testes only had a few cells in S phase during the same period (Fig 16C, arrow).

In order to quantify this proliferation defect, we counted the number of cysts of 16 germ cells that were in S phase during the BrdU labeling period in $chic^{11}$ homozygotes and $chic^{11}$ heterozygote control testes. The result of one such experiment is summarized in Fig. 16B and 16D. Whereas 66 % (20/30) of wild-type testes had one or more cysts of

16 germ cells in S phase during the labeling period, this was the case in only 17 % (4/20) of *chic*^{11} testes. Similar numbers were found by counting the number of cysts with 8 labeled germ cells (data not shown). Counts on cysts containing 4 or 2 labeled germ cells were not performed, as such cysts are close to the hub, in an area densely packed with cells in S phase, which makes it difficult to assign particular labeled nuclei to a given cyst.

An even more dramatic reduction in germ cell proliferation was observed in males homozygotes for the more severe *chic*^{37} allele, as virtually no cyst of 16 or 8 germ cells were found in S phase (data not shown). A proliferation defect for *chic*^{37} during oogenesis and spermatogenesis has been suggested by others (Verheyen and Cooley, 1994).

Our data demonstrate that *chic* is required for the proliferation of germ cells during spermatogenesis. However, they do not ascertain whether the proliferation defect occurs in stem cells or in the mitotically amplifying progeny, although the deficit in labeled cysts of 8 germ cells is suggestive of a prior defect.

Germ line stem cells are present and altered in a severe *chickadee* allele

We next wanted to examine the fate of germ line stem cells in *chic* mutant testes. We could not use marker lines in *chic*^{11} flies, as they themselves carry a P-element driving ubiquitous lacZ expression in the early stages of spermatogenesis. Instead, we turned to the more severe *chic*^{37} allele. A marker line revealed that germ line stem cells were usually present (8/12 in one experiment) in *chic*^{37} testes (Fig. 16F, arrow). However, mutant stem cells typically expressed lacZ at much lower levels than in wild-type (compare Fig. 16E and 16F, arrows). Even though we need to extend this observation to other stem cell markers (but see M5-4 below), we infer that stem cells are present in most *chic*^{37} testes. Thus, *chic* does not appear to be required for the specification of stem cells. However, *chic*^{37} mutant stem cells fail to fully express at least

one stem cell marker, and are probably not dividing properly, as revealed by the lack of germ cell proliferation.

Fasciclin III expression is altered in *chickadee* mutant testes

As *chic*^{37} mutant testes appear to have non-functional stem cells, we investigated whether this correlated with cell fate changes in the neighbouring somatic cells, as we observed in agametic testes devoid of germ cells (see Chapter 3).

By using a marker line labeling the cells of the hub (Fig. 17A, arrowhead), as well as germ line stem cells (Fig. 17A, arrow), we first found that the hub in *chic*^{37} mutant testes was typically larger than in the wild-type (Fig. 17B, arrowhead, compare with Fig. 17A, arrowhead). Some cells occasionally found near the hub expressed lacZ at a very low level (data not shown). These cells are probably germ line stem cells which express the marker at lower than wild-type levels, as had been observed with the stem cell specific marker. Interestingly, the hub was variable in size (Fig. 17B shows an average one) and shape, and was not located at the very apex of *chic*^{37} mutant testes, reminiscent of what was observed in agametic testes.

We have shown that fasciclin III expression is often altered in two ways in agametic testes: while expression is not properly maintained in the hub, fasciclin III is also ectopically expressed in cyst cells. We wanted to find out whether fasciclin III expression was similarly altered in *chic* mutant testes. Just like in the wild-type (Fig. 17C, arrow), fasciclin III was strongly expressed in the hub of most *chic*^{11} testes (data not shown). However, occasional *chic*^{11} testes had weaker fasciclin III expression in the hub (data not shown), while others displayed expanded fasciclin III expression beneath the hub (Fig. 17D, arrow). The severity of these alterations increased with stronger *chic* allelic combinations. Thus, *chic*^{37} mutant testes had no or reduced expression in the hub (Fig. 17E, arrowhead), and over half of them (see below) had a dramatic expansion of fasciclin III expression beneath the hub (Fig. 17E, arrow). Similar alterations in fasciclin

III expression were observed when *chic*^{37} was put in trans to a deficiency (Fig. 17F, arrow).

We wanted to ascertain that expanded fasciclin III expression in *chic*^{37} testes occured outside of the hub domain. To this end, we followed the fate of the cells of the hub along with fasciclin III expression in a double-labeling experiment, in which *chic*^{37} mutant testes (n=15) fell into three classes. First, 5 mutant testes had no fasciclin III expression at the tip, and 2 of those were also negative for the hub marker (data not shown). Second, 2 mutant testes, which were the less severely atrophied from the set, had weak fasciclin III expression in the hub (data not shown). Finally, 8 mutant testes had a dramatic expansion of fasciclin III expression beneath the hub. In 3 of these 8 cases, there was still some weak fasciclin III expression in the hub per se (just like in the testis shown in Fig. 17E). In the remaining 5, fasciclin III protein could no longer be detected in the hub (Fig. 17G and 17H, arrowheads), while it was ectopically expressed by neighbouring cells (Fig. 17G and 17H, arrows). Thus, just like for the agametic testis, cells beneath the hub, most likely cyst cells, often ectopically express fasciclin III in *chic* mutant testes.

chickadee^{11} males lack profilin in germ cells

chickadee encodes *Drosophila* profilin (Cooley et al., 1992), a cytoplasmic actin binding protein which regulates actin filament polymerization (reviewed by Machesky and Pollard, 1993; Theriot and Mitchison, 1993).

Two alternative promoters regulate *chic* transcription during oogenesis. Whereas one promoter directs chic mRNA expression in germ cells, the second drives expression in somatic cells. *chic* female-sterile mutants do not express the transcript specific to germ cells and have no detectable chic protein in nurse cells (Cooley et al., 1992; Verheyen and Cooley, 1994).

chic^{11} females are sterile as well (see Table 4), and the P-element in *chic*^{11} inserted into a small restriction fragment which always contains the P-element in

mutations affecting the transcript that is specific to germ cells in females (Cooley et al., 1992; E. Verheyen and P.G. unpublished observations).

We compared the distribution of chic protein in wild-type and *chic*^{11} mutant testes, in order to address whether the *chic* spermatogenesis phenotype is similarly correlated with a deficit of profilin expression in the germ line. chic protein was detected in all cell types and stages during wild-type spermatogenesis (data not shown). In the wild-type germ line, the cytoplasmic localization of profilin was best observed in cells in the growth phase (Fig. 18A, arrow). In contrast, we failed to detect chic protein in the germ cells of *chic*^{11} testes (Fig. 18B, arrow), while expression in cyst cells was unaffected (Fig. 18B, arrowhead). Thus, a male-sterile *chic* mutation correlates with a lack of profilin expression in germ cells. This suggests that *chic* is autonoumously required for the proper proliferation of germ cells, although we have not yet performed mosaic analyses to address this directly.

chickadee^{11} germ cells have no obvious defect in actin filaments

A defect in the polymerization of actin filaments was readily apparent in the cytoplasm of nurse cells in *chic* mutant egg chambers (Cooley et al., 1992).

In contrast, the distribution of actin filaments in *chic*^{11} mutant testes was indistinguishable from wild-type. In wild-type testes, actin filaments were abundant in the cells of the hub (Fig. 18C, 18D and 18E, arrowheads), and were present at much lower levels at the cortex of germ line stem cells (Fig. 18C and 18D, arrows). However, the most conspicuous actin filaments were found in more mature germ cells, within a branched structure apparently connecting germ cells within a cyst (Fig. 18F). Owing to its unique architecture, this structure most likely corresponds to the fusome. The presence of filamentous actin in the fusome was unexpected, as it has not been detected in the analogous structure in ovaries (Warn et al., 1985). Thus, although structurally similar, the fusome in females and males is made up of different proteins. In *chic*^{11} mutant testes,

the hub and stem cells appeared as in the wild-type (data not shown), as did the fusome in germ cells in the growth phase (Fig. 18H). While more subtle differences may have gone unoticed, germ cells in *chic*^{11} mutant testes do not have an obvious defect in the distribution of actin filaments.

_ms(2)916_ restricts the proliferation of germ cells during spermatogenesis

ms(2)916 homozygous males usually had somewhat thin and short testes (Fig. 15I). Interestingly, they contained little balls visible at the dissecting microscope (Fig. 15I, arrow), which were reminiscent of cysts of dying germ cells following overproliferation in *bam* and *bgcn* mutant testes (Fig. 15H, arrow).

Live preparations of germ cells confirmed that *ms(2)916* mutant testes contained cysts with more than 16 germ cells (Fig. 19B), which were never observed in the wild-type (Fig. 19A). While some *ms(2)916* cysts contained 64 or more small germ cells (19B, arrow), other mutant cysts contained 32 larger germ cells with a normal growth phase morphology (Fig. 19B, arrowhead). Thus, there is variability in the extent of overproliferation, and *ms(2)916* germ cells that have undergone only one extra round of mitotic division appear to progress through the growth phase. In addition, *ms(2)916* testes also contained regular cysts of 16 germ cells in the growth phase (Fig. 19B, thin arrowhead). These normal-looking germ cells probably execute the meiotic divisions and undergo post-meiotic differentiation, as sperm tails were often observed in *ms(2)916* testes (Fig. 15I, arrowhead), and mutant males were only semi-sterile (Table 3).

We next examined the BrdU incorporation pattern in *ms(2)916* testes to analyze the proliferative behavior of mutant germ cells. Similarly to *bam* and *bgcn*, *ms(2)916* testes contained cysts of more than 16 germ cells undergoing S phase in synchrony (Fig. 19D, arrows), in an area devoid of proliferation in the wild-type (Fig. 19C).

In performing these experiment, we also noticed that proliferating mutant germ cells often appeared to emanate from a region slightly away from the very tip of the testis.

This led us to test whether the hub of somatic cells was correctly positioned in *ms(2)916* testes. Staining with an anti-fasciclin III antibody demonstrated that, whereas the hub was always located at the very tip of the wild-type testis (Fig. 19E, arrow), it was often displaced in *ms(2)916* testes (Fig. 19F, arrow). In one experiment, the hub was located at the correct, apical-most position, in only 6/21 testes. The hub was displaced from the tip less than 50 μm in 4/20 testes, betwen 50 and 100 μm in 6/20 cases, and over 100 μm in 5/21 testes. A mutant testis with a hub displaced just over 100 μm from the tip is shown in Fig. 19F.

The *ms(2)916* overproliferation phenotype is variable, but reminiscent of that observed in *bam* and *bgcn* mutant testes. However, in contrast to those mutations, *ms(2)916* does not affect oogenesis, as no overproliferating germ cells were detected in ovaries and females were fully fertile. Thus, *ms(2)916* is a unique locus required for restricting the proliferation of germ cells during spermatogenesis.

Germ cells undergoing five mitotic divisions complete spermatogenesis

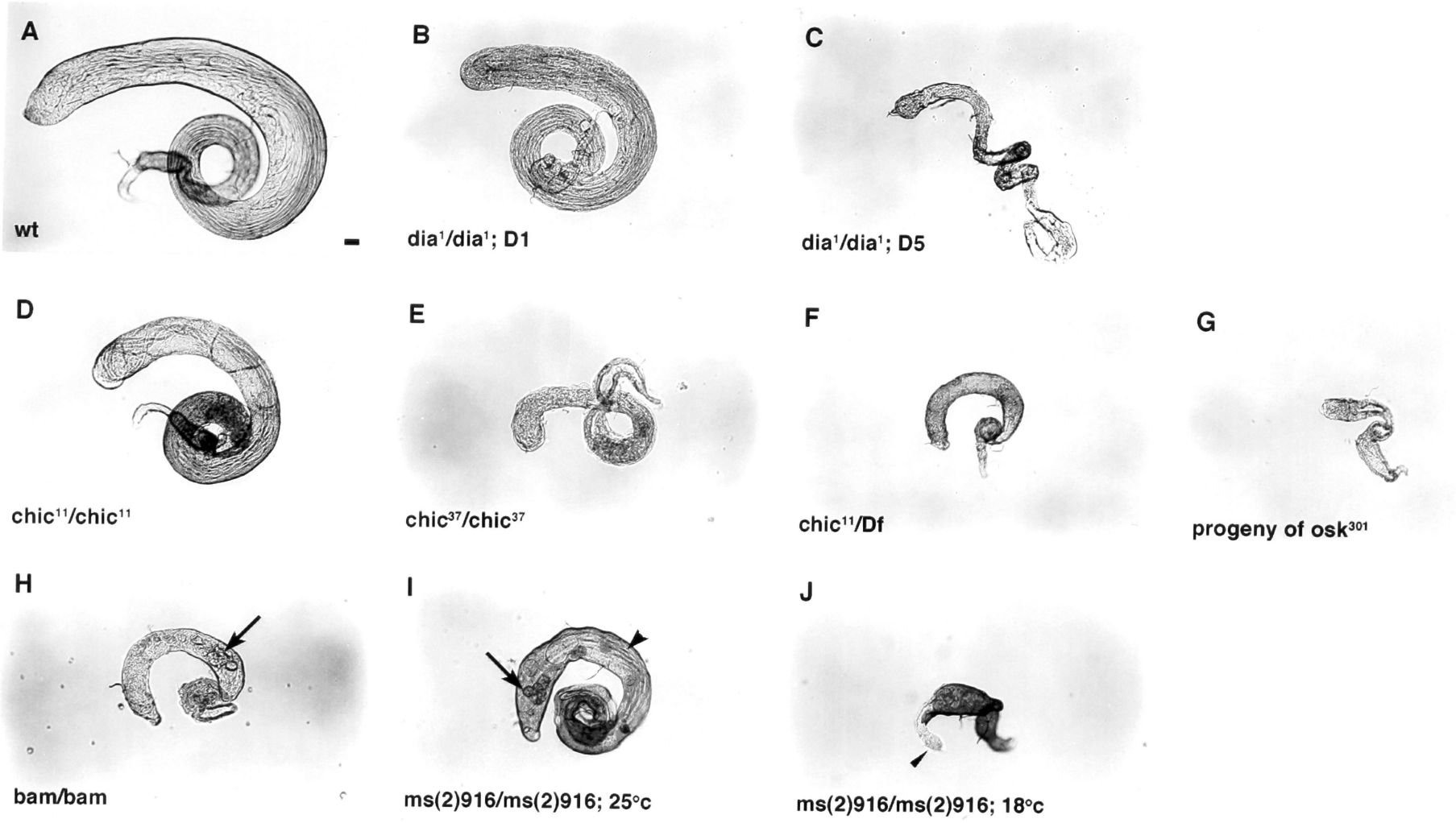
In the process of analyzing various *roughex* (*rux*) alleles (see Chapter 8), we noticed that a weak and male-fertile allele, rux^2, also occasionally had a cyst in the growth phase that contained 32 germ cells, instead of the usual 16 (Fig. 20A). The 32 germ cells had the same size and appearance as wild-type ones, and therefore likely resulted from an additional complete cell cycle after the usual 4, rather than from just an additional M phase without accompanying S phase.

This occasional fifth mitotic cell cycle probably does not reflect a role for *rux* in restricting the number of divisions to 4, since severe or null *rux* alleles don't display this phenotype (data not shown). Nevertheless, this unusual allele gave us the opportunity to test whether having an additional mitotic cell cycle interferes with progress through the remainder of spermatogenesis. As rux^2 males contained a majority of cysts with 16 germ cells and were fertile, we could not assay whether cysts with 32 germ cells differentiated

into fertile spermatozoae. Instead, we cut cross-sections through *rux*^{2} testes and examined advanced stages of post-meiotic differentiation by electron microscopy (Fig. 20B). Along with cysts that contained the regular number of 64 sperm tails (Fig. 20B, cyst 1), we occasionaly found cysts that, instead, contained 128 sperm tails (Fig. 20B, cyst 2), which were identical to those seen in the regular cysts. Therefore, cysts of germ cells undergoing 5 mitotic divisions, instead of the usual 4, nevertheless progress through the growth phase, undergo the meiotic divisions and reach the last stages of post-meiotic differentiation in a manner indistinguishable from wild-type.

Fig. 15 Germ cell proliferation mutants have altered testis morphology. Testes are from newly eclosed males, except for panel C. Testes were dissected and placed in a drop of Ringer's on a slide for immediate viewing, without fixation. **(A)** *dia1/+* testis exhibiting wild-type morphology. Proliferating germ cells are present at the testis tip, while growing spermatocytes are found along the inside wall of the testis through the first coil. Elongated sperm tails can be seen extending from the base to almost the tip of the testis. **(B)** *dia1/dia* testis, somewhat smaller than wild-type, yet with some differentiating cysts, as evidenced by the presence of sperm tails **(C)** *dia1/dia1* testis, 5-day old male. Testis is very thin and appears devoid of germinal contents. **(D)** $chic^{11}/chic^{11}$ testis, somewhat smaller than wild-type, but still exhibiting some elongated sperm tails **(E)** $chic^{37}/chic^{37}$ testis, smaller and thinner than $chic^{11}/chic^{11}$ testis. Some short sperm tails are visible. **(F)** $chic^{11}$/Df(2)GpdhA testis, shorter still, with severely reduced germinal contents. **(G)**. Testis from progeny of osk^{301}/osk^{301} mother. This agametic testis is severely stunted. **(H)** $bam^{\Delta 86}/bam^{\Delta 86}$ testis. Although germ cells overproliferate in *bam* mutant testes, the testis is short, probably as a result of the degeneration of germ cells after a few rounds of extra divisions. Cysts of dying germ cells are visible as little refractile balls (arrow). **(I)** *ms(2)916/ms(2)916* testis; flies grown at 25 ^{o}C. Testis is somewhat thinner and shorter than wild-type. There is variability in the size of the testis; an average testis is shown. Cysts of dying germ cells are visible (arrow). Not all germ cells overproliferate, and some progress through post-meiotic stages, as evidenced by the presence of sperm tails (arrowhead). **(J)** *ms(2)916/ms(2)916* testis; flies grown at 18 ^{o}C. Although there is variability in the size at 18 ^{o}C as well, the testis is typically smaller than at 25 ^{o}C; post-meiotic stages and sperm tails are no longer observed. Note that the sheath of the testis is not covering the apical region of the testis (thin arrowhead), a phenotype often observed in *ms(2)916/ms(2)916* mutant testes at both temperatures, and first noticed by Erika Matunis in the lab. Bar=50 μm; all panels are at the same magnification.

Figure 15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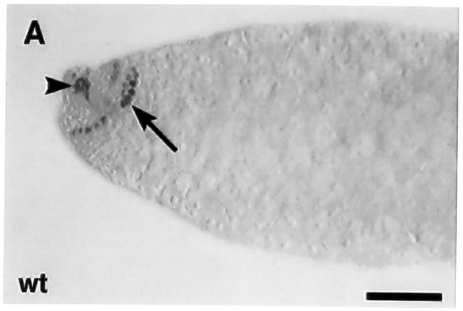
G
progeny of osk301
H
bam/bam
I
ms(2)916/ms(2)916; 25°c
J
ms(2)916/ms(2)916; 18°c

Fig. 16 Mutations in *chickadee* prevent germ cell proliferation. **(A, C)** 30 minute BrdU incorporation experiments. **(A)** Apical region of a wild-type testis; immunoperoxidase reaction with anti-BrdU and anti-fasciclin III antibodies. Some nuclei in the vicinity of the hub (arrowhead) have incorporated BrdU, while germ cells at the 4-, 8- and 16- (arrow) cell stage have undergone S phase as cysts move away from the hub **(C)** $chic^{11}/chic^{11}$ testis; immunoperoxidase reaction with anti-BrdU antibody. BrdU incorporation is only detected in two nuclei (arrow); no cysts containing several labeled germ cells is seen. Other $chic^{11}/chic^{11}$ testes had some germ cells in S phase (not shown, but see panel D). **(B, D)** Occurence of cysts of 16 labeled germ cells during a 30 min BrdU incorporation period in $chic^{11}/+$ control testes (n=30) and $chic^{11}/chic^{11}$ mutant testes (n=20). Cysts containing 16 or 8 labeled germ cells were counted at the compound microscope with a 40x lens under bright-field optics; each testis had 0, 1, 2 or 3 (X axis) cysts of 16 labeled germ cells; percentage of testes falling within each of these classes is given (Y axis). The proliferation deficit in $chic^{11}/chic^{11}$ is apparent by the shift of the distribution towards the left. Similar distributions were obtained by plotting the occurence of cysts with 8 labeled germ cells (not shown). We noticed some variability in the extent of S phases even among wild-type flies taken on different days or from different bottles. Thus, when assesing the extent of germ cell proliferation in a given mutant, it is important to compare the numbers with those obtained from control males from the same bottle and processed at the same time. **(E, F)** A marker line (S3-40) reveals the presence of stem cells in $chic^{37}/chic^{37}$ testes; immunoperoxidase staining with anti-β–galactosidase antibody. While wild-type stem cells express β–galactosidase (E, arrow), $chic^{37}/chic^{37}$ stem cells do so only at extremely low levels (F, arrow); note how stem cells are arranged in a rosette around the hub in the wild-type, while they are clustered on the side in the mutant. Bars=50 μm; all panels are at the same magnification.

Figure 16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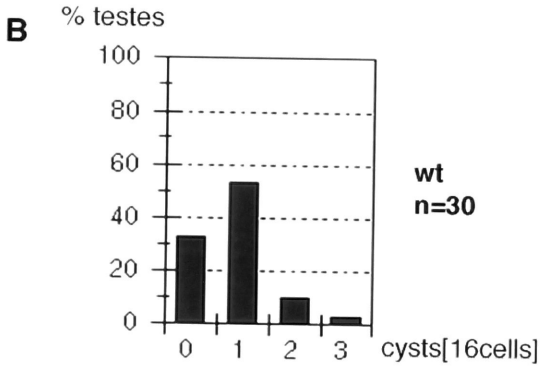
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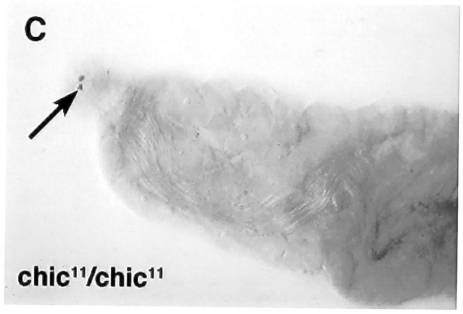
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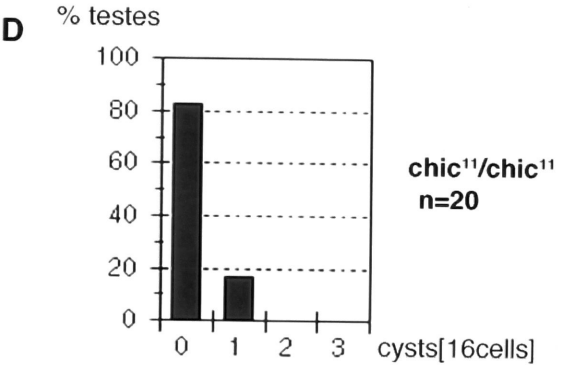
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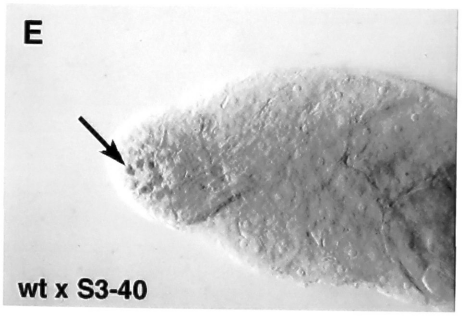
chic11/chic11
n=20


E
wt x S3-40

F
chic11/chic11 x S3-40

Fig.17 Somatic cell fates are altered in *chickadee* mutant testes. **(A, B)** Immunoperoxidase staining with anti-β–galactosidase antibody. A marker line (M5-4/CyO) expressed in the wild-type hub (A, arrowhead) and very early germ cells (A, arrow) demonstrates the presence of an enlarged hub in $chic^{37}/chic^{37}$ testes (B, arrowhead); note that no germ cells appear to express lacZ in this particular mutant testis, while they did so weakly in others. **(C, D, E, F)** Fasciclin III expression is altered in *chic* mutant testes; immunoperoxidase staining with anti-fasciclin III antibody. **(C)** Apical region of wild-type testis. Fasciclin III is expressed in the cells of the hub (arrow) **(D)** Apical region of $chic^{11}/chic^{11}$ testis. Fasciclin III expression expands in a small region beneath the hub (arrow); the hub does not appear to be stained in this particular testis. This is an unsual $chic^{11}/chic^{11}$ testis, as the majority had normal fasciclin III expression in the hub, while a few had lower levels of expression (not shown) **(E)** Apical region of a $chic^{37}/chic^{37}$ testis. Fasciclin III expression expands dramatically into a domain of cells (arrow) below the hub; note that there is still weak fasciclin III expression in the hub in this particular testis (arrowhead). Other $chic^{37}/chic^{37}$ testes failed to express fasciclin III in the apical region all together (not shown). **(F)** Apical region of a $chic^{37}$/Df(2L)Gpdh(A) testis. Just like for $chic^{37}/chic^{37}$, fasciclin III expression expands into a domain of cells (arrow) below the hub. Other $chic^{37}$/Df(2L)Gpdh(A) testes failed to express any fasciclin III in the apical region of the testis (not shown) **(G, H)** Fasciclin III is ectopically expressed in *chic* mutant testes; indirect immunofluorescence of a $chic^{37}/chic^{37}$ testis carrying the marker M5-4, which labels the cells of the hub and very early germ cells (see panel A); anti β–galactosidase and anti-fasciclin III antibodies visualized by indirect immunofluorescence with rhodamine and fluorescein conjugated secondary antibodies, respectively **(G)** β–galactosidase staining reveals the cells of an enlarged hub (arrowhead), and possibly some stem cells as well. **(J)** Fasciclin III is ectopically expressed in a domain of cells (arrow) below the hub (arrowhead); these cells are most likely cyst cells. Bars=50 μm.

Figure 17

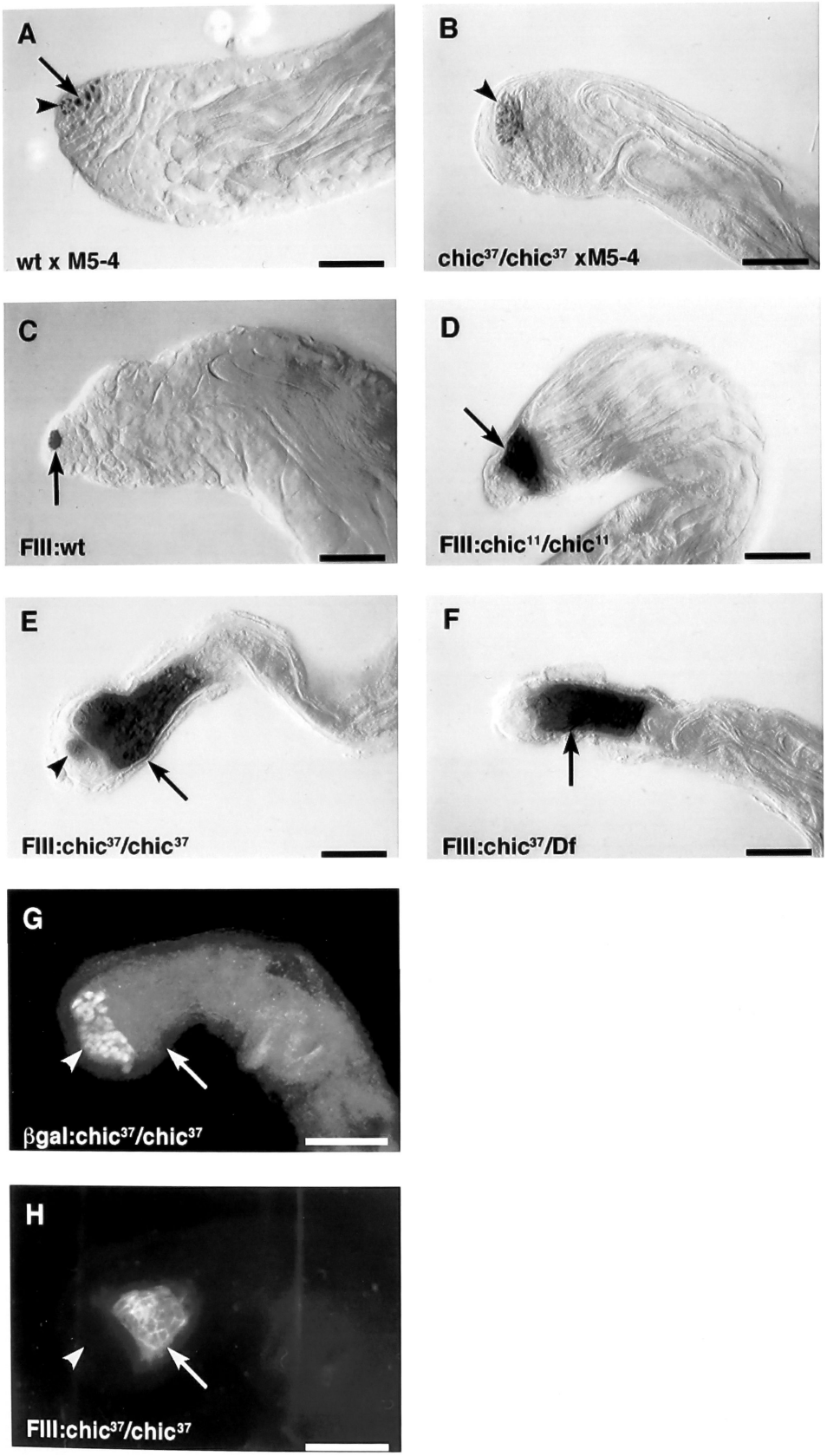

A
wt x M5-4
B
chic37/chic37 xM5-4
C
FIII:wt
D
FIII:chic11/chic11
E
FIII:chic37/chic37
F
FIII:chic37/Df
G
βgal:chic37/chic37
H
FIII:chic37/chic37

Fig. 18 *chic*^{11} mutant testes lack profilin in germ cells but have no detectable alteration in filamentous actin distribution. **(A, B)** chic protein distribution in wild-type and *chic*^{11} testes; immunoperoxidase staining with anti-chickadee antibody. **(A)** Mid-portion of a wild-type testis, illustrating the presence of chic protein in the cytoplasm of germ cells in the late growth phase (arrow); chic protein is also found in the cytoplasm of cyst cells and other somatic cell types (not visible). **(B)** Apical region of a *chic*^{11}/*chic*^{11} testis. chic protein is not detected in the germ line; germ cells in the early growth phase are shown (arrow), recognizable by their large size and typical nucleolus. chic protein is still present in the cytoplasm of cyst cells (arrowhead), which surrounds the non-staining germ cells. **(C, D, E, F, G, H, I)** Filamentous actin network in wild-type and *chic*^{11} mutant testes. **(C, F, H)** Rhodamine-phalloidin stains **(D, G, I)** Counterstain with the DNA dye Hoechst **(E)** Anti-fasciclin III antibody visualized by indirect immunofluorescence with fluorescein conjugated secondary antibody. **(C, D, E)** Cells from the apical region of a wild-type testis. Filamentous actin is abundant in the cells of the hub (C, arrowhead), which are recognizable by their characteristic staining pattern with Hoechst (D, arrowhead), and by their expression of fasciclin III (E, arrowhead). Filamentous actin is also present at much lower levels in what appears to be the cortex of germ line stem cells (C, arrow), which are recognizable by their position around the hub; the arrow in D points to the nucleus of such a stem cell. **(F, G)** Cyst of 16 wild-type germ cells in the growth phase. The most prominent filamentous actin distribution in germ cells in the testis is in a branched structure linking the 16 germ cells within a cyst (panel F); this most likely corresponds to the fusome. The position of the 16 germ cell nuclei is revealed in panel G. **(H, I)** Cysts of *chic*^{11}/*chic*^{11} germ cells in the early growth phase. Filamentous actin distribution is not altered in mutant germ cells. In particular, filamentous actin in the fusome is as in wild-type (compare panel H with panel F); the fusome is somewhat smaller than in wild-type because germ cells at an earlier stage of the growth phase were chosen for this illustration. The position of the germ cell nuclei is visible in panel I. Additionally, no difference between *chic*^{11} and wild-type testes was detected in the distribution of filamentous actin at the hub or in germ line stem cells (not shown). Bars=50 μm.

Figure 18

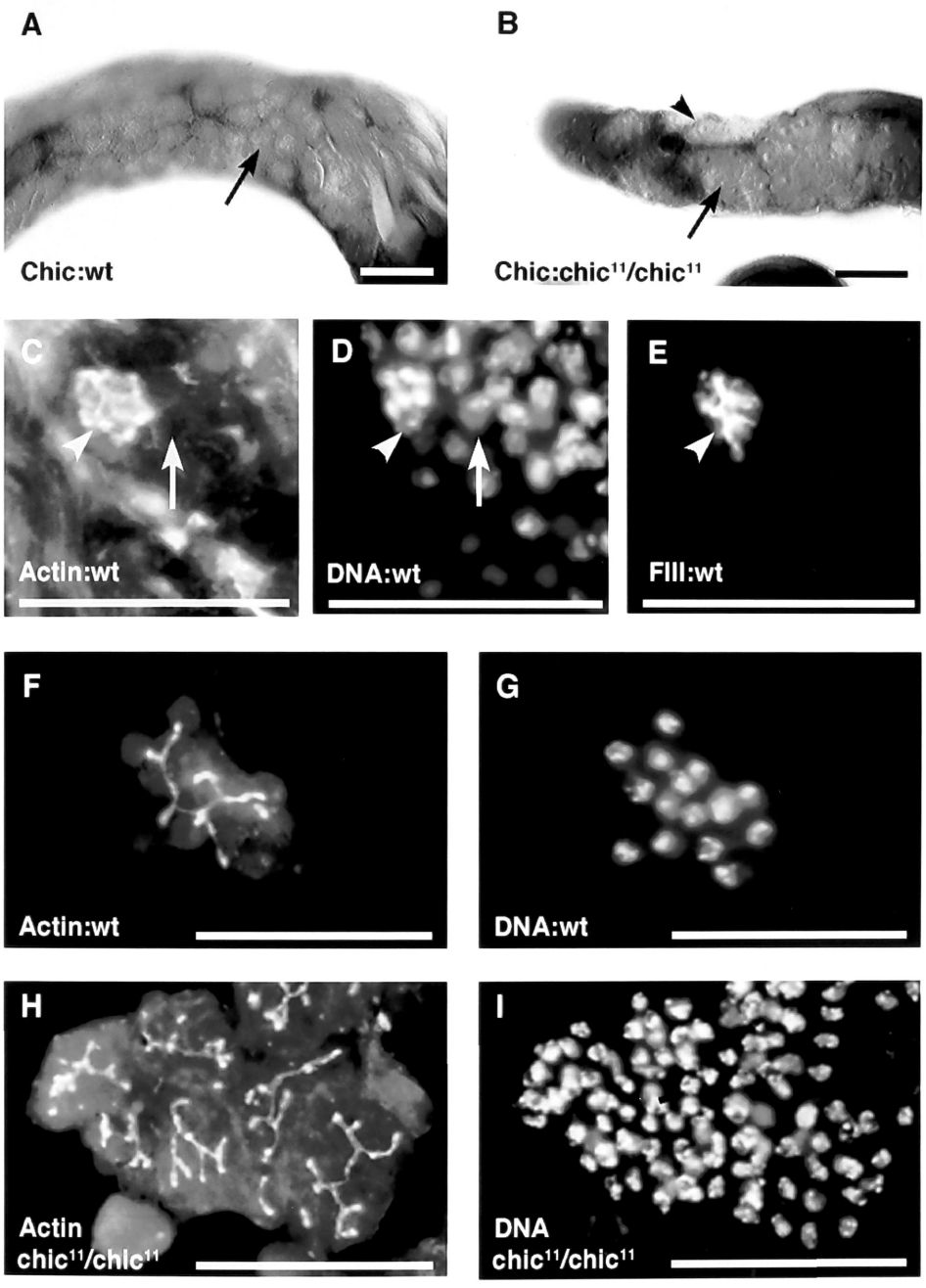

A
Chic:wt
B
Chic:chic11/chic11
C
Actin:wt
D
DNA:wt
E
FIII:wt
F
Actin:wt
G
DNA:wt
H
Actin
chic11/chic11
I
DNA
chic11/chic11

Fig. 19 *ms(2)916* mutant testes contain cysts of overproliferating germ cells and have a displaced hub. **(A, B)** Live testis squash **(A)** wild-type (*bgcn*/+); only cysts of 16 germ cells are observed (arrows); the refractile cyst cell cytoplasm can be seen in intact cysts (thin arrowhead). **(B)** *ms(2)916/ms(2)916*; some cysts contain many more than 16 small germ cells (arrow), while others contain 32 (arrowhead) or 16 (thin arrowhead) germ cells whose size and morphology is typical of the growth phase (compare to panel A).**(C, D)** 30 minute BrdU incorporation experiments. **(C)** Apical region of a wild-type testis; immunoperoxidase reaction with anti-BrdU and anti-fasciclin III antibodies. Nuclei in the vicinity of the hub (arrowhead) have incorporated BrdU; germ cells at the 4-, 8- and 16- (arrow) cell stage have undergone S phase in synchony within a cyst. Note that no nuclei are labeled below the cyst of 16 germ cells undergoing premeiotic S. **(D)** *ms(2)916/ms(2)916* testis; immunoperoxidase reaction with anti-BrdU antibody. Germ cells overproliferate in sychrony within a cyst; the arrows point to two cysts containing probably 32 and 64 germ cells undergoing S phase, respectively. **(E, F)** The hub is displaced in *ms(2)916/ms(2)916* testes; immunoperoxidase staining with anti-fasciclin III antibody. **(E)** Wild-type testis. Fasciclin III identifies the position of the hub, invariably at the very tip of the testis (arrow); fasciclin III is also expressed in the terminal epithelium joining the base of the testis to the seminal vesicle (arrowhead). **(F)** *ms(2)916/ms(2)916* testis. The hub (arrow) is displaced just over 100 μm from the very tip (thin arrowhead) in this mutant testis. There is some variability in the extent of hub displacement (see Text); a severely affected testis is shown. Fasciclin III expression in the terminal epithelium is not altered (arrowhead). Bar=50 μm.

Figure 19

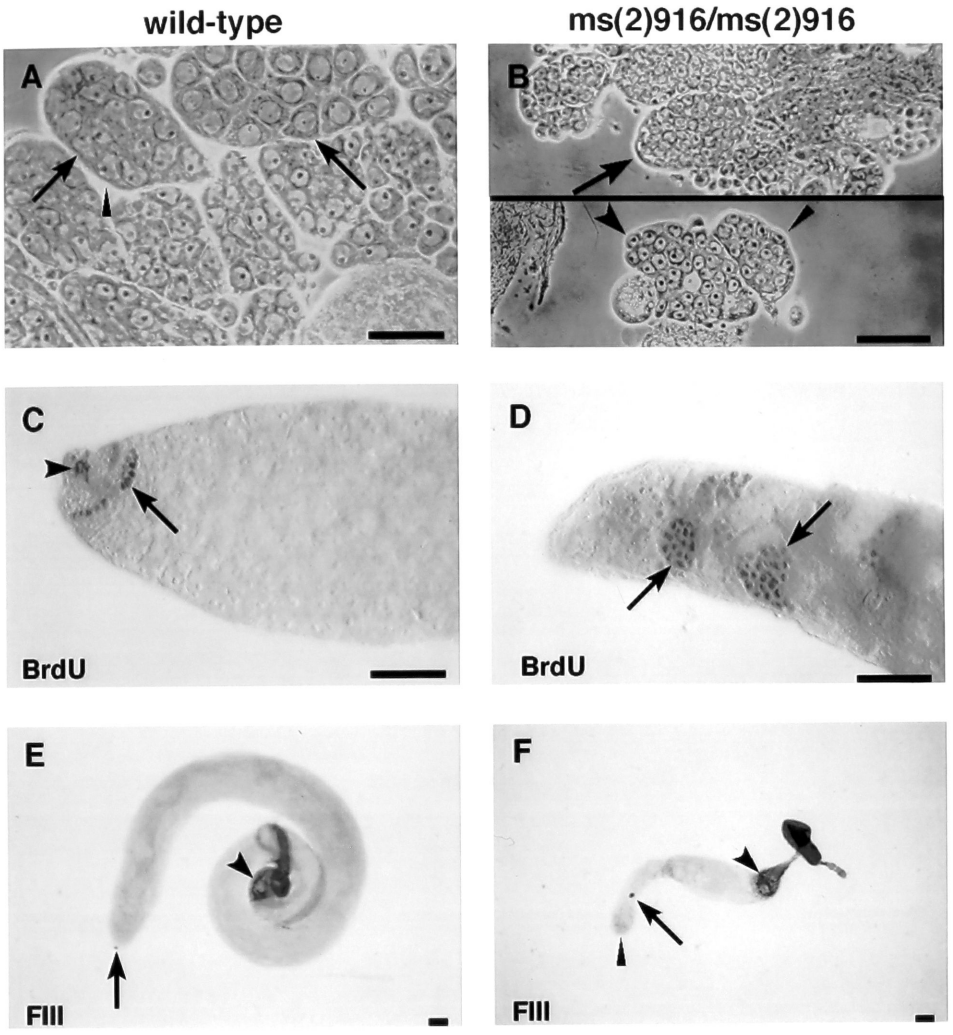
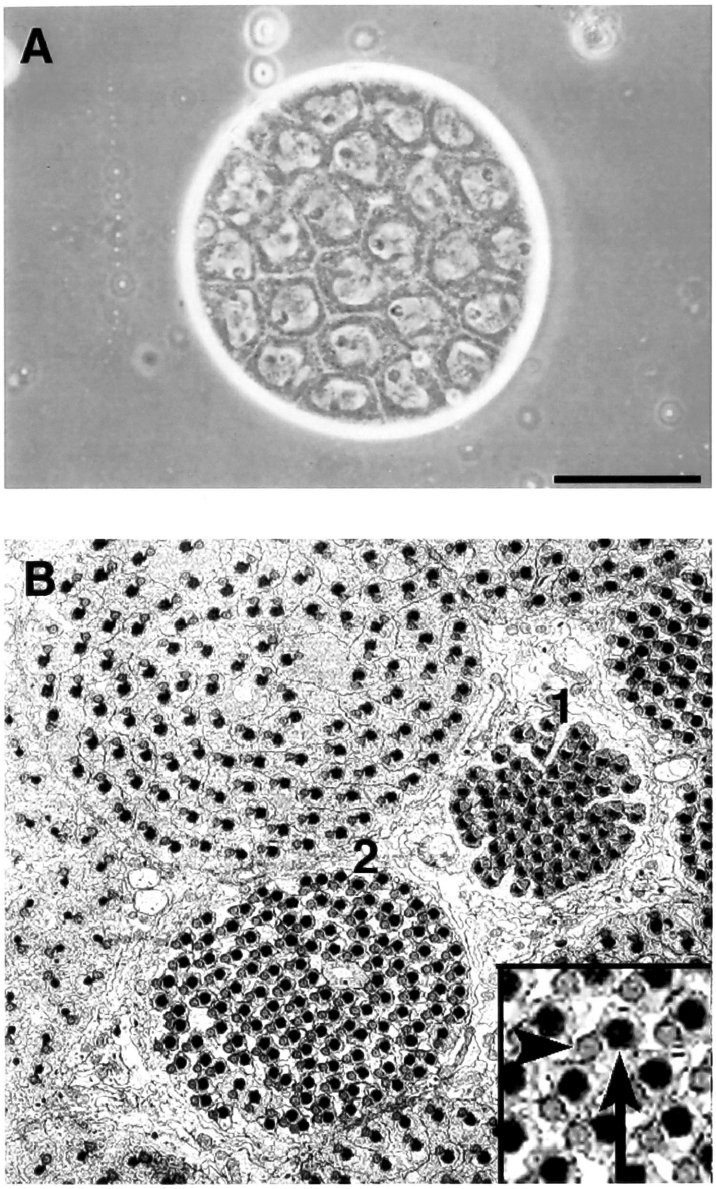


Fig. 20 Germ cells that undergo five rounds of mitotic divisions instead of four still complete spermatogenesis. **(A)** Live testis squash, *rux*^{2}/Y. Occasional cysts contain 32 germ cells, rather than the usual 16. In this intact cyst, 25 germ cells in the growth phase are visible in the plane of focus; note the refractile cystoplasm of the enclosing cyst cells. Bar=50 μm **(B)** Electron microscopy of a cross-section through sperm tails of *rux*^{2} testes. Cysts of sperm tails at various stages of post-meiotic differentiation are seen; in the most mature stages, spermatids have become individualized and are densely packed within the cyst (Tokuyasu et al., 1972a). Each individual spermatid (inset) has an axoneme (arrowhead) and a dark major mitochondrial derivative (arrow). Two cysts (1 and 2) at the individualisation stage are visible; while cyst 1 contains the regular number of 64 spermatids, cyst 2 contains 128 spermatids that are indistinguishable from those in cyst 1. Magnification: x4200; inset: x17200.

Figure 20



DISCUSSION

The controled proliferation of stem cells and their progeny is key in maintaining the appropriate production of differentiated cells in a variety of lineages. To learn about the components regulating these processes, we have explored the phenotype of three mutations which alter germ cell proliferation during *Drosophila* spermatogenesis. First, we have shown that mutations in *chic* abolish the proliferation of germ cells, possibly by preventing stem cell division. As *chic* encodes the actin binding protein profilin, this opens the door to investigating the mechanisms by which the actin network regulates stem cell function. Second, we have characterized a novel mutation, *ms(2)916*, which results in the overproliferation of germ cells during spermatogenesis, but not oogenesis. Third, we have shown that germ cells that undergo 5 mitotic divisions, instead of the usual 4, can still complete their subsequent differentiation program. Therefore, there is some flexibility in the counting of mitoses within the amplifying progeny of stem cells.

Profilin is required for the proliferation of germ cells

Profilin is a ubiquitous actin monomer binding protein that regulates filamentous actin polymerization (reviewed by Machesky and Pollard, 1993; Theriot and Mitchison, 1993). While profilin was initially proposed to inhibit filament polymerization by sequestring actin monomers (Carlsson et al., 1977), more recent experiments demonstrated that it can actually promote actin polymerization (Goldschmidt-Clermont et al., 1991b). Profilin stimulates nucleotide exchange on actin monomers, which results in accelerated actin polymerization if ATP is in excess of ADP, as monomeric actin bound to ATP polymerizes faster and has a lower critical concentration than that bound to ADP (Mockrin and Forn, 1980; Pollard and Cooper, 1986; Goldschmidt-Clermont et al., 1991b).

In addition to regulating actin assembly, profilin may also play a role in signal transduction, through its binding to phosphatidylinositol 4,5 bisphosphate (PIP_2). Profilin binding inhibits the conversion of PIP_2 into the second messengers diacylglycerol and inositol trisphosphate by unphosphorylated phospholipase C-γ1 (Goldschmidt-Clermont et al., 1991a). When this enzyme becomes phosphorylated, the inhibition is overcome and both second messengers and profilin are released in the cytoplasm. Profilin only then becomes available for binding to actin, and this mechanism thus may provide a link between a signal transduction cascade and cytoskeletal reorganization. In addition, profilin can also interact with the Ras pathway in yeast, as overexpression of profilin rescues a strain deficient for CAP, an associated protein required for Ras-mediated adenylate cyclase activation (Vojtek et al., 1991).

We have shown that mutations in *chic*, which encodes the *Drosophila* profilin, disrupt germ cell proliferation during spermatogenesis. Although we have shown that $chic^{11}$ mutant testes lack profilin in germ cells, we have not been able to correlate this deficit with a defect in the distribution of filamentous actin. We believe that a change in filamentous actin distribution in the fusome of mutant germ cells in the growth phase would have been noticed. However, germ cells in the vicinity of the hub are much smaller, and subtle differences may have been missed there. Alternatively, although profilin expression is lacking in the germ line of $chic^{11}$ mutants, *chic* function may still be required in somatic cells for proper germ cell proliferation. Our preparations did not allow us to distinguish the actin network in cyst cells. Generating clones of cyst or germ cells with null alleles should permit us to address the lineage requirement of *chic* function during spermatogenesis.

In contrast to the situation in male germ cells, a defect in the polymerization of actin filaments is apparent in the cytoplasm of nurse cells in *chic* mutant egg chambers (Cooley et al., 1992). As a consequence, nurse cell nuclei become delocalized and block

the ring canals leading to the oocyte, thus preventing the normal flow of cytoplasm in that direction.

Stronger *chic* allelic combinations have too few nurse cells, most of which are binucleate, while *chic*^{37} homozgous females appear to be also defective in germ cell proliferation (Verheyen and Cooley, 1994). A variable number of nurse cells suggests that mitoses are inappropriately regulated, while the presence of binucleate cells indicates that cytokinesis of the last mitotic division is prevented. Interestingly, *Tetrahymena* profilin is associated with the cleavage furrow at cytokinesis (Edamatsu et al., 1992). Moreover, yeast cells lacking profilin are unable to divide normally (Haarer et al., 1990). It is possible that *chic* is required for cytokinesis in germ cells during oogenesis (Verheyen and Cooley, 1994), and it is tempting to speculate that this may be the case as well during spermatogenesis, possibly in stem cells.

Interestingly, *diaphanous*, the other locus mutating to an underproliferation phenotype identified among our male-sterile strains, has been shown to be generally required for cytokinesis in *Drosophila* (Castrillon and Wasserman, 1994). *dia* encodes a protein with regions of homology to Bni1p, the product of a gene involved in cytokinesis in *S. cerevisiae*. Although the extent of proliferation or the fate of stem cells has not been directly assayed in *dia*^{1} testes, underproliferation of early germ cells may thus frequently correlate with defects in cytokinesis.

Defects in cytokinesis do not block the nuclear cycle. For instance, preventing cytokinesis by injection of anti-myosin antibodies in echinoderm embryos results in the continued division of nuclei, which eventually fuse and become polyploid (Kiehart et al., 1982). In *S. pombe*, mutants unable to form a division septum also continue their growth and nuclear cycle (Nurse et al., 1976). Finally, a *Drosophila* mutant in the regulatory light chain of non-muscle myosin is defective in cytokinesis, and contains multinucleate, as well as mononucleate polyploid cells (Karess et al., 1991). Therefore, if *chic* and *dia* indeed prevented cytokinesis in germ line stem cells, and nuclear cycling continued just

as in these other systems, one might expect to find polyploid stem cells in mutant testes. Although we need to address this more carefully, preliminary observations suggest that this is not the case for *chic*, as stem cells in *chic*^{37} mutant testes do not appear enlarged. Why would nuclear cycles be blocked along with cytokinesis in *chic* mutant stem cells ? One formal possibility is that a factor normally expressed in the gonial daughter, and responsible for inhibiting proliferation of germ cells after 4 mitotic cell cycles in the wild-type, is leaked into the abnormally connected stem cell in the mutant, thus arresting its nuclear cycle. However, this hypothesis is probably incorrect, as it predicts that stem cell would still undergo 4 nuclear cycles before being arrested, which is in contradiction with the apparent normal ploidy of germ line stem cells in *chic*^{37} mutant testes. It remains to be investigated how a potential defect in cytokinesis is coupled to a probable block of the nuclear cycle in germ line stem cells.

Alternatively to being required for cytokinesis, *chic* may regulate germ cell proliferation by some other mechanism, perhaps by modulating signal transduction during the early stages of spermatogenesis.

In addition to regulating germ cell proliferation, *chic* may also play a role in the signalling between germ cells and somatic cells, which maintains and restricts fasciclin III expression at the hub (see Chapter 3). Interestingly, although germ line stem cells are still present in most *chic*^{37} mutant testes, fasciclin III expression is nevertheless more severely affected than in agametic testes. For instance, while 8/23 agametic testes with a hub had ectopic fasciclin III expression beneath it, this was the case in 8/13 *chic*^{37} testes with a hub. Moreover, ectopic expression in *chic*^{37} mutants is in a larger domain of cells. This dramatic response in somatic cells is probably not merely due to the absence of the mitotically amplifying germ cells, as fasciclin III expression is not similarly affected in *pitzi*, a recently identified underproliferation mutant, which also retains some stem cells in the testis (John Tran and Steve DiNardo, unpublished observations). This raises the

possibility that *chic* function is required for proper signalling from the germ line to the soma.

An overproliferation mutant of the male germ line

ms(2)916 is a homozygous recessive mutation that results in a variable overproliferation of germ cells during spermatogenesis, but to no discernable phenotype during oogenesis. With the possible exception of the temperature-sensitive lethal mutation *l(1)55*, which has a similar phenotype during spermatogenesis, but whose consequences on oogenesis have not been analyzed (Lifschytz and Yakobovitz, 1978), *ms(2)916* could define the first locus mutable to an overproliferation phenotype in males, but not females. A separate recently identified locus, *ms(3)ods*, apparently also mutates to an overproliferation phenotype in males only (Schäfer, cited in Gateff, 1994). Loci conversely mutating to an overproliferation phenotype exclusively in females have been described, and identify for the most part components of the germ line sex determination pathway (reviewed by Steinmann-Zwicky, 1992; Spradling, 1993; see Chapter 4). Although the early stages of gametogenesis in both sexes share similarities, there are also differences, which provide ample opportunity for sex-specific regulation of germ cell proliferation. For instance, while the gonial daughter of the stem cell is immediatedly surrounded by two somatic cyst cells during spermatogenesis, somatic follicle cells cover germ cells only at the 16-cell stage during oogenesis (reviewed by Spradling, 1993). If germ line-soma interactions were to regulate germ cell proliferation, it would thus likely be in a different manner in males and females. Moreover, while the number of mitotic divisions during oogenesis is 4 in all *Drosophila* species examined, this number is species-specific during spermatogenesis (Liebrich, 1984). Thus, the counting of mitoses may be regulated differently in females than in males. As we have a single allele of *ms(2)916*, the sex-specificity of the overproliferation phenotype is tentative. More severe alleles may also affect oogenesis or display additional phenotypes.

Interestingly, the *ms(2)916* mutation is somewhat cold-sensitive. Mutant testes tend to be smaller at 18 ^{o}C than at 25 ^{o}C, although there is variability in the size at both temperatures. Moreover, there is a striking deficit of homozygous mutant flies eclosing during the first few days from bottles kept at 18 ^{o}C, which is not the case at 25 ^{o}C. Finally, flies raised at 18 ^{o}C usually have a scutellar bristle phenotype, which is not observed at 25 ^{o}C either. A cold-sensitive mutation may indicate that *ms(2)916* affects a microtubule-based process, as microtubules are known to be destabilized by cold.

Unfortunately, the *ms(2)916* phenotype is not due to the resident P-element. Excision of the P-element fails to revert the male-sterile phenotype, and *ms(2)916* is fertile in *trans* to a cytologically visible deficiency uncovering the P-element. The *ms(2)916* mutation is not uncovered either by any of the deficiencies from the *Drosophila* stock center deficiency kit, which covers over 50 % of the second chromosome (S. Gjnatic, P. G., and S. DiNardo, unpublished observations). We also verified that *ms(2)916* is not allelic to *bgcn*, which is on chromosome II. Erika Matunis in the lab is meiotically mapping *ms(2)916* , and has recombined away the P-element from the male-sterility in the process of doing so. As the original P-element led to lacZ expression in germ cells, we had not been able to use marker lines to address the fate of overproliferating *ms(2)916* germ cells, as we have done for *bam* and *bgcn*. However, the new mutant chromosome devoid of the P-element should enable us to learn about the identity of overproliferating germ cells in *ms(2)916* testes.

ms(2)916 testes have an additional novel phenotype, that of having a displaced hub. This could reflect two distinct requirements during spermatogenesis, one for restricting the proliferation of germ cells, and the other for proper placement of the hub. More likely perhaps, the two phenotypic manifestations may depend on one another. For instance, stem cells away from the tip of the testis, as a consequence of following a displaced hub, may no longer be able to restrict their proliferation in this ectopic location. However, a displaced hub is not a prerequisite for overproliferation, as the hub is correctly located in

bam and *bgcn* testes. Reciprocally, a displaced hub does not appear to always result in overproliferation, as some *ms(2)916* germ cells progress normally through spermatogenesis. Interestingly, in *C. elegans*, when the distal tip cell is displaced to an ectopic location on the side of the gonad, mitoses are ectopically induced in neighbouring germ cells (Kimble and White, 1981). It is a formal possility that an ectopic hub in *ms(2)916* testes similarly recruites neigbouring germ cells into a proliferation program.

We also occasionally observed a displaced hub in testes of severe *fumble* alleles, another male-sterile mutation from our collection (see Table 5). Interestingly, such testes also often contained overproliferating germ cells, as well as germ cells with other proliferation defects, such as polyploid nuclei (data not shown, but see Appendix at the end of Materials and Methods). Further phenotypic and molecular characterization of *ms(2)916* and *fumble* should shed light on the intriguing correlation between a displaced hub and defects in germ cell proliferation.

On the counting of mitoses

Germ cells can undergo 3 or 2 rounds of mitotic divisions instead of the usual 4, and still mature to the latest stages of post-meiotic differentiation (Lifschytz, 1978). We show here that they can do so also if they undergo 5 rounds of mitotic divisions. Peacock et al. (1975) mentioned the presence of occasional bundles with 128 spermatids in a study of males carrying an X-chromosome inversion, but did not report on the extent of differentiation of these spermatids. Our results demonstrate that precise counting of mitoses is not an absolute requirement for the completion of spermatogenesis in *D. melanogaster*. Nevertheless, it will be still of interest to investigate how this counting is achieved. For instance, one may ask whether the counting is autonomous to the germ line, or imposed by the neighbouring cyst cells. This can be addressed by performing reciprocal trans-species pole cell transplants, for instance between *D. hydei* and *D. melanogaster*, and counting the number of germ cell mitoses in chimeric animals (John

Tran and Steve DiNardo, in progress). Additionally, by screening for *D. melanogaster* mutants altering this number (see Chapter 7), one might get at the mechanisms determining the extent of proliferation in the amplifying progeny of stem cells.

Short testes are indicative of defects in germ line proliferation and growth

Mutations in which there is an underproliferation of germ cells, or an overproliferation followed by degeneration, alter testis morphology. Mutant testes from males homozygous for *dia*, *chic*, *bam* and *ms(2)916* are substantially reduced in size. Moreover, additional mutants in which there is degeneration of the germ cells following developmental arrest, such as *boule, cueball or l(2)26Ab* (see Table 5), also have testes reduced in size. That the size of the testis is highly correlated with the fate of germ cells is also evident in the tiny agametic testes from the progeny of osk^{301} females.

The size of the testis is also an excellent indicator of the extent of germ line proliferation and growth in other species. For example, mice with mutations at the *White* locus lack germ cells and have severely atrophied testes (Mintz and Russel, 1957). Gonads of various *C. elegans* mutants deficient in germ cells are similarly shorter than wild-type (Austin and Kimble, 1987; Capowski et al., 1991; Beanan and Strome, 1992).

In *Drosophila*, the short testis phenotype could greatly facilitate the isolation of mutations in loci required for regulating germ cell proliferation. The phenotype is obvious upon dissection of mutant males, and one could thus envisage to conduct visual screens bypassing labor-intensive fertility tests.

CHAPTER 7: DESIGNING A FLP/FRT-BASED SCREEN TO IDENTIFY NOVEL GERM CELL OVERPROLIFERATION MUTANTS

INTRODUCTION

Mutations characterized by germ cell overproliferation may identify loci regulating gonial cell fate, the counting of mitotic divisions or the entry into meiosis (see Chapter 3). Such mutations are extremely rare. *bam* and *bgcn* are the only two known loci that can mutate to an overproliferation phenotype in both male and female germ cells (Gateff, 1982; McKearin and Spradling, 1990; reviewed by Fuller, 1993; Spradling, 1993; Gateff, 1994). In a screen for female-sterile mutations on the second chromosome that was conducted to near-saturation, *bgcn* was the only locus identified which mutated to this phenotype (Schüpbach and Wieschaus, 1991). It is thus unlikely that many more loci which limit germ cell proliferation in both sexes will be recovered by screening for female- and male-sterile strains.

However, additional loci restricting the proliferation of germ cells during spermatogenesis probably exist, and may not have been detected for several reasons. First, some loci may be involved in the control of germ cell proliferation strictly in the male germ line. As discussed in Chapter 6, *ms(2)916* could be just such a locus. *ms(2)916* or loci like it would not have been noticed in the extensive screens directed at recovering female-sterile mutations. Second, as revealed by *ms(2)916* as well, some mutations can result in the overproliferation of some cysts of germ cells, but not all. If such a non-fully penetrant mutation had no additional phenotype during spermatogenesis, it would not be recovered in a male-sterile screen. Third, while *bam* and *bgcn* do not play a role outside of gametogenesis, other loci involved in the control of germ cell proliferation may in

addition be required in cells at other times and places during development, and thus be essential for viability. It might be difficult to generate non-lethal alleles of some of these loci.

In this Chapter, we report the design of a FLP/FRT based mutant sreen that should permit us to cirumvent these difficulties. The results of a reconstruction experiment with a *bgcn* mutant chromosome demonstrate that the screen should enable us to identify novel loci restricting the proliferation of germ cells during spermatogenesis.

RESULTS

Design of an FRT-based screen for overproliferation mutants

To design a screen that could test chromosomes carrying lethal mutations, we made use of the FLP/FRT site-specific recombination system, which has been developed in *Drosophila* by several labs (Golic and Lindquist, 1989; Golic, 1991; Siegfried et al., 1992; Harrison and Perrimon, 1993; Xu and Rubin, 1993). This system utilizes the *S. cerevisiae* 2 μm plasmid FLP-recombinase and its recombination targets (FRT's) (reviewed by Cox, 1988), which have been introduced on appropriate constructs into the fly genome. First, the FLP recombinase has been placed under the control of the hsp-70 promoter, and thus is heat-inducible. Second, lines with FRT sites inserted close to the centromere on each chromosome arm have been generated. If flies carrying both hsp70-FLP and FRT sites are given a heat-pulse, site-specific mitotic recombination can be induced by the recombinase at the FRT's. In this manner, entire chromosome arms distal to the FRT sites can be made homozygous at a high frequency, and be clonally expanded.

The design of our proposed FLP/FRT based screen for overproliferation mutants during spermatogenesis is outlined in Figure 21. A given mutation (denoted by an X) is generated on a chromosome arm with an FRT site (hatched rectangle) near the centromere (disk). This chromosome is put in *trans* to another one that has just an FRT site at the identical location. After duplication of the chromosomes during S phase and the subsequent G2, sister chromatids segregate to opposite poles of the division spindle at mitosis. Both resulting cells have the same heterozygote genotype as their parent, and, therefore, have no mutant phenotype (Fig. 21A). If, on the other hand (Fig. 21B), these cells also carry the hsp70-FLP construct and a heat-pulse is administred, the FLP recombinase catalyzes site-specific recombination between homologous chromatids at the FRT sites. After segregation of the recombinant chromatids to opposite poles of the mitotic division spindle, the resulting two cells have different genotypes: while one no

longer carries the mutation, the other is homozygous mutant. If the recombination event happens in a germ line stem cell, and if the self-renewing stem cell is the cell that becomes homozygous mutant, then all subsequent progeny of this stem cell will be mutant as well. If the given mutation results in an overproliferation phenotype, then one would expect to detect cysts of overproliferating germ cells after a few days (Fig. 21B).

Based on our experience with *ms(2)916*, we were hoping that mutant clones of overproliferating germ cells would be already detectable at the dissecting microscope as little refractile balls within the testis (see Fig. 15I, arrow). Alternatively, overproliferating germ cells can be unambiguously identified at the compound microscope after fixation and staining with the DNA dye Hoechst. Hoechst-stained nuclei of germ cells in the proliferation phase of wild-type spermatogenesis are bright (Fig. 22B, bracket), while those of germ cells in the subsequent growth phase are much duller (Fig. 22B, right of the bracket). In a *bgcn* mutant testis, overproliferating germ cells with brighly staining nuclei fill the entire testis (Fig. 22D, bracket), including the area where cysts of germ cells are degenerating (Fig. 22C, arrow). Hoechst staining also invariably identified cysts of overproliferating germ cells in *ms(2)916* testes (data not shown), and, therefore, should also allow us to recognize FLP-induced clones of overproliferating germ cells.

A reconstruction experiment with *bgcn* to test the design of the screen

To verify that our design could potentially lead to the isolation of novel overproliferation mutants, we tested whether it would have allowed us to recover a mutation in *bgcn*. We recombined a *bgcn* mutation (located on the tip of chromosome arm 2R) onto a second chromosome carrying an FRT site at the base of 2R. We then put this chromosome in *trans* to another one with an FRT site at the identical location, but which did not carry the *bgcn* mutation. We also crossed in a third chromosome carrying a construct with the FLP recombinase under the control of hsp70 (see Materials and Methods for further details).

We carried out the reconstruction experiment by heat-shocking adults or third instar larvae for one hour at 38 ^{o}C, and then waiting 3, 5 or 7 days before dissection and scoring. Non heat-shocked animals of the same genotype, as well as heat-shocked animals not carrying the FLP-recombinase, were included as controls. We scored testes both at the dissecting microscope for the presence of refractile balls and at the compound microscope after fixation and Hoechst staining for the presence of giant cysts of brightly staining germ cells. We kept track of individual testes going through both scoring procedures in order to determine the accuracy with which we could recognize an overproliferation phenotype under the dissecting scope alone. The combined data for the reconstruction experiment are given in Fig. 23, while representative examples are shown in Fig. 22.

When controls flies not carrying the FLP recombinase were examined at the dissecting microscope 5 days after the heat-shock, they never (0/240) contained little refractile balls. Examination of these testes after fixation and Hoechst staining confirmed that they were wild-type in apperarance, having brightly staining nuclei restricted to the tip of the testis (Fig. 22F, bracket). In contrast, when flies carrying the FLP recombinase were analyzed at the dissecting microscope 5 days after the heat-shock, they often contained one or more little refractile balls, suggestive of the presence of overproliferating germ cells. This was almost always (74/76) confirmed at the compound microscope after fixation and Hoechst staining. Cysts of brightly staining germ cells could be readily distinguished in a region normally devoid of proliferating cells (Fig. 22H, arrow). These cysts of *bgcn* homozygous mutant cells were also detectable under DIC optics (Fig. 22G, arrow). A BrdU incorporation experiment verified that germ cells were proliferating in synchrony within giant cysts, as expected from *bgcn* mutant cells (Fig. 22I, arrow).

Overall, mutant clones were observed in 35 % of testes (n=215) examined at the compound microscope 5 days after a heat-shock was given to young adults (Fig. 23). In 74 % of these cases, the clone of overproliferating germ cells was already detected at the

dissecting scope, while it was missed at that level the rest of the time. The frequency of clones observed at the compound microscope almost doubled, to 59 % of testes (n=32), when the heat-shock was administred to third instar larvae instead of adults. Clones of overproliferating cells were observed in only 11 % of testes (n=120) when the animals were examined 3 days after the heat pulse, and in 18 % of testes (n=179) when they were examined 7 days after the regimen. Mutant clones were never observed in flies that carried the FLP recombinase, but that were not heat-shocked (n=74), while they were present in a single testis among flies that did not carry the enzyme altogether (n=546).

In summary, we tested the design of our screen by inducing clones of homozygous mutant *bgcn* cells by FLP-mediated recombination. These clones were usually already recognized at the dissecting microscope, which should facilitate the screening of a large number of chromosomes.

Fig. 21 Principle of a FLP/FRT-based screen for overproliferation mutants during spermatogenesis. Flies are heteroygous for a mutation (denoted by an X) that causes overproliferation of germ cells in a cell-autonomous manner; the mutation has been induced by either X-rays or EMS at the previous generation. The mutation is on a chromosome arm carrying an FRT site (hatched rectangle) just distal to the centromere (disk). The homologous chromosome carries an FRT site at the identical location. **(A)** Homologous chromosomes will normally not undergo recombination, and unaltered sister chromatids segregate to opposite poles of the mitotic division spindle. This gives rise to two genotypically identical cells, heterozygote for the mutation just like the parental cell. Such germ cells undergo the regular number of mitotic divisions in the testis, enter the growth phase and complete spermatogenesis. **(B)** If a heat-shock is administred, FLP recombinase is produced, and the enzyme catalyzes site-specific homologous recombination at the FRT sites. At mitosis, recombined sister chromatids usually segregate to opposite poles of the division spindle (Pimpinelli and Ripoll, 1986), giving rise to two cells with different genotypes at the locus of interest, one being wild-type, the other homozygous mutant. If the recombination event happens in a stem cell, and the resulting homozygous mutant cell is the self-renewing stem cell, all subsequent progeny of that stem cell will be mutant as well. Such germ cells are expected to overproliferate. For the proposed screen, heterozygote males will be given a pulse of heat-shock at eclosion to induce FLP expression. They will then be allowed to mate for 5 days with females carrying appropriate balancers permiting the recovery of chromosomes of interest. Males will then be sacrificed for testis analysis. Clones of overproliferating germ cells are usually visible at the dissecting microscope as little refractile balls, and always detected at the compound microscope as cells with bright nuclei after fixation and Hoechst staining (see Figs. 22 and 23). Candidate chromosomes will then be recovered from the progeny of the mated females.

Figure 21

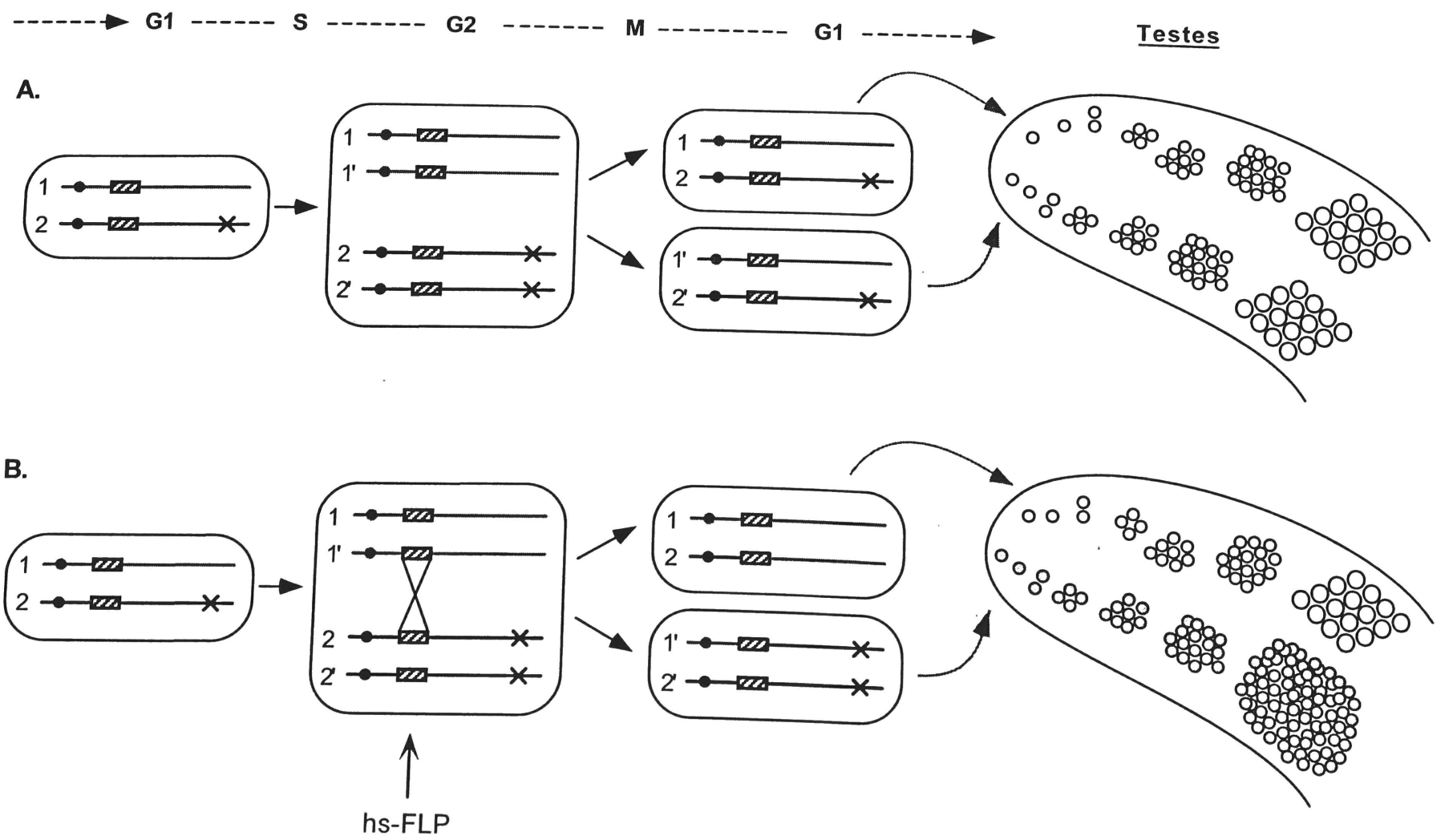


Fig. 22 FLP-induced *bgcn* mutant clones can be identified in a reconstruction experiment. **(A, C, E, G)** Apical region of fixed testes from 6 day old males viewed by DIC optics **(B, D, F, H)** Counterstain of panels **(A, C, E, G)** with the DNA dye Hoechst. **(A, B)** *bgcn*^{QS2}/+ testis. Brightly staining, proliferating, germ cells are restricted to the tip of the testis (B, bracket); note that germ cells in the subsequent growth phase are much duller. **(C, D)** *bgcn*^{QS2}/*bgcn*^{QS2} testis. Brightly staining, proliferating, germ cells fill the entire mutant testis (D, bracket). Note that cysts of degenerating germ cells, which are very apparent under DIC optics (C, arrow), are present further down in the testis; such cysts are also visible under the dissecting microscope (not shown). **(E, F)** FRT(43D)-*bgcn*^{QS2}/FRT(43D) testis. Without FLP recombinase, these control testes were identical to wild-type, and had brightly staining, proliferating, germ cells restricted to the tip (F, bracket). **(G, H)** FRT(43D)-*bgcn*^{QS2}/FRT(43D); FLP99-MKRS/+ testis; the male was heat-shocked for one hour at 38 ^{o}C on day 1. Clones of overproliferating germ cells were readily detected as little balls in the growth phase region both at the dissecting microscope (not shown) and under DIC optics at the compound microscope (G, arrow). Clones were also identified under epifluorescence as giant packets of brigthly staining cells (H, arrow), in an area which normally only contains duller growth phase germ cells. **(I)** 30 minute BrdU incorporation experiment performed on a FRT(43D)-*bgcn*^{QS2}/FRT(43D); FLP99-MKRS/+ testis 4 days after heat-shock; immunoperoxidase reaction with anti-BrdU antibody. In addition to the nuclei that have incorporated BrdU in the immediate vicinity of the hub, a giant cyst containing over 100 germ cells (arrow) has been caught in S phase during the labeling period. Bars=50 μm.

Figure 22

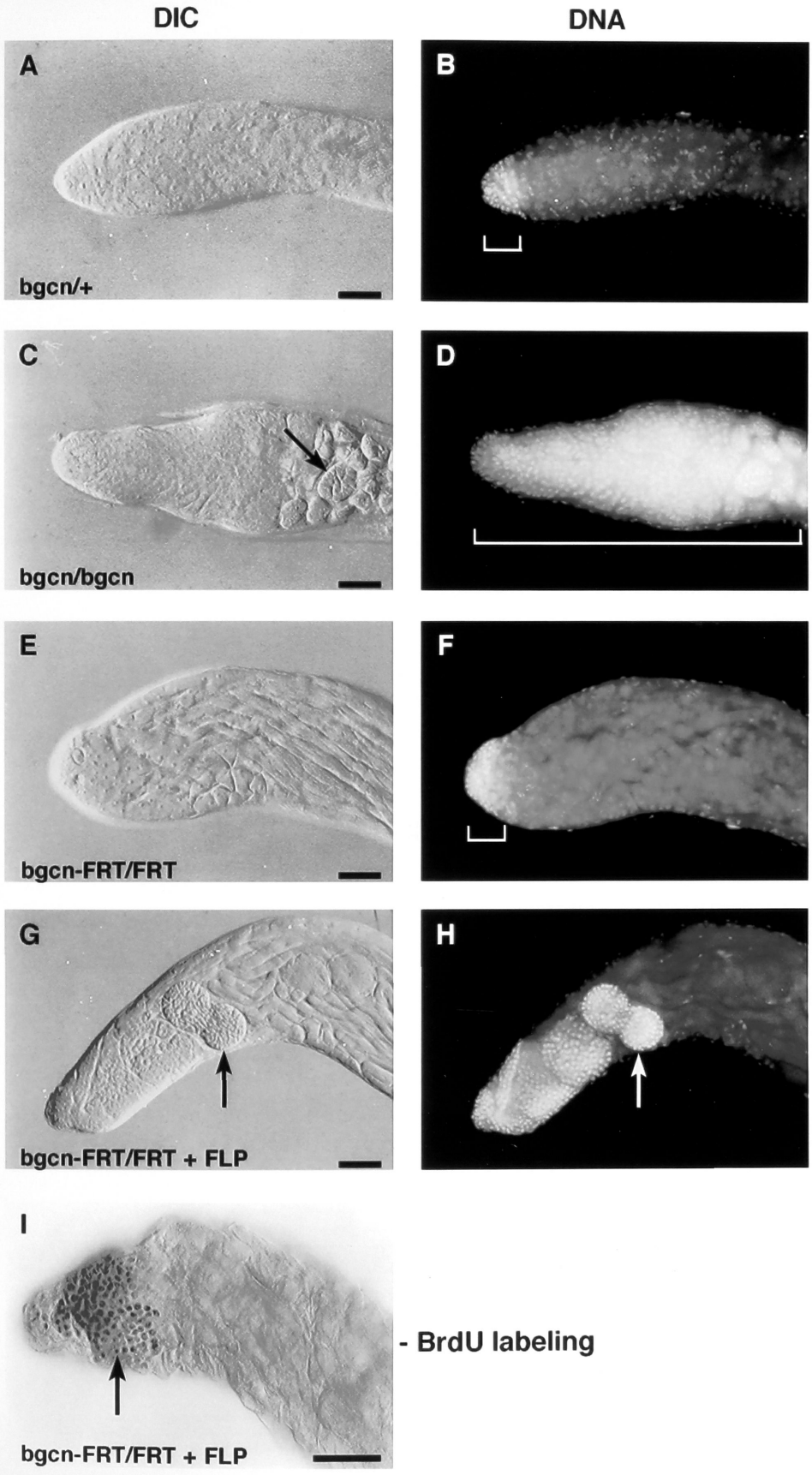
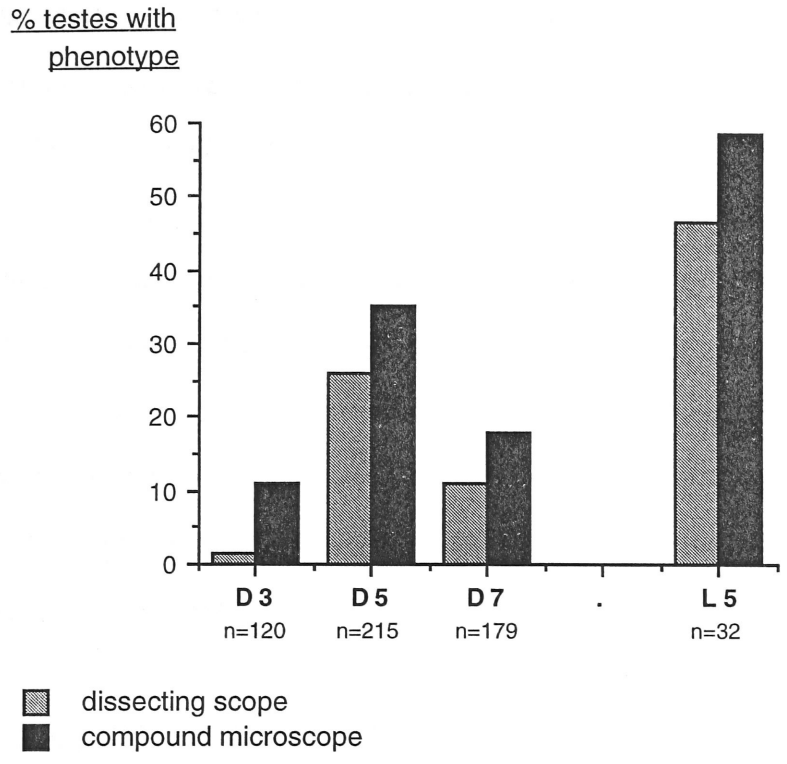

DIC
DNA
A
B
bgcn/+
C
D
bgcn/bgcn
E
F
bgcn-FRT/FRT
G
H
bgcn-FRT/FRT + FLP
I
bgcn-FRT/FRT + FLP
- BrdU labeling

Fig. 23 Frequency of *bgcn* overproliferating clones induced in the reconstruction experiment. FRT(43D)-*bgcn*^{QS2}/FRT(43D); FLP99-MKRS/+ adults were given a pulse of heat-shock for one hour at 38 ^{o}C, on the day of eclosion, and testes were analyzed 3 (D3), 5 (D5) or 7 (D7) days later for the presence of clones of overproliferating germ cells. Third instar larvae were also heat-shocked and analyzed 5 days later (L5). Percentage of testes showing a visible phenotype under the dissecting microscope (light bars) or the compound microscope (dark bars) are given. We first scored the presence of clones at the dissecting microsope, where they were often apparent as little refractile balls. We then scored them more reliably at the compound microscope after fixation and staining with the DNA dye Hoechst, which reveals brightly staining proliferating germ cells (see Fig. 22). Testes were tracked individually throughout the two scoring procedures, so that we could assess the accuracy of our scoring at the dissecting microscope. The difference in the height of the light and the dark bar thus represents testes with overproliferating germ cells that were missed at the dissecting microscope. Flies were treated in small batches over the course of two weeks, and there was a significant variation between batches treated on different days, probably owing to subtle temperature fluctuations in the water bath. The percentages given in the graph represent the average of all the data collected from the different batches.

Figure 23


% testes with phenotype
60
50
40
30
20
10
0
D 3
n=120
D 5
n=215
D 7
n=179
.
L 5
n=32
dissecting scope
compound microscope

DISCUSSION

We designed a FLP/FRT based screen to identify novel loci restricting the proliferation of germ cells during spermatogenesis. This approach has several features that should permit the isolation of loci which would not have been easily recovered in previous screens. First, we will visually screen for the mutant phenotype in males, thus sampling both mutations that affect males but not females, as well as those that would not result in a male-sterile phenotype. Second, we will induce homozygous mutant clones in a heterozygote fly, thus allowing the recovery of mutations that may be detrimental to other developmental processes. Third, as heterozygote flies can be analyzed, this screen is one generation faster than classic F3 screens, and may allow us to reach saturation for loci mutating to an overproliferation of germ cells during spermatogenesis.

The *bgcn* reconstruction experiment

We tested the feasibility of such a screen in a reconstruction experiment, for which a *bgcn* mutation was recombined onto an FRT-bearing chromosome. After providing a source of FLP recombinase, *bgcn* mutant clones were induced and identified a few days later by quick inspection at the dissecting microscope or more complete analysis at the compound microscope. 35 % of testes examined 5 days after heat-shocking young adults contained overproliferating germ cells. The fraction of testes with mutant clones varied as a function of the number of days separating FLP induction and dissection, and was higher if larvae, rather than adults, were heat-shocked.

Only 11 % of testes examined 3 days after heat-shock had clones of overproliferating germ cells. These mutant cysts were located still close to the tip and were mostly missed at the dissecting scope. This is not surprising, considering that they were not even apparent under DIC optics at the compound microscope. In the wild-type, it takes an estimated 2 days for the daughter of the stem cell to undergo the 4 mitotic

divisions and initiate the growth phase (Lindsley and Tokuyasu, 1980). Therefore, 3 days after heat-shock, mutant clones may have just transited through the regular 4 mitotic divisions and begun to overproliferate. This would explain both the proximity of the clones to the tip, and their apparent low frequency, as most may still be hidden in the proliferation region among cysts of heterozyote germ cells with brightly staining nuclei.

Only 18 % of testes examined 7 days after heat-shock had clones of overproliferating germ cells. This number may seem surprisingly low compared to the 35 % of testes having clones 5 days after heat-shock, as mutant stem cells are expected to generate clones of progeny throughout the life of the fly. However, it has been suggested that the cycling time of stem cells increases in older males (Hardy et al., 1979), as their number appears unchanged, and yet older males are less fecund (Ashburner, 1989a). A slower stem cell cycle in older males may explain the diminished recovery of clones after 7 days, since induced cysts of overproliferating germ cells eventually degenerate, just as in *bam* and *bgcn* mutant testes.

Close to 60 % of testes examined 5 days after third instar larvae were heat-shocked contained clones of overproliferating germ cells. This increased number was expected, as about twice as many stem cells are present in the third instar larval gonad as in the adult testis (Hardy et al., 1979). Therefore, the number of cells potentially subject to FLP activity is probably similarly increased. Despite this increase, generating mutant clones in larvae in the course of an actual screen may not be desirable, as it could lead to the underrepresentation of chromosomes carrying mutations in genes essential for further development of the animal.

Planning an actual screen

In the reconstruction experiment, an average of 35 % of testes examined 5 days after heat-shocking adults had overproliferating mutant cells. We treated flies in small batches and observed significant variations between batches heat-shocked on different

days, probably owing to subtle temperature fluctuations in the water bath. Therefore, we believe that the overall frequency of clones recovered could be increased by another 5 to 10 % if the temperature were optimized. Even if we were missing mutant chromosomes in 65 % of testes, the probability of not detecting a mutation of interest in one fly goes down to 42 %, as each fly has two testes [$(0.65)^2$=0.42]. Thus, 58 % of potential mutations should be detected by analzying a single fly. This number drops to about 43 % [74 % of 58 %] if one relies solely on the detection of little refractile balls at the dissecting microscope. The design of the screen could be easily adapted so that more than one fly with a given chromosome gets analyzed, ensuring the recovery of most potential chromosome carrying an overproliferation mutation.

From past experience, we know that one person can score 500 pairs of testes at the dissecting microscope per full-time day. As this number goes down significantly -perhaps to 100- if one verifies all testes at the compound microsope, we think it will be more efficient to score simply at the dissecting microscope in an actual screen. One could thus score 5000 mutant chromosomes arms over the course of two weeks. Although the time required for setting up the crosses and verifying candidate chromosomes also needs to be taken into account, the fact remains that a relatively large number of chromosomes could be rapidly analyzed. For comparison, just over 7000 EMS-mutagenized chromosomes were tested for female-sterility in a near-saturation screen on the second chromosome (Schüpbach and Wieschaus, 1989; Schüpbach and Wieschaus, 1991).

We plan on starting our mutant screen with chromosome arms 2R and 3R, where *bgcn* and *bam* are respectively located. The recovery of mutations in these two loci will serve as a positive control. Their occurence may give us some estimate of the number of loci that can mutate to an overproliferation phenotype, and thus help us decide how many chromosomes need to be screened to reach saturation.

Generating the mutation on an FRT chromosome can allow one to readily test autonomy of gene function, for instance by placing the mutant chromosome in *trans* to

one carrying the same FRT site as well as a distally located, ubiquitously expressing P-lacZ. In this case, mutant cells are X-gal$^-$, and it should be straightforward to determine whether non-staining cyst cells or germ cells are associated with clones of overproliferating germ cells.

Instead of the "classical" FRTs, we may use a modified set of FRT chromosomes, as they become reliable and available at the base of most chromosome arms (Harrison and Perrimon, 1993). This set results in lacZ expression upon mitotic recombination, following the joining of a ubiquitous tubulin promoter adjacent to the FRT on one chromosome with the lacZ coding sequence adjacent to the FRT on the other. In this case, all mutant cells should be marked, and it may be possible to visually screen for additional categories of mutants after fixation and X-gal staining, such as those failing to proliferate or undergoing a different number of mitoses.

Testing other candidate loci for the control of proliferation

We can also use the FLP/FRT system to test the function, during spermatogenesis, of essential genes known to regulate cell proliferation in other cases. For instance, the segment polarity gene *wingless* is required for proliferation of cells in the Malpighian tubules (Skaer and Martinez-Arias, 1992). Intriguingly, wingless may be expressed during the early stages of spermatogenesis, as an enhancer trap line inserted at *wingless* drives lacZ expression in cyst progenitor cells and early cyst cells (data not shown). It will thus be of interest to determine whether removal of *wingless* function in the cyst cell lineage using the FLP/FRT system will affect the proliferation of neighbouring germ cells.

CHAPTER 8: *roughex* IS A DOSE-DEPENDENT REGULATOR OF MEIOSIS II

INTRODUCTION

During spermatogenesis in all organisms, germ cells execute two meiotic divisions in quick succession, and then undergo extensive cellular differentiation as haploid cells to produce functional sperm. The regulation of the two meiotic divisions and their coupling to the subsequent differentiation program is poorly understood.

In *Drosophila*, the meiotic divisions can be studied through the isolation of male-sterile mutants, some of which affect meiosis (Lifschytz and Hareven, 1977; Lifschytz and Meyer, 1977; Hackstein, 1991; Castrillon et al., 1993; reviewed by Fuller, 1993). The majority of meiotic mutants exhibit defects during chromosome segregation or cytokinesis, suggesting that they identify structural components of the division apparatus. For instance, mutations in ß2-tubulin, a testis-specific ß2-tubulin isoform, result in failure to assemble the spindle of the first meiotic division (Kemphues et al., 1982). To date, only a few male-sterile mutants affecting the regulation of the meiotic divisions have been identified. Among these, *twine* and *pelo* are required for entry into meiosis I. In both mutants, germ cells fail to initiate chromosome segregation or cytokinesis, although they undergo some aspects of post-meiotic differentiation as 4N cells (Alphey et al., 1992; Courtot et al., 1992; Castrillon et al., 1993; White-Cooper et al., 1993).

twine encodes a homologue of *S. pombe* cdc25, a universal cell cycle regulator controlling entry into M phase (Russell and Nurse, 1986; Alphey et al., 1992; Courtot et al., 1992; reviewed by Millar and Russell, 1992). In all eukaryotic cells, the G2 to M transition is triggered by a kinase, composed of a cdc2 catalytic subunit and a G2 cyclin regulatory subunit (Draetta and Beach, 1988; Booher et al., 1989; Draetta et al., 1989; Giordano et al., 1989; Meijer et al., 1989; Pines and Hunter, 1989). Several steps are

involved in the activation of this kinase (reviewed by Nurse, 1990). Levels of cyclin proteins rise as cells progress through G2 (Evans et al., 1983). Cyclins associate with cdc2, but the complex is maintained inactive by phosphorylation of cdc2 (Gould and Nurse, 1989; Solomon et al., 1990; Krek and Nigg, 1991; Norbury et al., 1991). Entry into M is triggered by cdc25 which removes the inhibitory phosphorylation from cdc2 (Dunphy and Kumagai, 1991; Gautier et al., 1991; Millar et al., 1991; Strausfeld et al., 1991). The activated cdc2 kinase in turn triggers the early events of M, which include chromosome condensation, spindle formation and, in most species, nuclear membrane breakdown. For the completion of M phase, cdc2 kinase must be inactivated, which is accomplished by the destruction of the cyclin subunit through ubiquitin mediated proteolysis (Murray et al., 1989; Ghiara et al., 1991; Glotzer et al., 1991). Upon exit from M, chromosomes segregate and then decondense, cytokinesis occurs and microtubules redistribute into an interphase network.

Modifications of the cdc2 kinase cycle must exist at meiosis. First, in order to generate haploid gametes, a single round of DNA replication is followed by two successive divisions. In mitotically dividing cells, feedback controls ensure that M phase takes place only if S phase has been completed (reviewed by Hartwell and Weinert, 1989; Murray, 1992). These controls must be modified to allow meiosis II to be coupled to meiosis I without an intervening S phase. Second, in oocytes of many species, meiosis II occurs shortly after meiosis I, and germ cells do not return to a full interphase between the two divisions. Chromosomes remain condensed and the nuclear envelope does not reform, despite a drop in cdc2 kinase activity (Dorée et al., 1983; Gerhart et al., 1984; Draetta et al., 1989). It has been proposed that a kinase activated at meiosis I prevents a full interphase by maintaining the phosphorylation of some cdc2 substrates between the two meiotic divisions (Hunt and Murray, 1993b).

It is not known what specific modifications of the cdc2 kinase cycle may be required for the meiotic divisions during *Drosophila* spermatogenesis. cdc2 kinase is

likely to be essential for meiosis I, since the cdc25 homologue *twine* is required at this transition. By extension, the two characterized fly G2 cyclins, cyclin A and cyclin B (Lehner and O'Farrell, 1989; Whitfield et al., 1989; Lehner and O'Farrell, 1990; Knoblich and Lehner, 1993), are probably also involved in meiosis I. The regulation of meiosis II is not understood, however, because of the lack of mutants that specifically affect this division.

In this report, we demonstrate that the gene *roughex* is a dose-dependent regulator of meiosis II during *Drosophila* spermatogenesis. We find that the decision to execute meiosis II is made at meiosis I, and suggest that cyclin A-cdc2 kinase at meiosis I activates an unidentified target necessary for meiosis II.

RESULTS

From the set of male-sterile mutations that we generated on the X chromosome (see Chapter 5), we recovered an insertion in *roughex* (*rux*) (see Materials and Methods), a locus initially identified on the basis of its rough eye phenotype. The *rux* gene has been recently cloned and is predicted to encode a 335 aa protein with no homologies to other proteins in the data base (Thomas et al., 1994).

We designated our P-element induced male-sterile allele as rux^9. Southern blot analysis indicated that the P-element in rux^9 inserted into a 6 kb restriction fragment containing the *rux* transcription unit (Fig. 24A and 24B). This insertion event severely disrupted rux mRNA production, as rux^9 mutant testes contained significantly less rux mRNA than control testes (Fig. 24C).

rux^9 is a strong allele, similar in severity to rux^8, a likely null encoding only the first 79 aa of the protein (Thomas et al., 1994). Both rux^8 and rux^9 males had a similarly severe eye phenotype (Fig. 24E and 24F; Thomas et al., 1994), as well as an essentially indistinguishable phenotype during spermatogenesis (see below and data not shown).

Taken together, these observations suggest that rux^9 (hereafter referred to as *rux*) is a strong loss-of-function allele of *rux*.

roughex mutant germ cells execute the two meiotic divisions

Spermatogenesis in *rux* mutant testes was essentially indistinguishable from wild-type until after the meiotic divisions.

At the tip of the wild-type testis (Fig. 25A), a germ line stem cell divides, generating a daughter gonial cell, which then undergoes four mitotic divisions. The resulting 16 germ cells, enclosed by two somatic cyst cells, form a cyst and undergo premeiotic S phase close to the testis tip (see Fig. 12C and 12E). An extended G2 period follows (Fig. 25A, region between arrowheads), during which cysts move to

approximately the first coil of the testis, where germ cells undergo the two meiotic divisions, generating cysts of 64 haploid germ cells.

The fidelity of the meiotic divisions can be scored by examining germ cells at the stage which immediately follows, termed the onion stage (Fig. 25B; Fuller 1993). If chromosome segregation has proceeded normally, each of the 64 cells in a cyst contains one nucleus, and all nuclei within a cyst are of equal size (Fig. 25B, arrows). These nuclei appear as pale spheres (Fig. 25C, arrows) and have decondensed chromatin (Fig. 25D, arrows). In addition, the microtubules of the second meiotic division spindle have redistributed to the cytoplasm (data not shown). These characteristics indicate that the germ cells have exited the second meiotic division. The mitochondria in each haploid cell have aggregated into a structure called the nebenkern, which appears as a dark sphere equal in size to the nucleus (Fig. 25C and 25D, arrowheads). If cytokinesis proceeded normally during the meiotic divisions, each of the 64 haploid cells received evenly distributed cytoplasmic contents and mitochondria. As a result, each cell has one nebenkern and all nebenkerne within a cyst have a uniform size (Fig. 25B).

In *rux* mutants, most onion stage cysts were indistinguishable from wild-type (Fig. 25E, compare with Fig. 25B). We occasionally observed onion stage cysts with nuclei of unequal size, suggesting a rare defect in chromosome segregation (data not shown; see Mateials and Methods). The majority of *rux* onion stage cysts appeared normal, since they contained 64 haploid cells, each with a nucleus and nebenkern of equal size, indicating proper execution of the two meiotic divisions. Just as in wild-type, the nuclei appeared as pale spheres (Fig. 25F, arrows) and had decondensed chromatin (Fig. 25G, arrows), indicating that the germ cells had exited the second meiotic division.

roughex **haploid germ cells attempt to undergo an additional division after meiosis II**

Following the onion stage, *rux* haploid germ cells undergo an aberrant sequence of events (Fig. 26), which represents the first consistent deviation from wild-type.

In aberrant *rux* haploid cells, the nucleus was no longer visible (Fig. 26B, arrows), and counterstaining with a DNA dye revealed that the chromosomes had recondensed (Fig. 26C, arrows). Aberrant cysts contained 64 cells (Fig. 26A), each with a nebenkern and one complement of chromatin, indicating that the germ cells had properly executed the two meiotic divisions. Staining with an anti-tubulin antibody showed that a spindle had reassembled (Fig. 26D, arrow). This spindle appeared monopolar, suggesting that the centriole had not duplicated. Loss of interphase nuclear morphology, reappearance of condensed chromosomes and reassembly of a spindle all indicate that *rux* haploid germ cells attempt to undergo an extra nuclear division.

In addition to cysts containing only aberrant cells (Fig. 26A), we also observed mixed cysts in which some cells were aberrant while others in the same cyst had a normal onion stage appearance (data not shown). However, no normal germ cells were found in later stage *rux* cysts. Therefore, the mixed cysts are most likely not caused by a partially penetrant phenotype. Rather, the mixed cysts probably reflect the normal asynchrony in germ cell development within a cyst (Fuller, 1993). We infer that the mixed cysts contain some cells which just completed meiosis II and have a normal onion stage appearance, as well as slightly older cells which have already attempted the extra nuclear division. This suggests that the extra nuclear division takes place shortly after meiosis II.

The haploid set of chromosomes appeared to distribute randomly during the extra nuclear division (Fig. 26C and 26E, arrows). Probably as a consequence, later mutant cysts contained cells with nuclei of various size and number (Fig. 26F, arrows). These aneuploid nuclei had regained an interphase morphology (Fig. 26F, arrows) and had decondensed chromatin (Fig. 26G, arrows).

In addition to undergoing an extra nuclear division, some *rux* cells also appeared to attempt an extra cytokinesis, as indicated by a stretching of the nebenkern (Fig. 26H,

arrowheads). This probably resulted in the occasional splitting of the nebenkern, since some cysts contained more than 64 nebenkerne of various sizes and shapes (Fig. 26I, arrowheads).

The attempted extra nuclear division and cytokinesis indicate that *rux* mutant germ cells progress through an additional M phase after meiosis II. Lack of BrdU incorporation demonstrated that the additional division is not preceded by a round of DNA replication (data not shown). In wild-type, both DNA replication and centriole duplication occur prior to meiosis I, but not prior to meiosis II (Tates, 1971; Fritz-Niggli and Suda, 1972). In *rux* mutants, there is no S phase and apparently no centriole duplication prior to the additional division. Thus, the preparation for the additional division resembles that for meiosis II. We conclude that *rux* mutant germ cells execute the two regular meiotic divisions, go into a brief interphase and then aberrantly undergo an extra division, the preparation for which is similar to that for the second meiotic division.

Increased dose of *roughex* results in failure to execute meiosis II

Since loss of *rux* function results in an additional division after the two meiotic divisions, we asked whether, conversely, an increased dose of *rux* might result in a failure to progress normally through the meiotic divisions. We were able to address this question with a strain bearing a genomic rescue construct containing only the *rux* transcription unit. Flies homozygous for this construct and wild-type at the endogenous locus have an increased dose of *rux* (see Materials and Methods).

Germ cells with excess *rux* function executed only one division at meiosis and exited the meiotic division program as cysts of 32 germ cells (Fig. 27A). These germ cells started to differentiate but failed to give rise to functional sperm, resulting in sterility. We investigated the segregation of chromosomes during this single division to determine whether it was a first or a second meiotic division. In wild-type meiosis I, the X and Y sex chromosomes, which are cytologically distinguishable (Cooper, 1950; Goldstein,

1980), segregate to opposite poles, as do homologous chromosomes. During meiosis II, sister chromatids separate. During the single division executed by germ cells with an increased dose of *rux* , the X and Y chromosomes separated and segregated to opposite poles (Fig. 27B). Thus, germ cells with an increased dose of *rux* undergo meiosis I, but fail to execute meiosis II.

While excess *rux* function prevents the execution of the second meiotic division, loss of *rux* function results in an additional division resembling the second meiotic division. Therefore, *rux* is a dose-dependent regulator of meiosis II during spermatogenesis.

Cyclin B expression does not appear to be regulated by *roughex*

It is likely that the two *Drosophila* mitotic G2 cyclins, cyclin A and cyclin B, regulate cdc2 kinase activity at meiosis, although this has not been directly tested. To investigate the mechanism by which *rux* regulates meiosis II, we thus compared the expression of cyclin A and cyclin B in wild-type to that in *rux* mutants and increased *rux* dose.

We first examined the expression of cyclin B. The available antibody did not allow us to reliably quantitate the level of cyclin B protein (see Materials and Methods). However, cyclin B protein expression was qualitatively identical in wild-type, *rux* mutants and increased *rux* dose (Fig. 28 illustrates cyclin B expression for *rux* mutants). In all three cases, cyclin B accumulated in the cytoplasm of spermatocytes during the G2 preceding the meiotic divisions (Figs. 28A and 28B, arrows). Most cyclin B was degraded during the first meiotic division (data not shown). Cyclin B was present in the brief interphase between the two meiotic divisions (Figs. 28A and 28B, arrowheads). The presence of cyclin B just prior to meiosis II is consistent with a role of cyclin B-cdc2 kinase in driving this division. Interestingly, cyclin B was also present at low levels in the post-meiotic onion stage (Fig. 28C and 28D, arrows). Since this stage just preceedes the

extra division in *rux* mutants, it is possible that cyclin B-cdc2 kinase plays a role in driving the additional division.

The absence of a detectable change in cyclin B expression when the dose of *rux* is altered does not necessarily rule out cyclin B as a target of *rux* function. There may yet be differences in levels or *rux* could affect cyclin B post-translationally.

Cyclin A is a target of *roughex* function during the G2 preceding the meiotic divisions

In contrast to cyclin B, cyclin A expression was altered both in *rux* mutants and with increased *rux* dose.

In wild-type, cyclin A accumulated in the cytoplasm of spermatocytes during the G2 preceding the meiotic divisions (Fig. 29A, arrow). As has been observed for mitosis (Lehner and O'Farrell, 1989), cyclin A moved to the nucleus as chromosomes condensed at prophase of meiosis I, and then rapidly disappeared (data not shown). Cyclin A protein was not detected after this stage. In particular, cyclin A was not found in the brief interphase between the two meiotic divisions (Fig. 29B and 29C, arrows). The absence of cyclin A just prior to meiosis II suggests that cyclin A-cdc2 kinase can not directly drive this M phase.

In *rux* mutants, cyclin A accumulated to higher levels in the cytoplasm of spermatocytes in the G2 preceding the meiotic divisions (Fig. 29D, arrow, compare with 29A). As in wild-type, cyclin A then moved to the nucleus at prophase of meiosis I, rapidly disappeared and was not found between the two meiotic divisions (data not shown). Importantly, cyclin A was not detected at the onion stage (Fig. 29E and 29F, arrows), just prior to the extra division executed by *rux* mutants. Thus, cyclin A-cdc2 kinase is unlikely to directly drive the extra division. Cyclin A reaccumulated in older stage cysts towards the base of *rux* mutant testes (Fig. 29D, arrowhead), in an area which is devoid of cyclin A protein in the wild-type (Fig. 29A). However, this reaccumulation

occurred after the additional M phase and therefore could not be its cause. Thus, the only alteration in cyclin A expression detectable *in situ* prior to the additional division in *rux* mutants is the increased level of cyclin A protein during the G2 preceding the meiotic divisions.

We performed a Western blot in order to verify that levels of cyclin A were indeed higher in *rux* mutant testes (Fig. 30A). This experiment first showed that *rux* mutants accumulate a slower migrating form of cyclin A. This form was present but barely detectable in wild-type (compare lanes 1 and 2) and co-migrated with the previously described slower migrating form seen in embryos (lane 3) (Lehner and O'Farrell, 1989). This form disappeared upon treatment with calf alkaline phosphatase (Fig. 30B) or potato acid phosphatase (data not shown), indicating that it is a phosphorylated form of cyclin A. Cyclins in other species have been shown to be transiently phosphorylated at the G2 to M transition (Standart et al., 1987; Meijer et al., 1989; Pondaven et al., 1990). Thus, *rux* mutants might accumulate a form of cyclin A normally transiently present at the G2 to M transition. In addition, the Western blot confirmed the presence of excess total cyclin A protein in *rux* mutant testes (Fig. 30A, compare the overall signal in lanes 1 and 2). However, since proteins from whole testes were used, we could not determine whether the higher level or the phosphorylated form of cyclin A was associated with the the G2 preceding the meiotic divisions or, alternatively, strictly with the reappearance of cyclin A towards the base of the testis.

If cyclin A protein were a target of *rux* function during the G2 preceding the meiotic divisions, cyclin A expression might also be altered when the dose of *rux* is increased. Western analysis showed that overall levels of cyclin A were identical to wild-type (data not shown). Interestingly however, cyclin A protein was found predominantly in the nucleus during the G2 preceding the meiotic divisions in testes with an increased dose of *rux* (Fig. 29G, arrow).

Thus, changes in the dose of *rux* affect cyclin A protein during the G2 preceding the meiotic divisions. Increasing the dose of *rux* alters the subcellular localization of cyclin A, while reducing *rux* function results in excess and possibly modified cyclin A. These observations suggest that cyclin A is a target of *rux* activity. However, the two effects on cyclin A are not simply opposite and therefore do not suggest an immediate explanation for the mechanism by which *rux* might act on cyclin A (but see Discussion).

Lowering the dose of *cyclin A* or *twine* rescues the *roughex* phenotype

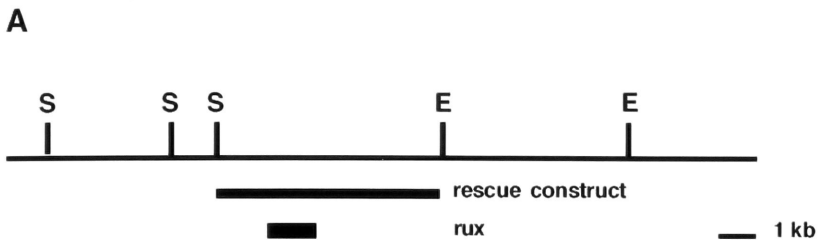
The additional division in *rux* mutants correlates with excess and possibly modified cyclin A during the G2 preceding the meiotic divisions. If these changes in cyclin A caused the extra division, then diminishing the amount of cyclin A may result in rescue of the *rux* mutant phenotype. Lowering the dose of *cyclin A* in a *rux* mutant background can be achieved by generating males mutant for *rux* and heterozygous for a mutation in *cyclin A*. Table 7 shows that such males were fertile, suggesting that the *rux* spermatogenesis phenotype is indeed due to excess or modified cyclin A present during the G2 preceding the meiotic divisions. The rescue to fertility also demonstrates that *rux* is not an obligatory component of post-meiotic differentiation.

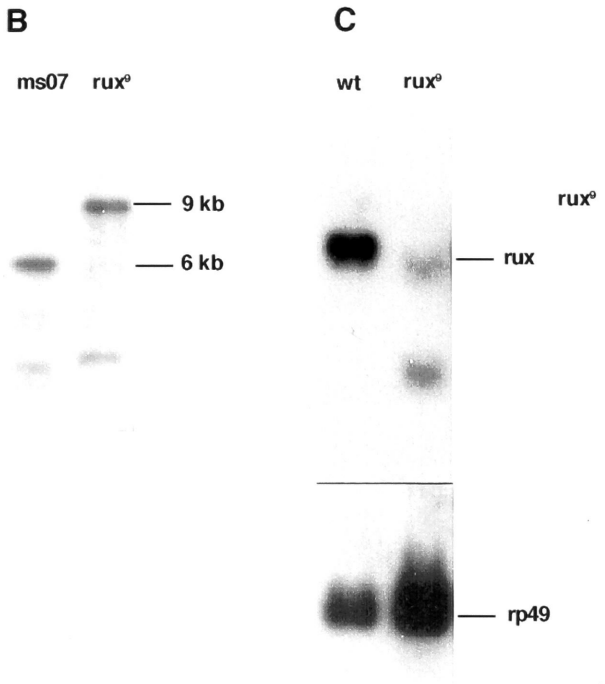
If the extra division is in fact due to excess or modified cyclin A-cdc2 kinase present at the G2 to M transition of meiosis I, then lowering the dose of *twine*, the cdc25 which triggers this transition, may also rescue the *rux* mutant phenotype. This was indeed the case, since most males mutant for *rux* and heterozygous for a mutation in *twine* were fertile (Table 7). By contrast, there was no rescue by lowering the dose of *string* (*stg*) (Table 7), which is the cdc25 used in mitotic cells (Edgar and O'Farrell 1989), showing that only lowering the dose of the specific cdc25 homologue used in meiotic cells rescues the *rux* spermatogenesis phenotype. Moreover, there was no rescue by lowering the dose of cdc2 (Table 7), which is thought to be a non rate-limiting component of cell cycle progression (Nurse and Thuriaux, 1980; Stern et al., 1993).

The presence of cyclin B at the onion stage raised the possibility that cyclin B-cdc2 kinase was involved in the additional M phase in *rux* mutants. Lowering the dose of *cyclin B* resulted in marginal rescue of the *rux* mutant phenotype (Table 7), consistent with some involvement of cyclin B-cdc2 kinase in progress through the extra division. However, since the rescue was much less than that observed with either *cyclin A* or *twine*, it is unlikely that cyclin B is the main target of *rux* function. Instead, excess or modified cyclin A-cdc2 kinase at meiosis I is likely responsible for the additional division that occurs after meiosis II in *rux* mutant germ cells.

Fig. 24 *roughex*^{9} is a severe loss-of-function allele. **(A)** Map of the *rux* genomic region (adapted from Thomas et al., 1994). The 6 kb genomic region into which the P-element in *rux*^{9} has inserted (see below), and which corresponds to the piece of DNA used as a *rux* rescue construct is indicated, as is the *rux* transcription unit. S=StuI; E=EcoRI. **(B)** Southern blot of DNA from control (*ms(2)S07*, isogenic strain) and *rux*9 males; the DNA was digested with StuI and EcoRI, blotted and probed with *rux* cDNA. The 6 kb restriction fragment present in the control DNA is replaced by a larger 9 kb piece in *rux*^{9} DNA, as a consequence of the insertion of the P-element into the 6 kb fragment. Weaker cross-hybridizing bands were also detected, and may represent related sequences in the genome. **(C)** Northern blot of RNA from wild-type and *rux*^{9} testes, probed with *rux* cDNA (top) or a ribosomal protein cDNA (rp49, bottom), as a loading control. An approximately 1.3 kb *rux* mRNA (Thomas et al., 1994) is detected in wild-type, and the transcript is severely diminished in levels in *rux*^{9} testes (at least 10 fold, as judged by the relative intensity of the rux signals, compared to the amount of RNA present, which is given by the rp49 signals). Moreover, a smaller, approximately 500 bp transcript is also detected in *rux*9 mutant testes, which may result from inappropriate transcript initiation within the P-element and read-through into the *rux* coding sequence, or premature transcription termination. **(D, E, F)** Scanning electron micrographs of **(E)** wild-type **(E)** *rux*^{9} and **(F)** *rux*^{8} eyes. The severity of the eye phenotype is similar in the two *rux* alleles. Magnification: x200.

Figure 24


A
S
S
S
E
E
rescue construct
rux
1 kb


B
ms07
rux⁹
9 kb
6 kb
C
wt
rux⁹
rux⁹
rux
rp49

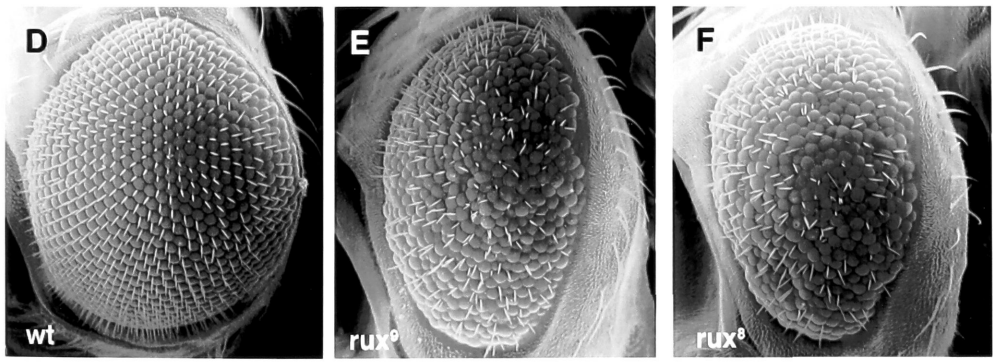
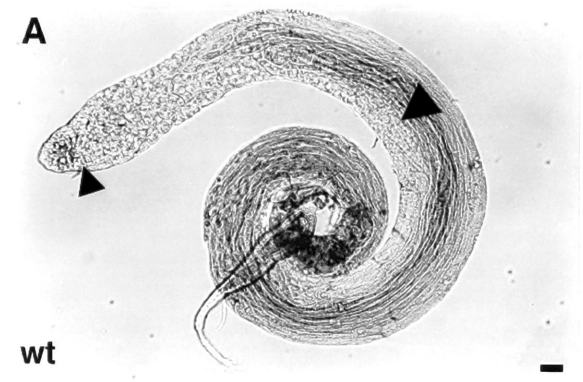
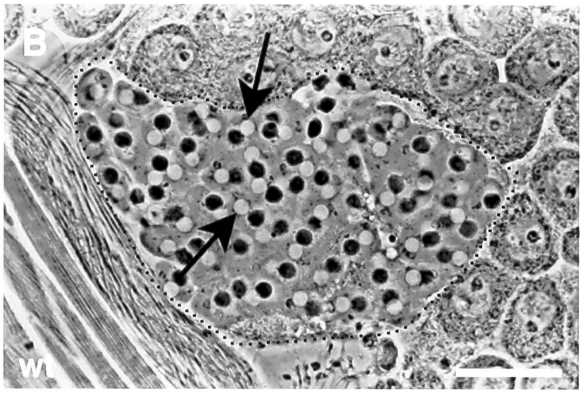
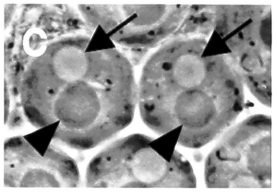

D
wt
E
rux⁹
F
rux⁸

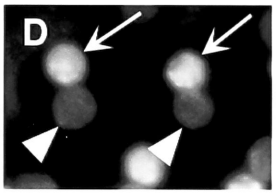
Fig. 25 *roughex* mutant germ cells execute the two meiotic divisions.**(A)** Wild-type testis. Spermatogenesis begins at the tip of the testis (left). Germ cells mature as they move towards the coiled region (right). Premeiotic S occurs near the tip (see Fig. 12E) and is followed by an extended premeiotic G2 (area between arrowheads). At approximately the first coil of the testis, germ cells undergo the two meiotic divisions. **(B, C, D, E, F, G)** Germ cells at the onion stage which immediately follows the meiotic divisions; dotted lines indicate the limits of intact cysts in this and subsequent figures. **(B, C, E, F)** Phase contrast. **(D, G)** DNA counterstain of panels **(C, F)**. **(B)** Wild-type and **(E)** rux^{9}: cyst of 64 haploid cells with nuclei (arrows) and nebenkerne (dark spheres) of equal size, indicating that proper chromosome segregation and cytokinesis have occurred during the two meiotic divisions. **(C, D)** Wild-type and **(F, G)** rux^{9}: nebenkerne (arrowheads), and nuclei (C and F, arrows) with decondensed chromosomes (D and G, arrows), indicating that germ cells have exited the M phase of the second meiotic division. Bars=50 μm. The field is 50 μm in small panels.

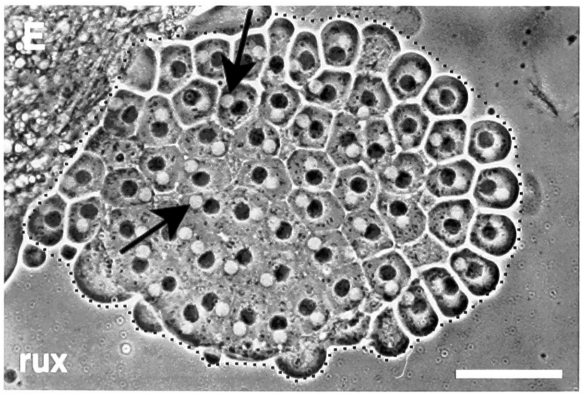
Figure 25

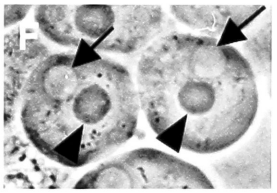












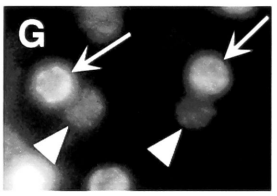


Fig. 26 *roughex* mutant germ cells undergo an attempted extra division after meiosis II. **(A, B, C, D, E)**: *rux*^{9} germ cells at the aberrant stage following the onion stage. **(A, B)** Phase contrast; **(C)** DNA couterstain of **(B)**. **(A)** Cyst of 64 haploid cells which properly executed the regular meiotic divisions, as seen by the presence, in each cell, of one nebenkern and one haploid chromosome complement (revealed by DNA counterstain, see panel C). **(B)** There no longer is a morphologically recognizable nucleus (arrows) next to the nebenkern (arrowheads) in each haploid cell. **(C)** The haploid complement of chromosomes has recondensed (arrows). **(D)** Indirect immunofluorescence with anti-tubulin antibody; **(E)** DNA counterstain of **(D)**. A spindle (D, arrow) reassembles in *rux* mutant germ cells as chromosomes have recondensed (E, arrow); this spindle appears monopolar, suggesting failure of centriole duplication prior to the extra division. **(F, G)** *rux*^{9} germ cells at an older stage. **(F)** Phase contrast; **(G)** DNA couterstain of panel **(F)**. Mutant cells in this older cyst have nuclei of variable size and number (arrows), probably as a result of the random distribution of chromosomes during the extra nuclear division. **(H, I)** Slightly older *rux*^{9} germ cells; phase contrast. **(H)** Cyst of 64 mutant cells, some of which have a stretched nebenkern (arrowheads), suggesting an attempted extra cytokinesis. **(I)** Cyst with more than 64 nebenkerne of variable size and shape (arrowheads), probably as a result of the splitting of the nebenkern during the attempted extra cytokinesis. Other mutant cysts at a similar stage contained only 64 nebenkerne, indicating some variability in the extent of the attempted extra cytokinesis (not shown). Bars=50 μm.

Figure 26

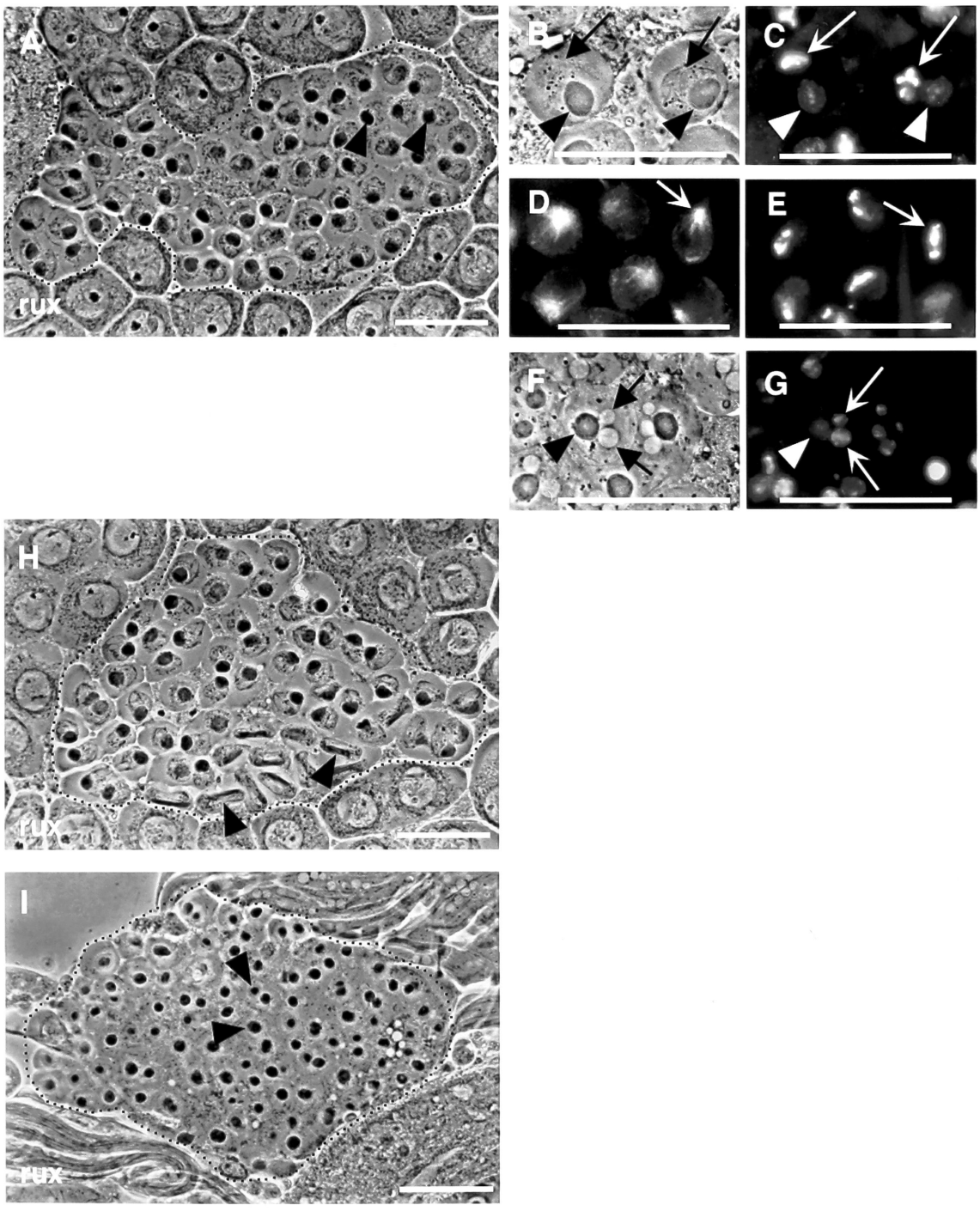


Fig. 27 Increased dose of *roughex* results in failure to execute meiosis II. **(A)** Early post-meiotic cyst of germ cells with increased *rux* dose; phase contrast. The cyst contains 32, rather than 64, germ cells. Each nucleus and nebenkern is larger than that seen in wild-type, when germ cells have executed both meiotic divisions (compare with Fig. 25B). Bar=50 μm. **(B)** Meiotic chromosomes from testes with increased dose of *rux*; early anaphase. Arrows point towards the poles of the division spindle. The X (bottom) and Y chromosome (top) are apposed and separating, as they are during wild-type meiosis I. Thus, excess *rux* results in a failure to execute meiosis II. The three autosomal pairs (right) have separated and are segregating towards the poles; the dot-like fourth chromosome pair is on the very right. There is a slight variation in the position of the chromosome pairs in relation to the metaphase plate, and also asynchrony in the onset of anaphase when comparing the different chromosome pairs. These features are also observed in wild-type (see Cooper 1950). Bar is 10 μm in this figure.

Fig. 28 Cyclin B protein is present between the meiotic divisions and at the onion stage. **(A, B, C, D)** rux^{9}. **(A, C)** Indirect immunofluorescence with anti-cyclin B antibody; **(B, D)** DNA counterstain of panels **(A, C)**. **(A, B)** Cells in premeiotic G2, recognizable by their size and typical decondensed DNA, accumulate cyclin B in the cytoplasm (A, arrow). Cyclin B is also present in germ cells between the two meiotic divisions; a cyst of 32 germ cells just entering meiosis II is visible (A and B, arrowhead). Note the age gradient within the cyst, with germ cells to the left being slightly more advanced, as best seen by the lower levels of cyclin B. **(C, D)** Cyclin B is also detected in post-meiotic germ cells at the onion stage (arrow); curiously, cyclin B was also found in the nebenkern in other young post-meiotic cysts (not shown). Bar=50 μm; all panels are at the same magnification.

Figure 27

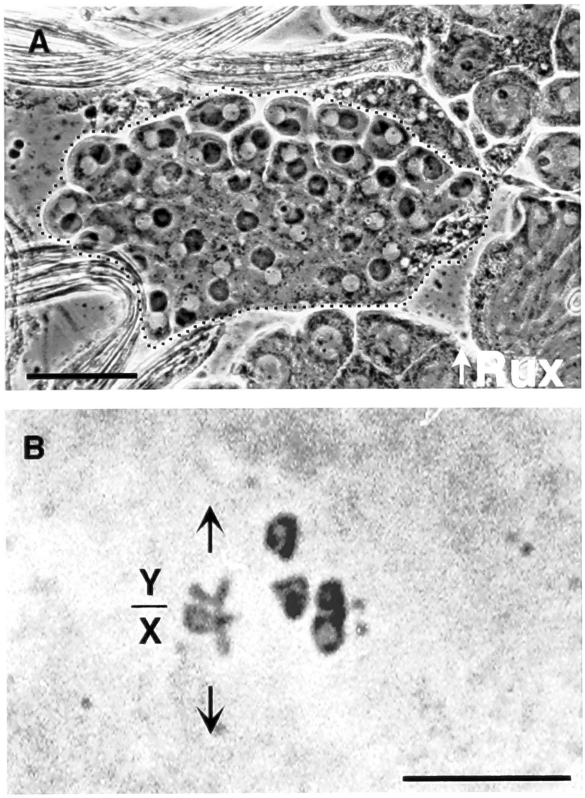


Figure 28

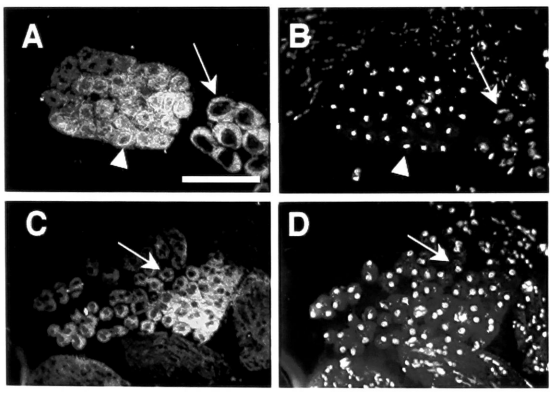


Fig. 29 Cyclin A protein is a target of *roughex* function. **(A, B, C, D, E, F, G)** Immunocytochemistry of whole-mount testes with anti-cyclin A antibody. **(A, D, G)** Premeiotic G2 area is shown. **(C, F)** DNA counterstain of panels **(B, E)**. **(A, B, C)** Wild-type. **(A)** During premeiotic G2, cyclin A accumulates in the cytoplasm (arrow); cyclin A is not detected after prophase of meiosis I. **(B, C)** Cyclin A is not detected prior to meiosis II; a cyst of 32 germ cells between the two meiotic divisions is visible (arrows). **(D, E, F)** rux^9. **(D)** Cyclin A accumulates to higher levels than in wild-type (arrow, compare with A). Note that cysts in premeiotic G2 do not extend as far along as in wild-type; this is likely due to the larger volume available for cysts in premeiotic G2 due to the lack of elongated sperm tails. Entry into meiosis I is not precocious in mutant testes, since 96-100 hours separate premeiotic S from metaphase of meiosis I in both wild-type and rux^9 mutant testes (data not shown). Cyclin A later reaccumulates in older stage cysts located towards the base of the testis (arrowhead). **(E, F)** Cyclin A is not detected at the onion stage prior to the extra division (E, arrow); haploid nuclei at the onion stage have decondensed chromatin (F, arrow). **(G)** increased *rux* dose. During premeiotic G2, cyclin A accumulates in the nucleus (arrow). Bars=50 μm.

Fig. 30 A phosphorylated form of cyclin A accumulates in *roughex* mutant testes. **(A)** Western blot with proteins from wild-type testes, rux^9 testes or 0-1 hour wild-type embryos, probed with anti-cyclin A antibody. *rux* mutants accumulate a slower migrating form of cyclin A (arrow) that is barely detectable in wild-type and that comigrates with the slower migrating form detected in embryos. **(B)** Proteins from rux^9 testes immunoprecipitated with anti-cyclin A antibody and either untreated, treated with calf alkaline phosphatase (CIP) or with heat inactivated calf alkaline phosphatase. The Western was probed with anti-cyclin A antibody. Treatment with phosphatase eliminates the slower migrating form seen in *rux* mutant testes, showing that it is a phosphorylated form of cyclin A.

Figure 29

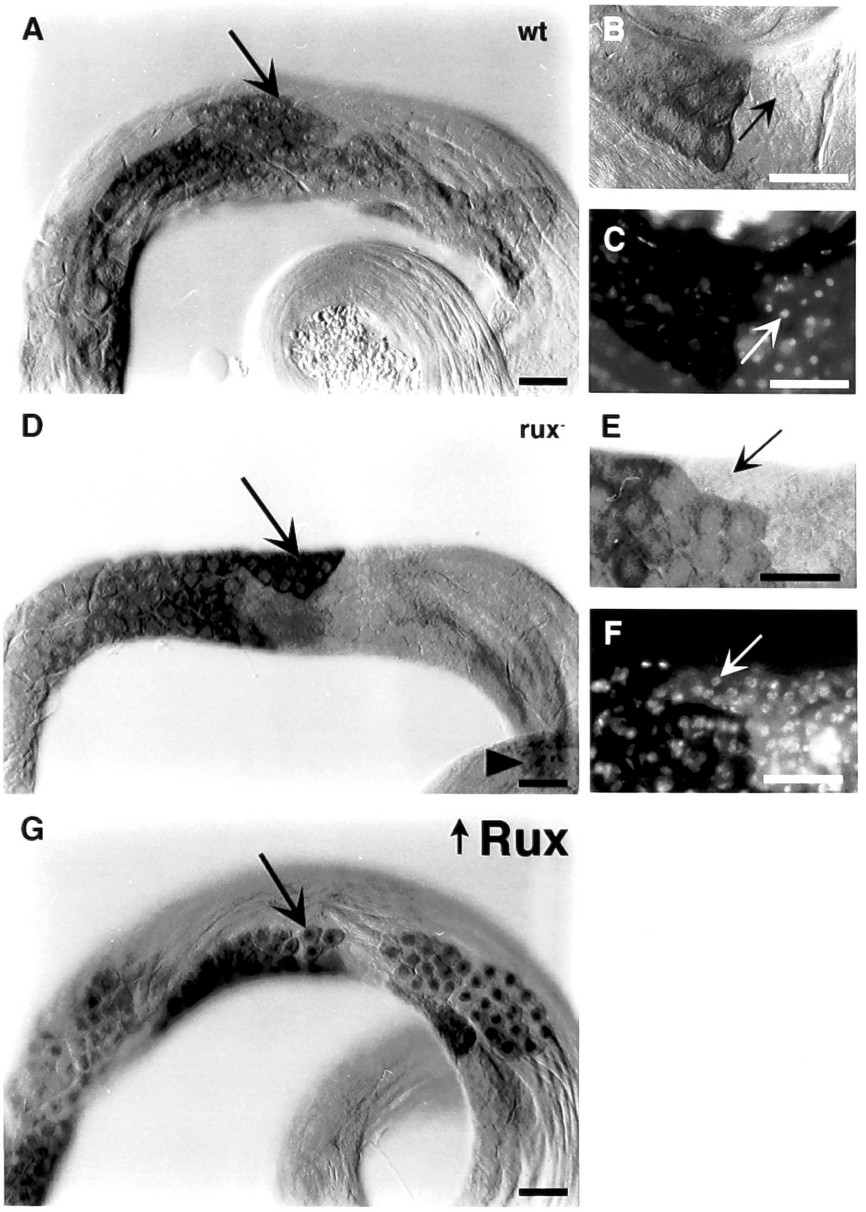


Figure 30

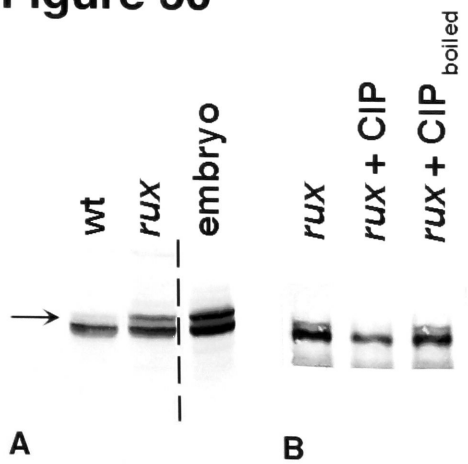


Table 7

Rescue of the *roughex* spermatogenesis phenotype

Genotype:	*rux*; + / +	*rux*;*cycA* / +	*rux*;*twi* / +	*rux*;*cycB* / +	*rux*;*cdc2* / +	*rux*; *stg* / +	wild-type
Fertile	0	25	12	1	0	0	25
Semi-sterile	1[a]	0	7	12	0	0	0
Sterile	24	0	6	12	25	25	0

Fertility assays performed with individual rux^9 males at 18 °C; rescue to fertility was reduced at 25 °C (see Material and Methods). Fertility of individual males was scored as one of the following: 1) fertile (over 100 progeny) 2) semi-sterile (less than 20 progeny) 3) sterile (no progeny). Mutations are referenced in Material and Methods. Lowering the dose of *cyclinA* or *twine* similarly rescued rux^8 males to fertility (data not shown).

[a] This was the only *rux* male to ever give rise to any progeny; it should be noted that this is an exceptional case (see for instance crosses to *stg* or *cdc2*).

DISCUSSION

In the absence of *rux* function, germ cells execute meiosis I and meiosis II, but then undergo an additional division as haploid cells. The preparation for this division resembles that for meiosis II, since it occurs without a preceding S phase. Excess *rux* function conversely results in a failure to execute meiosis II. Thus, *rux* is a dose-dependent regulator of meiosis II. A previously described male-sterile mutant also fails to execute meiosis II (Lifschytz and Meyer, 1977). In *(1)RA40*, however, defects are already apparent during meiosis I, when most cells arrest in early anaphase and have abnormal spindles. *(1)RA40* is thus probably required for both meiotic divisions. By contrast, an even further increase in the dose of *rux* still has no consequence on the execution of meiosis I (data not shown). Therefore, *rux* provides a unique experimental handle to analyze the regulation of the second meiotic division. Interestingly, our analysis reveals that *rux* regulates meiosis II by acting prior to meiosis I.

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Control of the second meiotic division

Cyclin A is a target of *rux* function and thus a central factor in the decision to execute meiosis II. However, cyclin A-cdc2 kinase is unlikely to directly drive meiosis II, since cyclin A protein is undetectable between the two meiotic divisions. *rux* acts during the G2 preceding the meiotic divisions, when cyclin A accumulates to higher levels in *rux* mutants, while it is mislocalized to the nucleus with excess *rux* function. Both alterations likely affect cyclin A-cdc2 kinase at the G2 to M transition of meiosis I. The *rux* mutant phenotype is rescued by lowering the dose of *cyclin A* or *twine*, suggesting that it is caused by excess cyclin A-cdc2 kinase. With excess *rux* function, perhaps the lack of cyclin A in the cytoplasm reduces the level of cyclin A-cdc2 available for activation by *twine*. Taken together, these data suggest that *rux* regulates meiosis II by modulating cyclin A-cdc2 kinase activity at meiosis I. Thus, the decision to execute meiosis II is

coupled to the execution of meiosis I. Furthermore, these data imply that a target of cyclin A-cdc2 kinase at meiosis I in turn triggers meiosis II.

Lowering the dose of a gene encoding the target of cyclin A-cdc2 kinase should fully rescue the *rux* mutant phenotype. Cyclin B-cdc2 kinase is therefore not the main target, since lowering the dose of *cyclin B* results in only marginal rescue of the *rux* mutant phenotype. Perhaps the target is a cdc2 kinase associated with a novel G2 cyclin. An additional B-type cyclin has been recently identified in *Drosophila* (Christian Lehner, personal communication), but its expression during spermatogenesis has not yet been characterized and no mutants are available. Alternatively, the target of cyclin A-cdc2 kinase could be a non-cdc2 kinase. MAP-kinase is such a candidate, since it can be activated by cdc2 kinase, and since it is present between the meiotic divisions in the frog oocyte (Ferrell et al., 1991; Gotoh et al., 1991; Gotoh et al., 1991; Posada et al., 1991). However, decreasing the dose of *rolled*, which encodes a *Drosophila* MAP-kinase (Biggs et al., 1994; Brunner et al., 1994), failed to rescue the *rux* mutant phenotype (data not shown).

Although cyclin B is not the target of cyclin A-cdc2 activity, cyclin B-cdc2 kinase probably participates in the execution of meiosis II, as indicated by the marginal rescue obtained by lowering the dose of cyclin B. We conclude that *rux* modulates cyclin A-cdc2 activity at meiosis I, and that a target of this kinase then acts in concert with cyclin B-cdc2 to trigger meiosis II. In *rux* mutants, we suggest that excess cyclin A-cdc2 kinase at meiosis I overstimulates this target, causing its activity to persist even after meiosis II. This in turn triggers an extra division, together with cyclin B-cdc2 kinase, which is normally present. With excess *rux* function, levels of cyclin A-cdc2 kinase at meiosis I may be too low to stimulate the target. As a consequence, meiosis II is not executed, despite the normal presence of cyclin B-cdc2. This implies that cyclin B-cdc2 kinase is not sufficient for meiosis II.

Two distinct activities are also required for an M phase in *A. nidulans*, where both cdc2 kinase and the NimA kinase are necessary for mitosis (Osmani et al., 1991). Interestingly, the expression of nek1, a mammalian homologue of NimA, is restricted to gonads (Letwin et al., 1992). In testes, nek1 mRNA is detected in meiotic and immediate post-meiotic germ cells. A non-cdc2 kinase may thus play a role in the meiotic divisions during mammalian spermatogenesis.

roughex acts during G2 to negatively regulate the following cell cycle

During eye development, *rux* also appears to regulate cyclin A-cdc2 kinase at a G2 to M transition. In the wild-type eye disc, cells in the morphogenetic furrow enter a G1 phase during which they receive inductive signals for patterning (Thomas et al., 1994). In *rux* mutants, these cells prematurely undergo the G1 to S transition and patterning is disrupted, resulting in a rough eye phenotype. The mutant phenotype correlates with increased expression of the *stg* gene in the preceding G2. The *rux* eye phenotype is suppressed by lowering the dose of *cyclin A* or *stg* (Thomas et al., 1994) suggesting that it is also caused by excess cyclin A-cdc2 kinase. Therefore, both during spermatogenesis and eye development, *rux* appears to act during G2 to negatively regulate a cell cycle transition in the following cell cycle. This distinguishes *rux* from other negative regulators of cell cycle progression, which block a transition in the phase of the cell cycle in which they act. Thus, Rb or Cip1 act during G1 to delay progress through the G1 to S transition (Mercer et al., 1990; Goodrich et al., 1991; Harper et al., 1993), while Wee1 or Mik1 are necessary during G2 to delay the G2 to M transition (Russell and Nurse, 1987; Lundgren et al., 1991; Parker et al., 1992).

Excess cyclin A-cdc2 kinase has different consequences on cell cycle progression during eye development and spermatogenesis. Whereas *rux* mutant cells in the eye disc undergo a premature G1 to S transition, mutant germ cells undergo an additional M phase after meiosis II. Perhaps this difference is due to distinct targets being activated by cyclin

A-cdc2 kinase at meiosis I and in the morphogenetic furrow. Alternatively, the same target may be activated, but lead to a different effect on cell cycle progression due to the special character of the meiotic cell cycle. Indeed, during meiosis, feedback controls must be modified to allow two M phases to occur without an intervening S phase. Thus, meiotic germ cells may only be competent to execute an M phase. Loss of *rux* function does not disrupt this control, since no S phase occurs prior to the extra division.

How does *roughex* function?

There appears to be a distinct target for *rux* activity during spermatogenesis compared to eye development. In the eye, loss of *rux* function affects the expression of *stg*, but not cyclin A, in the G2 prior to the G1 to S transition (Thomas et al., 1994). Reciprocally, during the G2 preceding the meiotic divisions, loss of *rux* function results in changes in cyclin A, but not in *twine* expression (this work; data not shown). It is possible that loss of *rux* function in either tissue causes the same defect, increased cyclin A-cdc2 activity, but that rate-limiting components leading to cdc2 activation differ between the eye and spermatogenesis. This could explain the altered expression of distinct cell cycle regulators for each tissue.

Increasing the dose of *rux* does not result in a phenotype in the eye (Thomas et al., 1994). Therefore, *rux* is a dose-dependent regulator only for meiosis II. Both excess and loss of *rux* function affect cyclin A protein during the G2 preceding the meiotic divisions. Thus, cyclin A is a strong candidate for a target of *rux* function during spermatogenesis. Understanding the effects of *rux* on cyclin A should help elucidate the biochemical activity of the *rux* protein.

rux mutant germ cells accumulate high levels of cyclin A. However, augmenting the amount of cyclin A protein in a wild-type background, by increasing *cyclin A* dose, does not result in a *rux*-like phenotype (data not shown). This implies that cyclin A protein in *rux* mutants is in some way modified. The rescue of the mutant phenotype by

lowering cyclin A dose can be explained if reducing the total pool of cyclin A protein shifts an equilibrium away from this modified form. It is possible that the modified form causing the phenotype is the observed phosphorylated form of cyclin A.

Further insight into how *rux* acts could come from understanding the cause of the nuclear mislocalization of cyclin A caused by excess *rux* function. Whatever may be causing this does not have a general effect on nuclear import, since, for instance, cyclin B remains cytoplasmic during the G2 preceding the meiotic divisions. Since cyclin A is required for S phase in some species (Girard et al., 1991; Pagano et al., 1992; Zindy et al., 1992), we considered whether the cells with nuclear cyclin A were undergoing DNA replication. However, this is not the case, since they do not incorporate BrdU (data not shown). Since cyclin A normally translocates to the nucleus at prophase of meiosis I, we also considered whether *rux* could be a positive regulator of this process, whose overexpression causes precocious nuclear import. This is unlikely, however, since nuclear translocation at meiosis I occurs normally in *rux* mutants. Alternatively, the abnormal nuclear localization raises the possibility that factors are required during the G2 preceding the meiotic divisions for anchoring cyclin A protein in the cytoplasm. *rux* could be a negative regulator of such a factor, thus explaining the nuclear translocation of cyclin A observed with excess *rux* function. In *rux* mutant testes, cyclin A might be better anchored in the cytoplasm, thus explaining the observed higher protein levels. The proposed cytoplasmic anchoring factor is reminiscent of the role played by *cactus* in inhibiting the nuclear translocation of the dorsal morphogen in the dorsal region of the *Drosophila* embryo (Roth et al., 1989; Rushlow et al., 1989; Steward, 1989). Finally, it is also possible that the nuclear mislocalization of cyclin A is an artifact of excess *rux* function and thus misleading in terms of *rux* activity. However, this seems unlikely, given that loss of *rux* function and increased *rux* dose have complementary consequences on the regulation of meiosis II.

Conclusion

rux function during spermatogenesis reveals a novel mode of regulation for meiosis II. We propose that cyclin A-cdc2 kinase at meiosis I activates a target which persists between the meiotic divisions and is required for meiosis II. Given that spermatogenesis is evolutionary conserved, it is possible that this mode of coupling the two meiotic divisions is also utilized in other species.

There is another case where targets activated at meiosis I have been postulated to play a role later during meiosis (Hunt and Murray, 1993). In many species, germ cells do not return to a full interphase after meiosis I. This is the case during spermatogenesis in other *Drosophila* species (Cooper, 1950), as well as during oogenesis in a variety of organisms, where it has been demonstrated that this occurs despite a drop in cdc2 kinase activity (Dorée et al., 1983; Gerhart et al., 1984; Draetta et al., 1989). Thus, kinases which share substrate specificity with cdc2 kinase may be active between the meiotic divisions to prevent germ cells from returning to a full interphase. Perhaps these unidentified kinases serve a dual function and also participate in the execution of meiosis II.

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CHAPTER 9: CONCLUSIONS AND PROSPECTS

Spermatogenesis in *Drosophila* offers a series of challenges and opportunities to developmental biologists. A succession of fundamental processes, including stem cell renewal, counting of mitoses, meiosis, and morphogenesis, are amenable to cellular analysis and genetic dissection. At the onset of our project, despite a comprehensive description of the sequence of events at the ultrastructural level, little was known of the mechanisms ensuring progress through spermatogenesis.

Almost six years later, we are still far from understanding these mechanisms in much detail. Nevertheless, our work has laid down the foundation of a molecular genetic dissection of spermatogenesis. Moreover, we have identified two loci controling germ cell proliferation, as well as a key regulator of meiosis II.

At the onset of our project, we needed to generate a set of reagents, in particular enhancer trap lines, to allow investigations of spermatogenesis at the light microscope level. We then decided to isolate P-element induced male-sterile strains, and characterize the mutant phenotype and the molecular nature of select loci regulating the early steps of spermatogenesis. Our achievements of these two aims is discussed below. We then suggest directions for the future that should pave the way for a molecular genetic dissection of spermatogenesis in *Drosophila*.

Marker lines to probe spermatogenesis

We retained a set of 89 marker lines after screening over 4000 enhancer trap lines for patterned lacZ expression in testes. Collectively, these strains identify all somatic cell types, and probably all stages of germ and cyst cell differentiation during spermatogenesis. For instance, by virtue of these marker lines, cyst cell differentiation can

be broken down into four successive stages prior to the meiotic divisions. Of course, novel and more refined domains of expression may still be found. For example, since different P-element constructs insert preferentially at distinct sites in the genome (see Kassis, 1990), novel expression patterns could be revealed by using another insertional vector. However, we note that various P-element constructs were used to generate the strains we have analyzed. Therefore, we believe that most possible lacZ expression patterns during spermatogenesis have been recognized and are represented in the set of 89 marker lines.

The marker lines are particularly valuable in following somatic cell fates. While the progress of germ cells through most stages of spermatogenesis can be analyzed by phase contrast microscopy, this is no so for somatic cells. Cyst cells are extremely thin and, therefore, difficult to recognize in such preparations. We have identified stage-specific cyst cell marker lines, with which cyst cells can be followed along their differentiation program. We have used such stage-specific markers in the agametic testis to show that cyst cells begin their differentiation in the absence of germ cells. We have also used such markers to demonstrate that cyst cells are blocked in their differentiation program, just like germ cells, in *bam* and *bgcn* mutant testes, raising the possibility that cyst and germ cells communicate during early spermatogenesis.

One could use the marker lines for the various somatic cell types to screen through male-sterile mutants and ask whether alterations in somatic cell fates have occured in some cases. We could not conduct such an analysis with our collection of P-element induced male-sterile mutants, as they themselves carry a P-lacZ insert, which is in most cases expressed in testes. Rather than crossing all possible somatic marker lines to all possible male-sterile mutants, one may instead probe the fate of specific somatic cells in subsets of mutant strains. For instance, the mutants which affect post-meiotic differentiation in a non-descript manner, and in which the primary defect has not been identified, could be probed with cyst cell markers to examine whether these somatic cells

are absent or altered in fate. A rarer class of male-sterile mutants have no apparent defect until the very last stages of spermatogenesis, and yet no sperm is found in the seminal vesicle. Although spermatogenesis in these mutants may be defective for a variety of reasons, one of them may be a faulty interaction between the head cyst cell and the terminal epithelial cell at the base of the testis. This interaction normally preceeds sperm individualization, coiling, and release in the seminal vesicle. The availability of marker lines for both head cyst cells and terminal epithelial cells should permit one to test this hypothesis.

A subset of marker lines is instrumental in investigating the position, size and shape of the hub in male-sterile mutants. The hub is diplaced in mutations at two loci identified among our male-sterile strains, *ms(2)916* and *fumble*. Interestingly, both mutations are associated with defects in germ cell proliferation. Although we followed the displaced hub with fasciclin III antibody in these cases, this may not be possible in other instances. Indeed, fasciclin III expression at the hub is deregulated under certain circumstances, for example in the absence of germ cells or in *chic* mutant testes. In cases such as these, it would be difficult to rely solely on fasciclin III staining to localize a displaced hub. The hub-specific marker lines, on the other hand, seem to have more persistant expression, possibly because ß-galactosidase is fairly stable in these post-mitotic cells. Therefore, hub-specific marker lines are a valuable counterpart to fasciclin III staining to examine the fate of the hub.

Even though the progress of germ cells through the growth phase, meiotic divisions and early stages of post-meiotic differentiation can be followed by phase contrast microscopy, this is not true of earlier stages of spermatogenesis. First, cells from early stages do not flow out from the dissected tip of the testis, and are thus less visible, being under the testis sheath. Second, germ line stem cells and their gonial daughters are morphologically indistinguishable (Hardy et al., 1979). Therefore, marker lines are also valuable to follow the fate of early germ cells. For instance, markers specific to the stem

cells and their immediate daughters allowed us to address the nature of overproliferating germ cells in *bam* and *bgcn* mutant testes. We also identified markers specific to male germ cells. As it is difficult to determine sexual transformation of XX germ cells based on morphological criteria alone, these male-specific markers, along with other markers of sexual fate such as the mode of Sxl RNA splicing, are an important asset for further deciphering germ line sex determination. In addition, as enhancer trap lines identify genes potentially expressed as the lacZ reporter, male-specific marker lines may identify transcription units specific to male, but not female, germ cells, and thus identify genes that are inactivated in XX germ cells as a result of *Sxl* expression.

Towards the molecular genetics of spermatogenesis

At the onset of this project, we had the choice between two kinds of mutageneses to isolate male-sterile mutants, among which we were hoping to identify loci regulating stem cell fate, germ cell proliferation and transit through meiosis. The first possibility would have been to use chemical mutagenesis to saturate at least one chromosome for male-sterile mutations. This would have had the advantage of giving an extensive knowledge of the kinds and frequencies of male-sterile mutants one can get. Moreover, a chemical mutagenesis would have generated different kinds of alleles, some of which could have been extremely useful for understanding gene function and determining epistatic relationships. However, we were worried that a saturation mutagenesis and the subsequent mapping and complementation analyses would take most of the time allocated, not leaving much for exploring the mutant phenotypes. Moreover, the prospects of molecularly characterizing any of the identified loci in the course of our work were dim.

Because of these reservations, we chose instead to isolate P-element induced male-sterile strains, despite two obvious limitations associated with a P-element mediated mutagenesis. First, insertion events occur preferentially in some genes, and not at all in

others, jeopardizing the identification of some loci of interest. Second, P-element induced mutations are almost always recessive loss-of-function alleles, and thus do not provide the genetic richness resulting from a chemical mutagenesis. However, P-element induced mutations can be rapidly located on the cytological map, thereby accelerating complementation and deficiency analyses. Moreover, new alleles, including nulls, can be readily generated by imprecise excision of the P-element. Finally, DNA flanking the insertion site can be easily recovered and lead to a rapid molecular characterization of the locus. We also felt that the small *Drosophila* spermatogenesis field was in need of molecular entry points that would, in turn, allow the design of more refined screens to identify interacting components.

Therefore, we established a collection of male-sterile strains that should help us and others dissect the genetic control of spermatogenesis. Ironically, however, we did not get the opportunity to exploit the presence of a P-element to determine the molecular nature of four loci that we chose to further analyze.

First, as we had rescued neighbouring genomic DNA and identified corresponding genomic phages, we realized that *ms(2)1112* ($chic^{11}$) was allelic to *chickadee*, whose molecular characterization had just been completed in Lynn Cooley's lab. Second, we did not clone *roughex*, as Larry Zipurski's lab had already made considerable progress in characterizing this gene. Importantly, however, owing to the localization of the P-element on the cytological map, we were able to rapidly suspect that our two male-sterile mutants were alleles of *chic* and *rux*, respectively. This may have taken more time with EMS induced alleles. Rapidly realizing that we had mutations in *chic* and *rux* was instrumental in using appropriate alleles from the Cooley and Zipurski labs to better understand the function of these genes during spermatogenesis.

In a third case, that of *ms(2)916*, the resident P-element was not responsible for the mutant phenotype, and was, therefore of course useless for the molecular characterization of this locus.

We initiated the molecular characterization of a fourth P-element induced male-sterile mutation, *fumble*, but have not completed the cloning of the gene yet. Our phenotypic analysis indicates that, while playing some role in restricting germ cell proliferation during spermatogenesis, *fumble* is also generally required for cytokinesis or an aspect of cell cycle progression linked to that process (an Appendix at the end of Materials and Methods details our phenotypic and molecular analysis of the *fumble* locus).

Besides the four loci already mentioned, our lab is also interested in at least two more P-element induced male-sterile mutants. The first is *ms(1)S07*, a tagged male-sterile on the X chromosome, in which germ cells do not progress past the growth phase, and then degenerate. *ms(1)S07* could thus be required for progress through an early stage of the G2 preceding the meiotic divisions. The second locus of interest is *pitzi*, an underproliferation mutant that we recognized among a set of male-sterile strains identified by Maurizio Gatti. *pitzi* is being currenty phenotypically and molecularly analyzed by John Tran in the lab.

While we did not take full advantage of having isolated P-element induced male-sterile mutations, our collaborators in the Wasserman lab did so in at least three cases. First, they showed that *diaphanous*, which appears to be required during spermatogenesis for germ cell proliferation and is essential for the meiotic divisions, encodes a Bni1p/formin homologue generally required for cytokinesis in *Drosophila* (Castrillon, 1994). Second, they found that *pelota*, which is required during spermatogenesis for entry into meiosis I, encodes a gene with similarities to a yeast gene required for sporulation (Eberhardt and Wasserman, in preparation). Third, they determined that *effete*, which is required for post-meiotic differentiation, encodes a ubiquitin-conjugating enzyme (Rawson et al., in preparation). In these three cases, a knowledge of the molecular nature of the gene has reinforced the phenotypic analysis or helped design hypotheses and tests for the function of the locus during spermatogenesis. For instance, the general role of *dia*

in cytokinesis, along with the postulated role of *chic* in germ line stem cell cytokinesis, has focused the attention on the relationship between cell division and stem cell function. In the case of *effete*, a double mutant with *bendless*, which encodes another ubiquitin conjugating enzyme (Muralidhar and Thomas, 1993), results in an earlier spermatogenesis phenotype, which could help address the role of ubiquitination in the progress through meiosis (Rawson et al., in preparation). These examples illustrate how P-element induced mutations can rapidly turn into molecular entry points that help decipher the regulation of spermatogenesis.

More screens

While it may still be worthwile to conduct a saturation screen for loci that can mutate to male-sterility, it may be more useful to use chemical mutagens to conduct directed screens, trying to enrich for loci regulating specific processes during spermatogenesis. This is especially true for the early stages, which are affected in only a tiny fraction of all male-sterile mutants. What follows are some examples of possible screens that may identify novel regulators of the early processes of spermatogenesis.

First, we have demonstrated the feasibility of a FLP/FRT-based visual screen for overproliferation mutants, and we obviously hope to conduct such a screen on a large scale in the near future. Such a screen may identify loci required for the fate of the gonial daughter cell, the restriction of mitotic divisions, or the entry into meiosis. As discussed previously, additional classes of mutants could be recovered in such a screen if it were conducted with the set of FRT sites that result in lacZ expression upon mitotic recombination. In this case, we may also identify mutations changing the number of mitotic divisions, as well as mutations abolishing the proliferation of stem cells or mitotically amplifying germ cells.

One could also devise a simple visual screen specifically to identify loci required for the maintenance of stem cell fate. In mutant testes, stem cells would be initially

present, but divide in such a way that both daughters would embark on the differentiation program, depleting the testis of stem cells over time. Although such mutants are expected to have an underproliferation phenotype, it is possible that they are missed in male-sterile screens, if the mutated gene has no other role during spermatogenesis. Indeed, even a single symmetric division of the 15 or so stem cells present in the larval gonad would generate about 2000 spermatozoae, probably more than enough to appear fully fertile. However, such mutants should be easily recovered by visual inspection of testes from 5-day old males, as mutant testes should be thin and devoid of germ cells in the apical region, yet have sperm in the seminal vesicle.

An alternative visual screen could rely on marker lines that we have identified. For instance, a screen could be envisaged in the background of a hub or cyst cell marker, in order to follow possible alterations in these two cell types. Such a visual screen could be either coupled to one for male-sterile mutants, or simply performed by following lacZ expression, without a prerequisite for sterility. This could be important, as some alterations in hub or cyst cells may not result in male-sterility. For instance, a displaced hub, which would be recognized in a simple visual screen, might alter division number without resulting in sterility.

Two other screens could be conducted in a sensitized background. First, we have preliminary observations that *fasciclin III* null mutants, which are viable and fertile, have a slightly odd-shaped hub, as revealed by hub-specific marker lines. Therefore, a *fasciclin III* null background could potentially be interesting in allowing the identification of loci required for the integrity of the hub. In such a sensitized background, it may be sufficient to halve the dose of a gene that is rate-limiting for maintaining hub integrity to observe a phenotype. The feasibility of such a haplo-insufficient enhancer screen could be initially explored by crossing the *fasciclin III* null to the panel of deficiencies from the stock center deficiency kit, which covers about 50 % of the genome.

The prospect of another haplo-insufficient enhancer screen stems from our observation that occasional cysts of overproliferating germ cells are found in testes heterozygous for *bam*, in the background of various marker lines. Overproliferating germ cells are never observed in the original *bam* strain, possibly because of differences in the genetic background or the presence of modifiers. Such cysts have never been observed in either the original *bgcn* strain, or in testes heterozygous for *bgcn* in the background of various marker lines. We do not believe that the occasional phenotype in the outcrossed heterozygous *bam* males is the result form the disruption of a gene by the P-element from the marker line, as this phenotype is observed with several, though not all, lines examined. Thus, *bam* heterozygotes may provide a favorable sensitized background to look for mutations that enhance an overproliferation phenotype.

The one powerful screen that alone would make spermatogenesis worth studying is reversion from sterility. Sterile mutations provide an ideal situation in which to look for heterozygous or homozyogus suppressors, as only chromosomes carrying mutations of interest give rise to progeny. For instance, halving the dose of *cyclin A* or *twine* can restore fertility to a *roughex* mutant male. Additional suppressors of the sterility of *rux* males may identify components controling *rux* function, and thus help elucidate the mechanisms by which *rux* regulates cyclin A protein during the G2 preceding the meiotic divisions. Moreover, such suppressors should also identify components downstream of cyclin A-cdc2 kinase activity at meiosis I, and which are required for driving meiosis II.

Reversion to fertility could be attempted in a number of male-sterile backgrounds, including *bam* and *bgcn*. One may envisage to generate FLP-induced clones of novel homozygous mutant chromosomes in flies homozygous for *bam* or *bgcn*. The possibility to screen an extremely large number of chromsosomes may allow for the recovery of even a very limited number of interacting loci.

For a dynamic analysis of spermatogenesis

The ultrastructural serial reconstructions were precious in identifying all cell types present in testes. However, they generated a static picture from which the dynamic behavior of cells could only be infered. Most crucial aspects of cellular dynamics in the early stages of spermatogenesis have thus not been addressed. First, although germ line stem cells are identically arranged around the hub, it is not known whether they are equivalent in their division behavior. Do some stem cells divide more than others ? For oogenesis, clonal analysis revealed bursts of divisions in some stem cells, while others were quiescent (Schüpbach et al., 1978; Wieschaus and Szabad, 1979). Second, the ultrastructural work in spermatogenesis revealed a two fold decrease in the number of germ line stem cells between the larval and adult stages (Hardy et al., 1979). However, the fate of the missing half has not been established. Conceivably, those stem cells may have died or undergone a symmetric division such that both daughters embarked on the differentiation program. Third, the cell cycling properties of individual germ line stem cells have not been determined and may vary during development.

To begin addressing some of these questions, we are using the modified FLP/FRT system which results in lacZ expression in cells following a mitotic recombination event at the FRT's (Harrison and Perrimon, 1993). If this happens in a germ line stem cell, and the self-renewing stem cell is the permanently marked cell, then stem cell behavior can be traced by following its marked progeny over time. We have just begun this kind of analysis and will only discuss some preliminary observations here. Three kinds of germ line stem cells were revealed in an experiment where clones were analyzed 14 days after FLP induction. In most cases, a labeled germ line stem cell was present at the hub, and a succession of progressively older labeled cysts testified of the self-renewing activity of that stem cell over the two week period. In some other cases, one labeled germ line stem cell was found at the hub, but no corresponding labeled progeny was observed in the subsequent stages of spermatogenesis. Thus, this class of stem cells appeared to have divided at least once, as it became labeled, but did not divide again in the remainder of

the experiment. A third set of testes contained a whole family of progressively older labeled cysts, but no labeled stem cell was present at the hub, and there were often no marked clones in the proliferation region either. Therefore, marked stem cells in such testes probably acted as bona fide self-renewing cells for most of the 2 week period, but then either left the hub or died. This observation is in apparent contradiction with the reported unchanged number of stem cells in older males (but n=1 testis in that analysis; Hardy et al., 1979). Such studies of stem cell behavior may thus reveal a more complicated picture than that of a set of equivalent self-renewing stem cells. This plasticiy may provide regulatory opportunities which were not recognized before, and which may be altered in some mutant conditions.

The actual sequence of cellular events during the initial formation of a cyst are similarly poorly understood. Two somatic cyst progenitor cells and a germ line stem cell located between them, are postulated to divide in concert, to form a cyst of two cyst cells surrounding a primary gonial germ cell (see Fig. 2). It is important to elucidate how the coordination between these cells may be established. Do the three cells undergo S phase or mitosis in synchrony, or are they simply coordinated at the stage of cell division ? Our observations with BrdU incorporation experiments suggest that germ line stem cells and neighbouring cyst progenitor cells do not always undergo S phase in synchrony (data not shown). In addition, we have observed mitotic figures in germ line stem cells that were not accompanied by detectable mitotic figures in neighbouring cells. These observations suggest that synchrony between the divisions in the two lineages may be only established at cell division, which could be delayed in one cell type until completion of the process in the other. These preliminary observations do not, however, distinguish whether it is the germ line stem cell or the cyst progenitor cells that undergo S phase first. An answer to this question may be obtained through experiments in which BrdU labeling is followed by colchicine treatment, which will arrest cells in M phase. For instance, let us imagine that germ line stem cells underwent S phase first. In that case, such an experiment should

show some triplet metaphase figures with one unlabeled germ cell, which completed S phase before the labeling period, surrounded by two BrdU positive cyst progenitor cell nuclei, which underwent S phase during the labeling period. Such a result would suggest that cyst progenitor cells are restrained from undergoing S phase until germ line stem cells tell them to do so. With this scenario, cyst progenitor cells might no longer be restrained from progressing through the cell cycle in the absence of germ line stem cells, which is one way to explain the observed increase in cyst cell S phases in agametic testes.

To probe the direction of signals that may coordinate the division of germ line stem cells and neighbouring cyst progenitor cells, one may attempt to determine whether forcing cell cycle progression in one cell, say the germ line stem cell, would in turn force the neighbouring cyst progenitor cells to do the same. As cyclinE is a necessary and rate-limiting component for progress through the G1/S transition in the fly (Knoblich et al., 1994; Richardson et al, submitted), overexpression of cyclinE in the germ line stem cells may force them prematurely into S phase and allow one to ask whether cyst progenitor cells would follow soon after. Cell-specific cyclinE overexpression could be achieved by using a FLP-out cassette, which would result in the overexpression of cyclinE in only a subset of cells upon FLP inducion.

Intriguingly, the ratio of cyst progenitor cells to germ line stem cells changes over time, being close to 2 in adults, but closer to 1 in third instar larval gonads (Hardy et al., 1979). How do the excess germ line stem cells in the larval gonad get surrounded by the correct number of cyst cells ? Maybe the immediate daughter of a larval cyst progenitor cell undergoes another division, and the germ line gonial cell gets surrounded by two clonally derived cyst cells only then.

Curiously, in adult testes, we have occasionally observed BrdU incoporation in a germ line stem cell and in one neighbouring cyst progenitor cell, but without concommitent labeling in a cyst progenitor cell located on the other side of the germ line stem cell. Such deviations from the postulated mechanims of cyst production may be

exceptional, or, alternatively, be more general, and ultimately lead to revising the current model.

In summary, stem cell dynamics and the mechanisms of cyst formation are poorly understood, and both are likely to be modulated throughout development. Understanding these cellular behaviors will be key in better characterizing mutant phenotypes and unraveling the regulatory logic of early spermatogenesis.

What about the hub ?

The hub is bound to play a crucial role in the regulation of the early steps of spermatogenesis. Germ line stem cells and cyst progenitor cells are in contact with the hub, which has, therefore, been postulated to play a role in maintaining stem cell fates (Hardy et al., 1979). Moreover, the cells of the hub are surrounded by basal lamina, and stem cells in a variety of systems are similarly in contact with a basement membrane. Remarkably, the hub is extremely conserved throughout insect spermatogenesis, either as a group of small cells, such as in *Drosophila*, or as a single giant apically located cell, such as in *Bombyx*. Moreover, the cessation of spermatogenesis, which occurs early in the life of some insects, is correlated with the disappearance of the hub (reviewed by Roosen-Runge, 1977).

A cell located at the tip of a tubule plays a fundamental role in regulating the proliferation of neighbouring cells in at least two instances. First, the distal tip cell is required during *C.elegans* gametogenesis for the continued proliferation of germ cells (Kimble and White, 1981). Second, a cell located at the tip of the developing Malpighian tubule in *Drosophila* and *Rhodnius*, is also required for the proliferation of neighbouring cells (Skaer, 1989; Skaer, 1992).

A potential role for the hub, however, remains to be tested. Stimulated by the ablation experiments in the worm gonad and insect Malpighian tubule, we made some ill-fated attempts towards physically ablating the hub. Erika Matunis in the lab is currently

trying to remove the hub in another way: she is screening P-GAL4 enhancer trap lines to identify some that express lacZ in the cells of the hub, and plans on crossing such lines to flies carrying a construct with UAS sequences upstream of a cold-sensitive diphteria toxin (Bellen et al., 1992). In this manner, diphteria toxin could selectively be expressed in the cells of the hub, and thus interfere with their function. Activating the conditional diphteria toxin at different times during development may reveal earlier roles for the hub, such as that of organizing the larval gonad. Interestingly, besides promoting mitoses in neighbouring germ cells, the *C. elegans* distal tip cell is also required earlier during development for elongating the hermaphrodite gonad (Kimble and White, 1981). We hope to soon learn what role the hub is playing in orchestrating early *Drosophila* spermatogenesis.

Conclusion

We set out to explore the molecular genetics of spermatogenesis in *Drosophila*, focusing on the early stages of this developmental program. We generated a collection of marker lines and P-element induced male-sterile mutants that provide a valuable starting material for further analysis. Moreover, we identified components regulating germ cell proliferation, as well as a key regulator of meiosis II. Although much remains to be done, our work has opened the door to investigations that should decipher the regulation of fascinating biological processes. The field is wide open.

CHAPTER 10: MATERIALS AND METHODS

Fly strains

Crosses were carried out at 25 °C, unless otherwise noted, in vials containing freshly yeasted cornmeal molasses agar. w^- flies were used as wild-type. The following mutations were of particular use: snf^{1621} is a hypomorphic allele of *sans-fille,* which interferes with germ line sex determination and results in overproliferating germ cells in ovaries (Gans et al., 1975; Gollin and King, 1981; Oliver et al., 1988; Steinmann-Zwicky, 1988); Sxl^{f4} is a point mutant which affects the germ line function of *Sex-lethal*, and results in a similar overproliferating phenotype (Salz et al., 1987; Bopp et al., 1993); $bam^{\Delta 86}$ removes most *bag-of-marbles* coding sequences (McKearin and Christerson, 1994); $bgcn^{QS2}$ is an EMS allele of *benign gonial cell neoplasm* ($peppercorn^{QS2}$ in Schüpbach and Wieschaus, 1991); $chic^{37}$ is a severe, homozygous viable allele of *chickadee* (Verheyen and Cooley, 1994), $cycA^{5}$ removes cyclin A coding sequences (Lehner and O'Farrell 1989); Df(2R)59AB removes the cyclin B coding sequence (Knoblich and Lehner 1993); Df(2L)J27 removes the cdc2 coding sequence (Stern et al. 1993); $twine^{HB5}$ is a point mutant in *twine* (Schüpbach and Wieschaus 1991; Alphey et al. 1992; Courtot et al. 1992); stg^{4} is an EMS allele of *string* which is described and referenced, along with all other mutations and marker chromosomes, by Lindsley and Zimm (1992).

Enhancer trap lines

For the first set of strains analyzed in Chapter 2, over 700 viable and fertile autosomal P-element enhancer trap lines were generously provided to us by the laboratories of Yuh-Nung and Lily Jan (Bier et al., 1989; 500 lines, preselected against

those with a staining pattern in the embryonic nervous system, 1a in Table 1), Margaret Fuller and Matthew Scott (150 lines, 1b in Table 1), Judy Kassis (Kassis, 1990; 60 lines, 1c in Table 1) and by Laurent Fasano (Fasano and Kerridge, 1988; 15 lines, preselected for patterns during oogenesis and cytologically mapped, 1d in Table 1) and the Bloomington *Drosophila* Stock Center (20 lines, preselected for patterns during spermatogenesis, 1e in Table 1). At least one line in this first set turned out to be homozygous lethal (line 254, Fig. 7A through 7G, an insertion at *esg*; Whitheley et al., 1992).

Most lines in the first set, except those from Judy Kassis and Laurent Fasano, carried the P-lacW construct described in Bier et al. (1989). Upon lacZ expression, these lines gave rise to nuclear X-gal staining in premeiotic germ cells (see for instance Fig. 3C, arrow) and in somatic cells (see for instance Fig. 6B, arrows), as expected from the presence of the nuclear targeting signal upstream of the lacZ reporter gene. ß-galactosidase was present in both the nucleus and the cytoplasm of post-meiotic germ cells, thereby showing up in the elongating sperm tails (see Fig. 3B and 3C, arrowhead). This is probably due to the reshaping of the spermatid nucleus whose diminished volume might not be able to efficiently retain the nuclear targeted ß-galactosidase. The lines from Laurent Fasano carried P[lac, ry^{+}]A (O'Kane and Gehring, 1987), which similarly contains a nuclear targeting signal.

The lines from Judy Kassis carried constructs with sequences from the *engrailed* (*en*) gene, including a fragment from the en promoter, fused to the lacZ reporter gene with an AUG codon (Kassis; 1990). In addition to the en-specific patterns discussed by Kassis (1990), many of these lines had expression patterns dependent on the insertion site in the genome, effectively behaving as enhancer traps. Owing to the lack of a nuclear targeting signal, lines expressing lacZ gave rise in this case to cytoplasmic X-gal staining (see Fig. 7A through 7F).

The origin of the second and third sets of enhancer trap lines analyzed for patterned lacZ expression in Chapter 2 is described below under "Single P-element insertion lines". All the lines from sets 2 and 3 had a lacZ reporter construct with a nuclear localization signal.

The majority of enhancer trap lines also expressed lacZ in other cells in the adult. However, since this did not interfere with their use as marker lines to probe spermatogenesis, the characterization of expression patterns outside the gonads was not pursued further.

We learned to better recognize the different cell types present in testes during the course of this work. Therefore, subtle expression patterns may have been disregarded in the initial phase of the study. In addition, some lines examined have been preselected as indicated above. Thus, the frequency of occurence for a particular staining pattern could be only approximated, and is indicated in the text when relevant.

Obtaining agametic males carrying P-lacZ

Homozygous *oskar*^{301} (*osk*^{301}; Lehmann and Nüsslein-Volhard; 1986) female virgins were collected, aged at 18 ^{o}C for 4 days and crossed to males from the different marker lines. At 18 ^{o}C, *osk*^{301} mothers give rise to viable progeny that lack pole cells, resulting in flies with agametic testes, and carrying one copy of P-lacZ.

FLP/FRT mediated induction of homozygous *bgcn* mutant clones

First, *bgcn*^{QS2} was recombined onto a chromosome carrying P[ry^{+}, hs-neo FRT]43D (Rubin lab). A stock that is w^{-}; P[ry^{+}, hs-neo FRT]43D, *bgcn*^{QS2}/CyO was established. For the reconstruction experiment, we first crossed males that were w^{-}/Y; P[ry^{+}, hs-neo FRT]43D, P[miniw^{+}, hs-ΠM]45F, 47F/ P[ry^{+}, hs-neo FRT]43D, P[miniw^{+}, hs-ΠM]45F, 47F (Xu and Rubin, 1993) to y^{-}w^{-} females carrying MKRS-FLP99 (Chou and Perrimon, 1992) on the third chromosome. The resulting male progeny, of genotype

y^-w^-/Y; P[ry^+, hs-neo FRT]43D, P[$miniw^+$, hs-ΠM]45F, 47F/ +; MKRS-FLP99/+ was then crossed to w^-/w^-; P[ry^+, hs-neo FRT]43D, *bgcn*^{QS2}/CyO; +/+ females. Two classes of male progeny resulting from this cross were utilized in the reconstruction experiment: 1) w^-/Y; P[ry^+, hs-neo FRT]43D, *bgcn*^{QS2}/ P[ry^+, hs-neo FRT]43D, P[$miniw^+$, hs-ΠM]45F, 47F; MKRS-FLP99/+. These males contained the FLP recombinase, and were either heat-pulsed (experimental animals) or not (control animals). 2) w^-/Y; P[ry^+, hs-neo FRT]43D, *bgcn*^{QS2}/ P[ry^+, hs-neo FRT]43D, P[$miniw^+$, hs-ΠM]45F, 47F; +/+. These males did not contain the FLP recombinase, and were heat-shocked as a second set of control animals. For the heat-shock, flies were sampled from the pad, placed in food vials, allowed to recovered for 15 min, trapped at the bottom of the vial by the cotton plug, and then tranfered to a 38 ^oC water bath for 60 min. Larvae were hand-picked and similarly placed at the bottom of a food vial for the duration of the heat-shock. Vials with larvae or flies were then placed in the 25 ^oC incubator until analysis 3, 5 or 7 days later.

Single P-element insertion lines

A first set of strains was generated by us as described in detail in Figure 14. An estimated 1015 lines containing new insertions of P-lacW (Bier et al., 1989) on a y^-w^- X chromosome were generated and balanced with FM7c. 14 male-sterile and semi-sterile strains were recovered from this set (see Table 2).

The second set of 28 autosomal male-sterile mutations (see Table 3) was identified among single P-element insertion lines carrying P[lac, ry^+]A (O'Kane and Gehring, 1987), which were generously provided to us by the UCLA fly groups. Insertions events were on an unmarked second chromosome or a *ry*^{506} third chromosome (see Bownes, 1990).

The set of male-sterile strains identified by the Wasserman lab came from a collection of approximately 8000 lines, generated by Spradling and collaborators (Spradling, 1993). The crossing scheme used for the generation of these strains is

described elsewhere (Karpen and Spradling, 1992). Insertiond of the P-element were on an isogenized *cn* second chromosome or a ry^{506} third chromosome.

Identification of male-sterile mutants

While the first set of male-sterile mutants, those on the X chromosome, were immediately recognized during the mutagenesis (see Figure 14), male-sterile mutants from the second and third sets were identified among P-element insertion lines by performing fertility assays. Eight to ten homozygous males were crossed with an equal or greater number of virgin wild-type females. Homozygotes were designated as sterile if there were no progeny from these crosses, and as semi-sterile if they produced fewer than 10% the number of progeny produced by heterozygous siblings.

Viability for each autosomal male-sterile line was measured among the progeny of a cross between balanced heterozygotes. Mutations were deemed to be semi-lethal if homozygotes were present at less than 30% the expected frequency.

Female fertility was assayed for the autosomal lines by crossing at least five homozygous females from a given line with an equal number of balancer sibling males.

Individual fertility assays (*roughex* rescue experiment)

Males of the relevant genotype were grown at 18 ^{o}C in uncrowded bottles. Two to five days old males were individually crossed to 3 virgin females and vials were checked 6 days later for the presence of larvae. Individual males were classified as fertile, semi-sterile, or sterile as indicated in Table 7. Rescue of the *rux* mutant phenotype was more modest at 25 ^{o}C. The better rescue at 18 ^{o}C is perhaps because the assembly of the additional division spindle is impaired at 18 ^{o}C, since microtubules are known to be destabilized at low temperatures.

In situ hybridization

Larvae for salivary gland squashes were simply grown in the case of enhancer trap marker lines, or generated by an outcross to wild-type in the case of male-sterile lines. After 2-3 days at 25 °C, bottles were transferred to 18 °C and supplemented with fresh yeast. *In situ* hybridizations and Giemsa staining were carried out essentially as described (Ashburner, 1989b). A digoxygenin labeled P[lArB] plasmid (Wilson et al., 1989) was used as probe.

Complementation and deficiency crosses

Complementation tests were performed by crossing each male-sterile mutation from the expanded collection of 83 autosomal male-sterile mutations to chromosomal deficiencies potentially spanning the insertion site, as well as mutations that mapped within two lettered polytene divisions, including: 1) other insertion mutations from the collection; 2) other cytologically localized, male-sterile mutations induced with P-elements (Cooley, Berg and Spradling, 1988); 3) alleles of *β2-tubulin* and *fruitless* (Kemphues et al., 1979; Gailey and Hall, 1989); and 4) mutations that are both male- and female-sterile from the collection of Schupbach and Wieschaus (1991).

Reversion analysis

Autosomal insertion-bearing chromosomes were brought together with a transposase source by crossing flies from the balanced mutant stocks to flies carrying the P[ry$^+$ Δ 2-3] transposase source on either the second or third chromosome (Robertson et al., 1988, M. Sanicola and W. Gelbart, personal communication). In the next generation, the P[ry$^+$ Δ 2-3] chromosome was segregated away and *ry*$^-$ derivatives of the original insertion bearing chromosome were selected and used to establish lines. The *ry*$^-$ derivatives were then made homozygous or put in *trans* to the original insertion for tests of male and female fertility.

For male-sterile mutations on the X chromosome, balanced females were crossed to males carrying the P[ry$^+$ Δ 2-3] transposase source (Robertson et al., 1988). The dominant Sb marker of the P[ry$^+$ Δ 2-3] chromosome was segragated away at the next generation. w^- derivatives of the original insertion bearing chromosome were tested in the subsequent generation for male fertility.

roughex* mutants and flies with an increased dose of *roughex

One of our P-element induced male-sterile mutants on the X chromosome (*ms(1)5C48*) had a unique phenotype following the meiotic divisions, as well as a severe rough eye phenotype. The insertion event was responsible for both phenotypes, since they reverted together upon excision of the P-element. The P-element mapped to cytological position 5D, in the vicinity of *roughex* (*rux*). The P-element induced mutant failed to complement the weak rough eye phenotype of three original *rux* alleles, rux^1, rux^2 and rux^{60d}. rux^1 had been originally described as male-sterile; however, in our hands, it was male-fertile. The P-element induced allele has been designated rux^9. Viability of rux^9 males was good, but decreased in crowded conditions. Females homozygous for rux^9 (obtained by crossing rux^9/FM7c females to fertile rux^9;cycA5/+ males) were semi-sterile, apparently due to a defect in egg laying.

Strains CI and DI (Thomas et al., 1994) carry a 6 kb genomic piece wich fully rescued the eye and spermatogenesis phenotypes of rux^9. CI/CI homozygous males were sterile; most DI/DI males were also sterile, but some gave rise to a few progeny. Fertility was regained in rux^9; CI/CI males, demonstrating that the sterility of CI/CI males is truly due to increased dose of *rux*, and not to the disruption of an unrelated gene by the insertion of the rescue construct.

Initial characterization of male-sterile phenotypes

For gross examination of reproductive organs, five homozygous or hemizygous mutant males, 1 to 7 day old, were dissected in modified *Drosophila* Ringer's solution (5 mM PIPES, pH 6.9; 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$. Seminal vesicles were torn open with forceps under a dissecting microscope and examined for the presence and motility of sperm. Testes from mutants that failed to produce motile sperm were analyzed by phase contrast microscopy and Hoechst staining, as described below. For those male-sterile mutants that produced motile sperm, transfer of sperm to females was assayed by dissection of the sperm storage organs of females with whom they had been mated. Mutants that failed to transfer their motile sperm to females were further analyzed for behavioral or anatomical defects.

Examination of live squash germ cells by phase-contrast microscopy

Testes from newly eclosed males were dissected and placed on a slide with 10 µl Ringer's solution containing 10 µg/ml of the vital DNA dye Hoechst 33342 (Sigma) to monitor the state of the chromatin. The sheath of the testis was peeled open and the contents of the testis were gently squashed under a coverslip and readily examined by phase contrast microscopy (Kemphues et al., 1980). Relatively intact cysts were obtained. However, cellular boundaries between cells within a cyst were not always maintained. This method is best for analyzing germ cells just before, during, and just after the meiotic divisions (Fuller 1993). Cytological analysis of much older stages is difficult and, for instance, did not allow us to determine whether *rux* germ cells undergo successive divisions, after the additional division described in Chapter 8.

In order to verify that chromosome segregation was occurring normally during the meiotic divisions in *rux* mutant cysts, we followed the distribution of chromatin during late anaphase or telophase of the first and second meiotic divisions. Defects in chromosome segregation result in uneven distribution of chromatin among the daughter cells. 16/16 mutant cysts in meiosis I and 17/19 mutant cysts in meiosis II were

indistinguishable from wild-type, showing that most *rux* cysts properly executed the two meiotic divisions. 2/19 mutant cysts in meiosis II showed some defect in chromosome segregation, in accordance with the occasional finding of onion stage cysts containing nuclei of unequal size.

Classification of male-sterile mutations

For the expanded collection (see Table 5), male-sterile mutants were first categorized with regard to whether they produced normal amounts of motile sperm in the seminal vesicle or were defective for spermatogenesis. The mutants were then grouped into seven general classes, five for those affected in spermatogenesis and two for those producing wild-type levels of motile sperm. Classification was based on the earliest stage at which defects were readily and reproducibly apparent in homozygotes.

Proliferation phase defect: a reduction or absence of growth phase spermatocytes is apparent at eclosion or a few days post-eclosion. Mutant testes are thin and in more extreme examples resemble those of agametic testes.

Growth phase defect: germ cells complete the proliferation stage and form cysts of 16 early spermatocytes, but undergo aberrant development during the primary spermatocyte growth phase.

Entry into the meiotic divisions defect: spermatogenesis is normal through the late spermatocyte stage, but the meiotic divisions do not occur.

Meiotic division defect: the meiotic divisions are initiated, but fail to give rise to the normal pattern of sixty-four spermatids.

Post-meiotic differentiation defect: mutants undergo normal meiotic divisions, but fail to produce mature sperm. Mutants in this category are readily distinguishable from wild-type by the absence of sperm in the seminal vesicle.

Behavioral defect: homozygous males produce motile sperm at wild-type levels, but fail to copulate with females during 30 minute observation periods (Gailey et Hall, 1989).

Sperm transfer defect: motile sperm are found in the seminal vesicle at wild-type levels, but no sperm transfer occurs. In one mutant, mating is observed but few or no sperm are found in the female storage organs. In other mutants, the external genitalia of homozygous males are aberrant or absent.

Naming of loci

The results of genetic tests governed the naming of loci from our expanded collection (see Table 5): 1) in cases where our mutations were found to be alleles of previously characterized loci, the preexisting name, if any, was adopted; 2) in cases where more than one allele was identified in our screen, or where alleles were identified among available male-sterile mutations of unnamed loci, we assigned the locus a name according to our phenotypic characterization; 3) we also gave names to any of the loci for which we isolated transposase-induced revertants or identified deficiencies that failed to complement the male-sterile phenotype; 4) all other single allele loci were given designations based solely on cytological location.

Meiotic chromosomes (increased dose of *roughex*)

Meiotic chromosomes were prepared and stained with orcein as described (Goldstein 1980). This allows the identification of the cytologically distinct X and Y chromosomes in favorable spreads. 11 cysts in anaphase of the single division observed with increased *rux* dose were examined. 30 cells could be scored unambiguously and, in all cases, the X and the Y chromosome separated and segregated to opposite poles of the division spindle.

X-gal staining

Testes from newly eclosed males (defined as day 1 males) or ovaries from day 3 virgin females, were dissected in Ringer's, transferred to microtiter plates and fixed for 15 min in 1 % glutaraldehyde (Fluka); 50 mM Sodium-Cacodylate. The tissues were rinsed three times with staining buffer (7.2 mM Na_2HPO_4, 2.8 mM NaH_2PO_4, 1 mM $MgCl_2$, 0.15 M NaCl), left at room temperature (RT) for 30 min and then incubated at 37 ^oC for 12 to 16 hours in staining buffer plus 5 mM each of potassium ferro- and ferri-cyanide and 0.2 % X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). Testes and ovaries were washed three times in PBS-1 mM EDTA at RT, and mounted in 80 % glycerol for observation, or dehydrated in ethanol solutions of increasing concentrations and mounted in 2:1 Canada Balsam:methyl/salicylate for photography.

Hoechst staining

To assess the shape and distribution of germ cell nuclei, testes were fixed for 20 min with 4% formaldehyde (EM grade, Polyscience) in PBX (PBS plus 0.1% Triton X-100), washed three times for 5 min in PBX, stained with 1 mg/ml Hoechst 33258 (Sigma) for 2 min in PBX, rinsed in PBX, washed for 30 min in PBS and mounted in 80 % glycerol or Fluoromount-G (Fisher).

Reagents for antibody stains

The rabbit anti-ß-galactosidase polyclonal antibody (usually used at 1:500 in PBX or PBX-2, which is PBX plus 2 % Normal Goat Serum (NGS)) was purchased from Cappel; the mouse anti-BrdU monoclonal antibody (used at 1:10) from Becton Dickinson, and the anti-α tubulin antibody (used at 1:500) from Amersham. The rabbit anti-ß3-tubulin polyclonal antibody (used at 1:100) was a gift from Elizabeth Raff and is described in Kimble et al. (1989); this antibody was preadsorbed at its final dilution against an equal volume of fixed 0- 3 hour embryos (Kimble et al.; 1989). The anti-

fasciclin III monoclonal antibody DA.1B6 (used at 1:500) utilized in early experiments (Fig. 7E only), was a gift from Danny Brower (Brower et al., 1981). All other anti-fasciclin III stains relied on the monoclonal antibody 7G10 from the Goodman lab (used at 1:50). The anti-ß-galactosidase monoclonal antibody (used at 1:50) was a gift from Alfonso Martinez-Arias. Monoclonal anti-chickadee antibody 4B (used at 1:2) was kindly provided by Lynn Cooley's lab (Verheyen and Cooley, 1994). Affinity-purified rabbit anti-cyclin A polyclonal antibody (used at 1:1000) was a generous gift from Christian Lehner (Lehner and O'Farrell 1989). Monoclonal anti-cyclin B antibody was also provided by Chrisitan Lehner, and was used undiluted (Lehner and O'Farell, 1990). Rhodamine- and fluorescein-conjugated goat or donkey anti-rabbit and goat or donkey anti-mouse secondary antibodies (usually used at 1:400) were purchased from Jackson Laboratories, and were typically preadsorbed at their final dilution for 2 hours against an equal volume of fixed embryos from an overnight collection. Occasionally, we also utilized affinity-purified fluorescein-conjugated sheep anti-mouse secondary antibody (used at 1:10), purchased from Boehringer Manheim. Biotin-conjugated goat anti-rabbit and horse anti-mouse secondary antibodies (used at 1:400) were purchased from Vector.

Whole-mount indirect immunofluorescence

All testes antibody stains were performed with day 1 males. For indirect immunofluorescence, testes or third instar larval gonads were dissected in Ringer's or PEM (0.1 M Pipes, 1 mM $MgCl_2$, 1 mM EGTA, pH 6.9), and fixed for 20 min in 4 % formaldehyde in PBX or PEX (PEM plus 0.1 % Triton X-100). The tissues were rinsed at least 3 times 5 min in PBX, and blocked for 90 min in PBX-2. Testes or gonads were incubated with primary antibodies 60 to 90 min at RT, or overnight at 4 ^{o}C, and then washed three times 10 min in PBX. The tissues were incubated with secondary antibodies for 60 to 90 min at RT, washed three times for 10 min in PBX. Finally, testes or gonads

were counterstained for 2 min in 1 μg/ml Hoechst 33258 in PBX, rinsed with PBX, washed for 20 min in PBS, and mounted in a drop of glycerol of Fluoromount-G.

Whole-mount immunocytochemistry

Embryos (Fig. 7F) were collected for 2 hours and aged for 13 hours at 25 °C, then permeabilized, fixed and devitellinized as described (Mitchison and Sedat, 1983). The embryos were stored in 100 % methanol at -20 °C. Prior to rehydration, they were treated for 15 min with 3 % H_2O_2 in methanol to block endogenous peroxidase activity.

Testes were dissected in Ringer's solution, fixed for 20-30 min in 4 % formaldehyde- PBX and washed three times, at least 5 min each, in PBX. The immunocytochemsitry procedure was a modified version of that described by Kellerman et al. (1990). Samples were blocked in Blotto (5 % powdered milk in PBX) for 60-90 min at RT or overnight at 4 °C, and incubated with primary antibodies in Blotto or 2 % NGS for 60-90 min at RT or overnight at 4 °C. The samples were washed three times for 10 min in PBX, incubated for 60-90 min with biotin-conjugated secondary antibody in Blotto, and washed three times for 10 min in PBX. Samples were then incubated for 50 min with avidin-biotinylated hoseradish-peroxidase (Vectastain ABC kit, Vector), or 30 min with 1:500 horseradish peroxidase-conjugated streptavidin (Chemicon) in earlier experiments, washed three times for 10 min and once for 30 min in PBX. Diaminobenzidine (Polysciences) immunocytochemistry was carried out as described in Kellerman et al. (1990). Finally, the samples were counterstained for 2 min in 1 μg/ml Hoechst 33258 in PBX, rinsed with PBX, washed for 20 min in PBS and mounted in a drop of glycerol.

For double-labeling experiments (Fig. 11C), agametic testes were incubated at once with both mouse anti-BrdU and rabbit anti-β-galactosidase primaries antibodies, followed by an anti-mouse secondary antibody, which led to reveal the BrdU signal first (brown precipitate). Next, agametic testes were incubated with anti-rabbit secondary antibody,

which allowed to reveal the β-galactosidase signal in a purple precipitate, owing to the addition of nickel chloride (0.2 % final) in the diaminobenzidine solution.

BrdU labeling

Testes were dissected in Ringer's, placed into a clean watchglass or a siliconized Eppendorf, and incubated, within 10 min of dissection (this needs to be rapidly done as cells rapidly loose their proliferative capacities), for 30 min at 25 °C in 10 mM BrdU (Boehringer Manheim). Testes were then rinsed with Ringer's, fixed for 60 min in 4 % formaldehyde-PBX (longer fixations are important for optimal signal), washed three times 5 min in PBX and blocked in 2 % NGS. Testes were incubated for 90 min at 25 °C with anti-BrdU primary antibody in 66 mM Tris pH 8, 2.66 mM $MgCl_2$, 1 mM β-mercaptoethanol, in the presence of DNAse I (23unit/ ml of incubating solution), in order to allow access for the antibody to the BrdU incorporated in the DNA during S phase. The rest of the staining procedure was as for that described for other antibodies (see above). We favored this method over that using HCl treatment to allow access to incorporated BrdU (Schubiger and Palka, 1987), as acid treatment damages other antigens and also results in poor Hoechst staining of nuclei.

Whole-mount indirect immunofluorescent anti-fasciclin III and anti ß-galactosidase staining of male gonads

Best results were obtained with the first anti-fasciclin III monoclonal antibody when staining was performed on unfixed tissue (Brower et al., 1981). The double-labeling experiment in which this antibody was utilized (Fig. 7D and 7E) involved two successive reactions: a first staining for fasciclin III, performed on live tissue, followed by fixation and a second staining for ß-galactosidase. Testes were dissected in Ringer's solution, incubated for 45 min with DA.1B6 mouse anti-fasciclin III antibody in PBT-2 (PBS plus 0.1 % Tween-20 and 2 % NGS), washed four times for 5 min at 4 °C in PBT. The gonads

were then incubated for 30 min at 37 °C with rhodamine-conjugated anti-mouse antibody in PBT-2, washed four times for 5 min at 4 °C in PBT, and fixed in 4 % formaldehyde-PBT for 30 min at 4 °C. The remainder of the experiment was carried out at RT. The gonads were washed three times for 5 min in PBT, incubated for 45 min in 1:5000 rabbit anti-ß-galactosidase antibody in PBT, and washed three times for 20 min in PBT. The samples were then incubated for 45 min with fluorescein-conjugated anti-rabbit antibody in PBT-2, washed three times for 20 min in PBT, counterstained for 2 min in 1 µg/ml Hoechst 33258 in PBT, rinsed with PBT and washed for 20 min in PBS. The apical tip of the testis was dissected and placed in a drop of Fluoromount-G under a coverslip. All other fasciclin III staining experiments were conducted with the antibody from the Goodman lab, which was utilized just like most other antibodies (see above)

Indirect immunofluorescence with anti-cyclin B and anti-tubulin antibodies

In some case, such as with anti-cyclin B or anti-tubulin antibodies, satisfactory results were not obtained on whole-mount preparations, and we used an alternative protocoll as follows. Testes were prepared as for examination of live squash germ cells by phase contrast microscopy, gently squashed under a siliconized coverslip and frozen in liquid nitrogen. The coverslip was flipped off with a razor blade and germ cells on the slide were processed for antibody staining (Bonaccorsi et al. 1988). The slide was immersed in a slide carrier 5 min in methanol precooled at -20 °C and 1 min in acetone precooled at -20 °C. The rest of the procedure was carried out at RT. The slides were immersed 10 min in 0.5% acetic acid, 1% Triton X-100 in PBS, washed three times 10 min in PBS, and incubated with 20 µl primary antibodies for 60 min in a humid chamber, with a siliconized coverslip over the tissue. The slides were then washed three times 10 min in PBS, and incubated as for the primary antibody with fluorescein-conjugated anti-mouse secondary antibodies. The slides were washed three times 10 min in PBS,

counterstained for 2 minutes with 1 µg/ml Hoechst 33258 in PBS, rinsed in PBS and mounted in Fluoromount-G under a coverslip.

This method proved useful for detecting the spindle during the extra division in *rux* mutant germ cells or for assessing the presence of cyclin B. However, this method proved not reproducible enough to draw quantitative conclusions, such as whether the levels of cyclin B were different in wild-type and *rux* mutant testes.

Rhodamine-phalloidin staining of filamentous actin

This procedure was adapted from Gary Hime (Fuller lab). Testes were dissected in TB1 (15 mM potassium phosphate (equimolar di- and mono-basic), pH 6.7, 80 mM KCl, 16 mM NaCl, 5 mM MgCl2, 1 % PEG 6000) and transfered to a 7 µl drop of TB1 on a regular glass slide. Testes were gently teased open in the area of interest (tip or growth phase) with a fine tungsten needle, and cysts of germ cells were allowed to flow out for a few seconds, before being flattened under a siliconized 18 mm^2 coverslip. The preparation was frozen in liquid nitrogen, and the coverslip was flipped off with a razor blade; usually, most of the tissue remained afixed to the slide. The slide was placed for 10 min in ethanol precooled at -20 °C. The cells were then covered with freshly prepared 3.7 % formaldehyde-PBS for 10 min, and rinsed in slide carriers successively with 0.1 M glycine (2 x 5 min) and PBS (2 x 5 min). The cells were then incubated with 20 µl primary antibody (if needed, such as in the fasciclin III double-labeling experiment) for 60 min at RT, under a coverslip, in a humid chamber. Washes were 3 x 5 min in PBS in a slide carrier, followed by a 60 min incubation in 20 µl of a solution containing Rhodamin-phalloidin (Molecular Probes) and secondary antibody, if needed. The rhodamin-phalloidin stock (approximately 6.6 µM) was prepared according to the manufactor's instructions, and stored in 50 µl aliquotes in methanol at - 20 °C. Before use, the methanol was evaporated, and rhodamin-phalloidin was resuspended in 200 µl PBX (final molarity, 1.65 µm). The slides were then washed 3 x 5 min in PBS,

counterstained for 2 min with 1 μg/ml Hoechst 33258 in PBS, and rinsed again in PBS. A drop of Fluoromount-G was placed onto the tissue, which was then covered with a coverslip.

Microscopy and photography

We used a Nikon Optiphot microscope equipped with Nomarski optics and epifluorescence. X-gal stains and immunocytochemistry were viewed with bright field optics for low magnifications (see for instance Fig. 3A) and Nomarski optics for higher power views (see for instance Fig. 3C), and photographed with Kodak Ektar 25, Ekatachrome 160 or T-Max 100 film. Indirect immunofluorescence was photographed with Kodak Tech Pan film set at ASA 800 for rhodamine and ASA 125 for fluorescein and Hoechst. Negatives and slides were scanned, and scans were treated in Adobe Photoshop 3.0, and Figures were assembled in Pagemaker 5.0.

Preparation of testes for electron microscopy

Testes from newly eclosed *rux*^{2} males were fixed and processed as described (Tokuyasu et al., 1972a) by the electron microscopy facility of the Rockefeller University (Helen Shio).

Preparation of eyes for scanning electron microscopy

We followed a standard protocol obtained from the Rubin lab. Briefly, flies were dehydrated through an ethanol series (25 %, 50 %, 75 %, 2 x 100 %, 12 hours each), and then through a Freon 113 series in ethanol (25 %, 50 %, 75%, 2 x 100%, 12 hours each). Flies were dried under vacuum, and mounted onto electron microscopy stubs. T.V. tube coating (Ted Pella) was first painted on the stubs, and flies were carefully positioned onto the stubs on small drops of additional coating. The stubs were then viewed by scanning electron microscopy at the Sloan Kettering electron microscopy facility (Nina Lampen).

RNA extraction from testes

Our protocoll was a modification of a published rapid RNA extraction method (Jowett, 1986). Testes were dissected in Ringer's, and pooled in an Eppendorf containing Ringer's and placed on ice. Once enough testes were gathered (typically 50 or 100 pairs), the Ringer's was replaced by 300 µl freshly made lysis buffer (7 M urea, 0.35 M NaCl, 0.1 M Tris pH 8.0, 0.01 M EDTA, 2 % SDS). Testes in the Eppendorf were homogenized for about 30 sec with a small glass pestle; 100 µl lysis buffer was pipetted onto the glass pestle to recover additional material, and the preparation was vortexed for 30 seconds. 200 µl phenol (pH 5.0, saturated with 0.2 M Na-acetate) and 200 µl chloroform were added, and the preparation was vortexed for another 30 seconds. After a 4 min centrifugation, the supernatant was carefully transfered to a new Eppendorf, and the extraction procedure was repeated two more times. 200 µl chroroform was added to the last aqueous phase, and the preparation was vortexed and centrifuged again. The aqueous phase was transfered to a new tube, and nucleic acids were precipitated by the addition of 1 ml EtOH. After 15 min at -70 ^{o}C, the nucleic acids were pelleted, dried, and resuspended in H_2O. Typically, 50 pairs of testes were resuspended in 5 µl H_2O, yielding enough material for one lane on a Northern (about 5-10 µg of RNA).

This procedure resulted in the efficient recovery of RNA, but some DNA was present in the end as well. This never presented a problem, as the RNA species we analyzed were fairly abundant, and as the Northern blot hybridization conditions favored RNA-DNA hybrids over DNA-DNA hybrids. In cases where the presence of DNA would be a problem, the resuspension could be treated with DNAseI.

Molecular biology

Fly DNA mini-preps, Northern and Southern blots were performed essentially as described in standard procedures (Ashburner, 1989c; Maniatis et al., 1989).

Western blot

Testes (100) from newly eclosed wild-type or *rux* mutants were dissected in Ringer's, resuspended in 50 µl SDS-PAGE loading buffer, homogenized with a glass pestle, and boiled for 10 min. Wild-type embryos (0-1 hour) were dechorionated and similarly prepared. Samples corresponding to 20 testes were loaded. Two sets of samples were run on a 8% SDS-polyacrylamide gel. Half of the gel was stained with Coomassie-blue to verify that similar amounts of protein had been loaded in the testes lanes. The other half was transferred to Immobilon-P (Millipore). The blot was then processed essentially as described (Ronchi et al., 1993), using 1:1000 affinity-purified rabbit-anti cyclin A polyclonal as primary antibody.

Immunoprecipitation and phosphatase treatment

Testes (240) from newly eclosed wild-type or *rux* mutants were dissected in Ringer's and resuspended in 200 µl IPTAG buffer (50mM Tris pH7.5, 50 mM NaF, 12.5 mM beta-glycerophosphate, 1% Triton-X, 0.5 mM PMSF, 5 mg/ml leupeptin, 2 mg/ml pepstatin, 15 mg/ml aprotinin) with 420 mM NaCl. The extracts were gently homogenized with a glass pestle, rotated for 30 min at 4 ^{o}C, and briefly spun; the supernatant was recovered. 400 µl IPTAG buffer was added, bringing the final salt concentration to 140 mM NaCl. The amount used per immunoprecipitation was 100 µl. The extracts were cleared with preimmune serum and then incubated for 60 min on ice with 1 µl of 1:6 affinity purified rabbit-anti cyclin A polyclonal antibody. Staphyllococus aureus beads (10 µl, Repligen), rinsed in IPTAG-150 buffer (IPTAG with 150 mM NaCl), was added and incubated overnight at 4 ^{o}C. The beads were recovered, washed three times in IPTAG-150, without NaF or β-glycerophosphate, resuspended in 55 µl of the same buffer, and incubated for 60 min at 37 ^{o}C with 200 units of calf alkaline phosphatase (CIP, Boehringer Manheim). A control sample received 200 units of CIP which had been previously heat-inactivated by boiling for 10 min. The beads were

washed five times with IPTAG-150, resuspended in 20 µl SDS loading buffer, and boiled for 10 min. Half of the reaction was loaded on a 7.5% SDS gel, which was processed as for the Western blot analysis.

Appendix: phenotypic and molecular analysis of *fumble*

The original *fumble* homozygous mutant phenotype is a failure of cytokinesis at meiosis: four haploid nuclei are associated with one giant nebenkern, revealing the lack of segregation of cytoplasmic components during the meiotic divisions (see Table 5). Moreover, the haploid nuclei are small and appear to still have condensed chromatin. When we put each of two original *fumble* alleles in trans to a deficiency, BrdU incorporation experiments demonstrated the presence of occasional cysts of overproliferating germ cells in older testes. This indicated that *fumble* may play a role in restricting the proliferation of germ cells during spermatogenesis. Therefore, we generated more severe alleles by imprecise excision of the P-element, and recovered two classes of stronger *fumble* alleles. From about 150 independent lines established, we isolated 3 severe male-sterile alleles, which have smaller testes, often with a displaced hub, and which also display the overproliferation phenotype in older testes. We also recovered 19 lethal *fumble* alleles. Interestingly, homozygous mutant third instar larvae from some of these lethal alleles have no or extremely reduced imaginal discs, and have polypoid neuroblasts in the brain. These observations suggest that *fumble* is generally required for cytokinesis or another aspect of cell cycle progression affecting that process.

In attempting to clone *fumble*, we have recovered neighbouring DNA by inverse PCR, and used that piece of DNA to pull out genomic phages, and characterize a 30 kb genomic region surrounding the P-element insertion site. Two ubiquitous transcripts were detected in this 30 kb interval, but neither was affected in level or size in testis RNA from the original *fumble* alleles, or larval RNA from the lethal alleles. Thus, we do not have a candidate for the *fumble* gene yet. It is still possible that one of these two transcripts is *fumble* indeed, as subtle size differences may not have been resolved. Alternatively, although most P-element insertion events alter the size or level of the affected mRNA, this is not always the case. For instance, the level and probably the size of bam mRNA

was not altered in the original P-element induced *bam* mutation, although this allele has a strong overproliferation phenotype, similar to that of a null (McKearin and Spradling, 1990). Thus, for *fumble*, it may be worthwile to generate genomic rescue contructs encompassing the location of the two transcription units to test whether they can rescue the male-sterile and lethal phenotypes. Alternatively, *fumble* might be located outside of the 30 kb region. Indeed, while P-elements preferentially insert into promoter regions, this is not always so, and insertions into enhancers located tens of kb away may disrupt gene function.

CHAPTER 11 : REFERENCES

Aboïm, A. N. (1945). Dévelopement embryonnaire et postembryonnaire des gonades normales et agamétiques de *Drosophila melanogaster*. Rev. Suisse de Zool. *52:* 53-154.

Adamson, J. W. and J. E. Brown (1978). Aspects of erythroid differentiation and proliferation. *In* Molecular Control of Proliferation and Differentiation. Symp. Soc. Dev. Biol. *35:* 161-179.

Allan, D. J., B. V. Harmon and S. A. Roberts (1992). Spermatogonial apoptosis has three morphologically recognizable phases and shows no circadian rythm during normal spermatogenesis in the rat. Cell Prol. *25:* 241-250.

Alphey, L., J. Jimenez, H. White-Cooper, I. Dawson, P. Nurse and D. M. Glover (1992). *twine*, a cdc25 homologue that functions in the male and female germ line of *Drosophila*. Cell *69:* 977-988.

Anderson, W. A. (1967). Cytodifferentiation of spermatozoa in *Drosophila melanogaster*: the effect of elevated temperature on spermiogenesis. Mol. Gen. Genet. *99:* 257-273.

Ashburner, M. (1989a). *In* Drosophila: a laboratory handbook. (Cold Spring Harbor, NY, Cold Spring Harbor Press). pp. 148-150.

Ashburner, M. (1989b). *In* Drosophila: a laboratory manual. (Cold Spring Harbor, NY: Cold Spring Harbor Press). pp. 50-52.

Ashburner, M. (1989c). *In* Drosophila: a laboratory manual. (Cold Spring Harbor, NY: Cold Spring Harbor Press). pp. 106-107.

Austin, J. and J. Kimble (1987). *glp-1* is required in the germ line for regulation of the decision between mitosis and meiosis in *C. elegans*. Cell *51:* 589-599.

Austin, J. and J. Kimble (1989). Transcript analysis of *glp-1* and *lin-12*, homologous genes required for cell interactions during development of *C. elegans*. Cell *58:* 565-571.

Bairati, A. (1967). Struttura ed ultrastruttura dell'apparato genitale maschile di Drosophila melanogaster. Meig. I. Il testiculo. Z. Zelforsch. *76:* 56-99.

Baker B. S. and J. C. Hall (1976). Meiotic mutants: genetic control of meiotic recombination and chromosome segregation. In The Genetics and Biology of Drosophila. (New York: Academic Press; Vol. 1A). pp. 351-434.

Baker, B. S., A. T. C. Carpenter, M. S. Esposito, R. E. Esposito and L. Sandler (1976). The genetic control of meiosis. Annu. Rev. Genet. *10:* 53-134.

Barrandon, Y. and H. Green (1987). Three clonal types of keratinocytes with different capacities for multiplication. Proc. Natl. Acad. Sci. USA *86:* 2302-2306.

Beanan, M. J. and S. Strome (1992). Characterization of a germ-line proliferation mutation in *C. elegans*. Development *116:* 755-766.

Bellen, H. J., D. D'Evelyn, M. Harvey and S. J. Elledge (1992). Isolation of temperature-sensitive diptheria toxins in yeast and their effects on *Drosophila* cells. Development *114:* 787-796.

Bellen, H. J., C. J. O'Kane, C. Wilson, U. Grossniklaus, R. K. Pearson and W. J. Gehring (1989). P-element mediated enhancer detection: a versatile method to study development in *Drosophila*. Genes Dev. *3:* 1288-1300.

Bellvé, A. R. (1979). The molecular biology of mammalian spermatogenesis. *In* The Oxford reviews for reproductive biology. (Oxford: Clarendon Press). pp. 159-261.

Berg, C. A. and A. C. Spradling (1991). Studies on the rate and site-specificity of P-element transposition. Genetics *127:* 515-24.

Bier, E., H. Vaessin, S. Shepherd, K. Lee, K. McCall, S. Barbel, L. Ackerman, R. Carretto, T. Uemura, E. Grell, L. Y. Jan and Y. N. Jan (1989). Searching for pattern and mutation in the Drosophila genome with a P-lacZ vector. Genes Dev. *3:* 1273-1287.

Biggs, W. H. I., K. H. Zavitz, B. Dickson, A. van der Straten, D. Brunner, E. Hafen and S. L. Zipursky (1994). The Drosophila *rolled* locus encodes a MAP kinase required in the Sevenless signal transduction pathway. EMBO J. *13:* 1628-1635.

Bonaccorsi, S., C. Pisano, F. Puoti and M. Gatti (1988). Y chromosome loops in *Drosophila melanogaster*. Genetics *120:* 1015-1034.

Bonaccorsi, S., M. Gatti, C. Pisano and A. Lohe (1990). Transcription of a satellite DNA on two Y chromosome loops of *Drosophila melanogaster*. Chromosoma *99:* 260-266.

Booher, R. N., C. E. Alfa, J. S. Hyams and D. H. Beach (1989). The fission yeast cdc2/cdc13/suc1 protein kinase: regulation of catalytic activity and nuclear localization. Cell *58:* 485-497.

Bopp, D., J. I. Horabin, R. A. Lersch, T. W. Cline and P. Schedl (1993). Expression of the *Sex-lethal* gene is controlled at multiple levels during *Drosophila* oogenesis. Development *118:* 797-812.

Bownes, M. (1990). Preferential insertion of P-elements into genes expressed in the germ-line of *Drosophila melanogaster*. Mol. Gen. Genet. *222:* 457-460.

Boyle, M. and S. DiNardo (1995). Specification, migration and assembly of the somatic cells of the *Drosophila* gonad. Develoment: *in press.*

Brand, A. H. and N. Perrimon (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development *118:* 401-415.

Braun, R. E., J. J. Peshon, R. R. Behringer, R. L. Brinster and R. D. Palmiter (1989). Protamine 3'-untranslated sequences regulate temporal translational control and subcellular localization of growth hormone in spermatids of transgenic mice. Genes Dev. *3:* 793-802.

Brinster, R. L. and J. W. Zimmermann (1994). Spermatogenesis following male germ-cell transplantation. Proc. Natl. Acad. Sci. USA *91:* 11298-11302.

Brookman, J. J., A. T. Toosy, L. S. Shashidhara and R. A. H. White (1992). The 412 retrotransposon and the development of gonadal mesoderm in *Drosophila*. Development *116:* 1185-1192.

Brower, D. L., R. J. Smith and M. Wilcox (1981). Differentiation within the gonads of *Drosophila* revealed by immunofluorescence. J. Embryol. Exp. Morph *63:* 233-242.

Brunner, D., N. Oellers, J. Szabad, W. H. I. Biggs, S. L. Zipursky and E. Hafen (1994). A gain-of-function mutation in Drosophila MAP kinase constituitively activates multiple receptor tyrosine kinase signaling pathways. Cell *76:* 875-888.

Capowski, E. E., P. Martin, C. Garvin and S. Strome (1991). Indentification of grandchildless loci whose products are required for normal germ-line development in the nematode *Caenorhabditis elegans*. Genetics *129:* 1061-1072.

Carlsson, L., L.-E. Nyström, F. Sundkvist, F. Markey and U. Lindberg (1977). Actin polymerizability is influenced by profilin, a low molecular weight protein in non-muscle cells. J. Mol. Biol. *115:* 465-483.

Castrillon, D. H., P. Gönczy, S. Alexander, R. Rawson, C. G. Eberhart, S. Viswanathan, S. DiNardo and S. A. Wasserman (1993). Toward a molecular genetic analysis of spermatogenesis in *Drosophila melanogaster*: characterization of male-sterile mutants generated by single P-element mutagenesis. Genetics *135:* 489-505.

Castrillon, D. H. and S. A. Wasserman (1994). *diaphanous* is required for cytokenesis in *Drosophila* and shares domains of similarity with the products of the *limb deformity* gene. Development *120:* 3367-3377.

Chasan, R. and K. V. Anderson (1993). Maternal control of dorsal-ventral polarity and pattern in the embryo. *In* The Development of Drosophila melanogaster. (Cold Spring Harbor, NY: Cold Spring Harbor Press). pp. 387-424.

Cheng, C. Y., J. Grima, M. S. Stahler, R. A. Lockshin and C. W. Bardin (1989). Testins are structurally related Sertoli cell proteins whose secretion is tightly coupled to the presence of germ cells. J. Biol. Chem. *264:* 21386-21393.

Chiquoine, A. D. (1954). The identification, origin and migration of the primordial germ cells in the mouse embryo. Anat. Rec. *118:* 135-146.

Clermont, Y. (1962). Quantitive analysis of spermatogenesis of the rat: a revised model for the renewal of spermatogonia. Am. J. Anat. *111:* 111-129.

Clifford, R., R. Francis and T. Schedl (1994). Somatic control of germ cell development in *Caenorhabditis elegans*. Sem. Dev. Biol. *5:* 21-30.

Cooley, L., R. Kelley and A. Spradling (1988). Insertional mutagenesis of the Drosophila genome with single P-elements. Science *239:* 1121-1128.

Cooley, L., E. Verheyen and K. Ayers (1992). *chickadee* encodes a profilin required for intercellular cytoplasm transport during *Drosophila* oogenesis. Cell *69:* 173-184.

Cooper, K. W. (1950). Normal spermatogenesis in Drosophila. *In* The biology of Drosophila. (New York and London: Hafner Publishing Company). pp. 1-61.

Courtot, C., C. Frankhauser, V. Simanis and C. F. Lehner (1992). The *Drosophila cdc25* homologue *twine* is required for meiosis. Development *116:* 405-416.

Cox, M. M. (1988). FLP site-specific recombination system of Saccharomyces cerevisiae. *In* Genetic Recombination. (Washington D.C.: American Society for Microbiology). pp. 429-443.

Dantschakoff, V. (1941). Der aufbau des Geschlechts beim höheren Wirbetier. (Jena: Gustav Fischer).

Dixon, K. E. (1994). Evolutionary aspects of primordial germ cell formation. *In* Germ line Development. (Chichester, England: John Wiley & Sons). pp. 92-120.

Dorée, M., G. Peaucellier and A. Picard (1983). Activity of the maturation-promoting factor and the extent of protein phosphorylation oscillate simultaneously during meiotic maturation of starfish oocytes. Dev. Biol. *99:* 489-501.

Draetta, G. and D. Beach (1988). Activation of *cdc2* protein kinase during mitosis in human cells: Cell cycle-dependent phosphorylation and subunit rearrangement. Cell *54:* 17-26.

Draetta, G., F. Luca, J. Westendorf, L. Brizuela, J. Ruderman and D. Beach (1989). cdc2 protein kinase in complexed with both cyclin A and B: evidence for proteolytic inactivation of MPF. Cell *56:* 829-838.

Dubois, R. (1969). Le méchanisme d'entrée des cellules germinales primordiales dans le réseau vasculaire, chez l'embryon de poulet. J. Embryol. Exp. Morphol. *21:* 255-270.

Dulic, V., W. K. Kaufmann, S. J. Wilson, T. D. Tisty, E. Lees, J. W. Harper, S. J. Elledge and S. I. Reed (1994). p53-dependent inhibition of cyclin-dependent kinase activities in human fibroblasts during radiation-induced G1 arrest. Cell *76:* 1013-1023.

Dunphy, W. G. and A. Kumagai (1991). The cdc25 protein contains an intrinsic phosphatase activity. Cell *67:* 189-196.

Dym, M. and Y. Clermont (1970). Role of spermatogonia in the repair of the seminiferous epithelium following X-irradiation of the rat testis. Am. J. Anat. *128:* 265-282.

Edamatsu, M., M. Hirono and Y. Watanabe (1992). *Tetrahymena* profilin is localized in the division furrow. J. Biochem. *112:* 637-642.

Egel, R. (1984). The pedigree pattern of mating type switching in *Schizosaccharomyces pombe*. Curr. Genet. *8:* 205-210.

El-Deiry, W. S., J. W. Harper, P. M. O'Connor, V. E. Velculescu, C. E. Canman, J. Jackman, J. A. Pietenpol, M. Burrell, D. E. Hill, Y. Wang, K. G. Wiman, W. E. Mercer, M. B. Kastan, K. W. Kohn, S. J. Elledge, K. W. Kinzler and B. Vogelstein (1994). WAF1/CIP1 is induced in p53-mediated G1 arrest and apoptosis. Cancer Res. *54:* 1169-1174.

El-Deiry, W. S., T. Tokino, V. E. Veculescu, D. B. Levy, R. Parsons, J. M. Trent, D. Lin, E. Mercer, K. W. Kinzler and B. Vogelstein (1993). WAF1, a potential mediator of p53 tumor suppression. Cell *75:* 817-825.

Ephrussi, A. and R. Lehmann (1992). Induction of germ cell formation by *oskar*. Nature *358:* 387-392.

Eppig, J., L. Kozak, E. Eicher and L. Stevens (1977). Ovarian teratomas in mice are derived from oocytes that have completed the first meiotic division. Nature *269:* 517-518.

Esposito, R. E. and S. Klapholz (1981). Meiosis and ascopore development. *In* The Molecular Biology of the Yeast *Saccharomyces*. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory). pp. 211-287.

Evan, G. I., A. H. Wyllie, C. S. Gilbert, T. D. Littlewood, H. Land, M. Brooks, C. M. Waters, L. Z. Penn and D. C. Hancock (1992). Induction of apoptosis in fibroblasts by c-myc protein. Cell *69:* 119-128.

Evans, G. S. and C. S. Potten (1991). Stem cells and the elixir of life. BioEssays *13:* 135-138.

Evans, T., E. T. Rosenthal, J. Youngbloom, D. Distel and T. Hunt (1983). Cyclin: a protein specified by maternal mRNA in sea urchin eggs that is destroyed at each cell division. Cell *33:* 389-396.

Evans, T. C., S. L. Crittenden, V. Kodoyianni and J. Kimble (1994). Translational control of maternal *glp-1* mRNA establishes an asymmetry is the *C. elegans* embryo. Cell *77:* 183-194.

Ewen, M. E., H. K. Sluss, L. L. Whitehouse and D. M. Livingston (1993). TGFβ inhibition of Cdk4 synthesis is linked to cell cycle arrest. Cell *74:* 1009-1020.

Fasano, L. and S. Kerridge (1988). Monitoring positional information during oogenesis in adult *Drosophila*. Development *104:* 245-253.

Fehon, R. G., P. J. Kooh, I. Rebay, C. L. Regan, T. Xu, M. A. T. Muskavitch and S. Artavanis-Tsakonas (1990). Molecular interactions between the protein products of the neurogenic loci *Notch* and *Delta*, two EGF-homologous genes in *Drosophila*. Cell *61:* 523-534.

Ferrell, J. J., M. Wu, J. C. Gerhart and G. S. Martin (1991). Cell cycle tyrosine phosphorylation of p34cdc2 and a microtubule-associated protein kinase homolog in Xenopus oocytes and eggs. Mol. Cell. Biol. *11:* 1965-1971.

Fialkow, P. J., J. W. Singer, W. H. Raskind, J. W. Adamson, R. J. Jacobson, I. D. Bernstein, L. W. Dow, V. Najfeld and R. Veith (1987). Clonal development, stem-cell differentiation, and clinical remissions in acute nonlymphocytic leukemia. N. Engl. J. Med. *317:* 468-73.

Francis, R., M. K. Barton, J. Kimble and T. Schedl (1995a). *gld-1*, a tumor suppressor gene required for oocyte development in *Caenorhabditis elegans*. Genetics *139:* 579-606.

Francis, R., E. Maine and T. Schedl (1995b). Analysis of the multiple roles of *gld-1* in germ line development: interactions with the sex determination cascade and the *glp-1* signaling pathway. Genetics *139:* 607-630.

Fritz-Niggli, H. and T. Suda (1972). Bildung und bedeutung der zentriolen: eine studie und neuinterpretation der meiose von *Drosophila*. Cytobiologie *5:* 12-41.

Fuller, M. T. (1993). Spermatogenesis. *In* The Development of Drosophila melanogaster. (Cold Spring Harbor, NY: Cold Spring Harbor Press). pp. 71-147.

Fuse, N., S. Hirose and S. Hayashi (1994). Diploidy of *Drosophila* imaginal cells is maintained by a transcriptional repressor encoded by *escargot*. Genes Dev. *8:* 2270-2281.

Gailey, D. A., B. J. Taylor and J. C. Hall (1991). Elements of the *fruitless* locus regulate development of the muscle of Lawrence, a male-specific structure in the abdomen of *Drosophila melanogaster* adults. Development *113:* 879-890.

Gans, M., C. Audit and M. Masson (1975). Isolation and characterization of sex-linked female-sterile mutants in Drosophila melanogaster. Genetics *81:* 683-704.

Gateff, E. (1982). Gonial cell neoplasm of genetic origin affecting both sexes of *Drosophila melanogaster*. *In* Embryonic Development, Part B: Cellular Aspects. (New York, Alan R. Liss Inc.). pp. 621-632.

Gateff, E. (1994). Tumor suppressor and overgrowth suppressor genes of *Drosophila melanogaster*. Developmental aspects. Int. J. Dev. Biol. *38:* 565-590.

Gausz, J., H. Gyurkovics, G. Bencze, A. A. M. Awad, J. J. Holden and D. Ish-Horowicz (1991). Genetic characterization of the region between 86F1,2 and 87B15 on chromosome 3 of *Drosophila melanogaster*. Genetics *98:* 775-789.

Gautier, J., M. J. Solomon, R. N. Booher, F. Bazan and M. W. Kirschner (1991). cdc25 is a specific tyrosine phosphatase that directly activates $p34^{cdc2}$. Cell *67:* 197-211.

Geer, B. W., T. D. Lischwe and K. G. Murphy (1983). Male fertility in *Drosophila melanogaster*: genetics in the *vermilion* region. J. Exp. Zool. *225:* 107-118.

Geigy, R. (1931). Action de l'ultra-violet sur le pôle germinal dans l'oeuf de Drosophile melanogaster. (Castration et mutabilité). Rev. Suisse Zool. *38:* 187-288.

Gergen, J. P. and E. Wieschaus (1986). Dosage requirements for *runt* in the segmentation of Drosophila embryos. Cell *45:* 289-299.

Gerhart, J., V. Wu and A. M. Kirschner (1984). Cell cycle dynamics of an M-phase-specific cytoplasmic factor in Xenopus laevis oocytes and eggs. J. Cell Biol. *98:* 1247-1255.

Ghiara, J. B., H. E. Richardson, K. Sugimoto, M. Henze, D. J. Lew, C. Wittenberg and S. I. Reed (1991). A cyclin B homolog in *S. cerevisiae*: chronic activation of the Cdc28 protein kinase by cyclin prevents exit from mitosis. Cell *65:* 163-174.

Giardina, A. (1901). Origine dell'ocite e delle cellule nel Dytiscus. Int. Mschr. Anat. Physiol. *18:* 417-484.

Gill, K. S. (1963). A mutation causing abnormal courtship and mating behavior in males of *Drosophila melanogaster* (abstract). Am. Zool. *3:* 507.

Giordano, A., P. Whyte, E. Harlow, B. R. J. Franza, D. Beach and G. Draetta (1989). A 60 kd cdc2-associated polypeptide complexes with the E1A proteins in adenovirus-infected cells. Cell *58:* 981-990.

Girard, F., U. Strausfeld, A. Fernandez and N. J. C. Lamb (1991). Cyclin A is required for the onset of DNA replication in mammalian fibroblasts. Cell *67:* 1169-1179.

Gloor, H. (1943). Entwicklungsphysiologie Untersuchung an den Gonaden einer Letalrasse (lgl) von Drosophila melanogaster. Rev. Suisse. Zool. *50:* 339.

Glotzer, M., A. W. Murray and M. W. Kirschner (1991). Cyclin is degraded by the ubiquitin pathway. Nature *349:* 132-138.

Goldschmidt-Clermont, P. J., J. W. Kin, L. M. Machesky, S. G. Rhee and T. D. Pollard (1991a). Regulation of phospholipase C-γ1 by profilin and tyrosine phosphorylation. Science *251:* 1231-1233.

Goldschmidt-Clermont, P. J., L. M. Machesky, S. G. Rhee and T. D. Pollard (1991b). Mechanism of the interaction of human platelet profilin with actin. J. Cell Biol. *113:* 1081-1089.

Goldstein, L. S. (1980). Mechanisms of chromosome orientation revealed by two meiotic mutants in *Drosophila melanogaster*. Chromosoma *78:* 79-111.

Goldstein, L. S., R. W. Hardy and D. L. Lindsley (1982). Structural genes on the Y chromosome of *Drosophila melanogaster*. Proc. Natl. Acad. Sci. USA *79:* 7405-7409.

Golic, K. G. (1991). Site-specific recombination between homologous chromosomes in Drosophila. Science *252:* 958-961.

Golic, K. G. and A. S. Lindquist (1989). The FLP recombinase of yeast catalyzes site-specific recombination in the Drosophila genome. Cell *59:* 499-509.

Gollin, S. M. and R. C. King (1981). Studies on *fs*(1)1621, a mutation producing ovarian tumors in *Drosophila melanogaster*. Dev. Genet. *2:* 203-218.

Gomperts, M., C. Wylie and J. Heasman (1994). Primordial germ cell migration. *In* Germ line Development. (Chichester, England: John Wiley & Sons). pp. 134-139.

Goodrich, D. W., N. P. Wang, Y. W. Qian, E. Y. Lee and W. H. Lee (1991). The retinoblastoma gene product regulates progression through the G1 phase of the cell cycle. Cell *67:* 293-302.

Gordon, M. Y., G. P. Riley, S. M. Watt and M. F. Greaves (1988). Compartmentilization of a haemopoietic growth factor (GM-CSF) by glycosaminoglycans in the bone marrow microenvironment. Nature *326:* 403-405.

Gotoh, Y., K. Moriyama, S. Matsuda, E. Okumura, T. Kishimoto, H. Kawasaki, K. Suzuki, I. Yahara, H. Sakai and E. Nishida (1991). Xenopus M phase MAP kinase: isolation of its cDNA and activation by MPF. EMBO J. *10:* 2661-2668.

Gotoh, Y., E. Nishida, S. Matsuda, N. Shiina, H. Kosako, K. Shiokawa, T. Akiyama, K. Ohta and H. Sakai (1991). In vitro effects on microtubule dynamics of purified Xenopus M phase-activated MAP kinase. Nature *349:* 251-254.

Gould, K. L. and P. Nurse (1989). Tyrosine phosphorylation of the fission yeast $cdc2^{+}$ protein kinase regulates entry into mitosis. Nature *342:* 39-45.

Gould-Somero, M. and L. Holland (1974). The timing of RNA synthesis for spermiogenesis in organ cultures of *Drosophila melanogaster* testes. Roux's Arch. EntwMech. Org. *174:* 133-148.

Grigliatti, T. (1986). Mutagenesis. *In* Drosophila: a practical approach. (Oxford, IRL Press Ltd.). pp. 39-58.

Grossniklaus, U., H. J. Bellen, C. Wilson and W. J. Gehring (1989). P-element-mediated enhancer detection applied to the study of oogenesis in *Drosophila*. Development *107:* 189-200.

Gu, W., J. W. Schneider, G. Condorelli, S. Kaushal, V. Mahdavi and B. Nadal-Ginard (1993). Interaction of myogenic factors and the retinoblastoma protein mediates muscle cell commitment and differentiation. Cell 72: 309-324.

Haarer, B. K., S. H. Lillie, A. E. M. Adams, V. Magdolen, W. Bandlow and S. S. Brown (1990). Purification of profilin from *Saccharomyces cerevisiae* and analysis of profilin-deficient cells. J. Cell Biol. *110:* 105-114.

Hackstein, J. H. (1987). Spermatogenesis in Drosophila. Results Probl. Cell Diff. *15:* 63-116.

Hackstein, J. H. P. (1991). Spermatogenesis in Drosophila. A genetic approach to cellular and subcellular differentiation. Eur. J. Cell Biol. *56:* 151-169.

Hackstein, J. H. P., H. Beck, R. Hochstenbach, H. Kremer and H. Zacharias (1990). Spermatogenesis in *Drosophila hydei*: a genetic survey. Roux's Arch. Dev. Biol. *199:* 251-280.

Hall, P. A. and F. M. Watt (1989). Stem cells: the generation and maintenance of cellular diversity. Development *106:* 619-633.

Handel, M. A. and J. J. Eppig (1979). Sertoli cell differentiation in the testes of mice genetically deficient in germ cells. Biol. Reprod. *20:* 1031-1038.

Hardy, R. W., K. T. Tokuyasu, D. L. Lindsley and M. Garavito (1979). The germinal proliferation center in the testis of *Drosophila melanogaster*. J. Ultrastruct. Res. *69:* 180-190.

Harper, J. W., G. R. Adami, N. Wei, K. Keyomarsi and S. J. Elledge (1993). The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. Cell *75:* 805-816.

Harrison (1983a). *In* Principles of Internal Medicine. (New York: McGraw Hill; 10th Edition). pp. 255-256.

Harrison (1983b). *In* Principles of Internal Medicine. (New York: McGraw Hill; 10th Edition). pp. 1885-1888.

Harrison, D. A. and N. Perrimon (1993). Simple and efficient generation of marked clones in *Drosophila*. Curr. Biol. *3:* 424-433.

Hartwell, L. H. and T. A. Weinert (1989). Checkpoints: controls that ensure the order of cell cycle events. Science *246:* 629-634.

Hay, B., L. Ackerman, S. Barbel, L. Y. Jan and Y. N. Jan (1988). Identification of a component of *Drosophila* polar granules. Development *103:* 625-640.

Hayashi, S., S. Hirose, T. Metcalfe and A. Shirras (1993). Control of imaginal cell development by the *escargot* gene of *Drosophila*. Development *118:* 105-115.

Heitzler, P. and P. Simpson (1991). The choice of cell fate in the epidermis of *Drosophila*. Cell *64:* 1083-1092.

Henderson, S. T., D. Gao, E. J. Lambie and J. Kimble (1994). *lag-2* may encode a signaling ligand for the GLP-1 and LIN-12 receptors of *C. elegans*. Development *120:* 2913-2924.

Heppner, G. H. (1984). Tumor heterogeneity. Cancer Res. *44:* 2259-2265.

Horvitz, R. H. and I. Herskowitz (1992). Mechanisms of asymmetric cell division: two B's or not two B's, that is the question. Cell *68:* 237-255.

Hoyt, M. A., L. Totis and B. T. Roberts (1991). *S. cerevisiae* genes required for cell cycle arrest in response to loss of microtubule function. Cell *66:* 507-517.

Huckins, C. (1978). The morphology and kinetics of spermatogonial degeneration in normal adult rats: an analysis using a simplified classification of the germinal epithelium. Anat. Rec. *190:* 905-926.

Huijser, P., C. Kirchoff, D. H. Lankenau and W. Hennig (1988). Retrotransposon-like sequences are expressed in Y-chromosomal lampbrush loops of *Drosophila hydei*. J. Mol. Biol. *203:* 689-697.

Hulsebos, T. J., J. H. Hackstein and W. Hennig (1984). Lampbrush loop-specific protein of *Drosophila hydei*. Proc. Natl. Acad. Sci. USA *81:* 3404-3408.

Hummel, K. P. (1966). Mouse News Letter *34:* 31-32.

Hunt, T. and A. Murray (1993a). Enzymes that control mitosis. *In* The Cell Cycle. (New York: W. Freeman and Company). pp. 42-65.

Hunt, T. and A. Murray (1993b). The meiotic cell cycle. *In* The Cell Cycle. (New York: W. Freeman and Company). pp. 167-182.

Hunter, T. and J. Pines (1994). Cyclins and cancer II: cyclin D and CDK inhibitors come of age. Cell *79:* 573-582.

Illmensee, K. and A. P. Mahowald (1974). Transplantation of posterior polar plasm in *Drosophila*: induction of germ cells at the anterior pole of the egg. Proc. Natl. Acad. Sci. USA *71:* 1016-1020.

Inaba, T., H. Matsushime, M. Valentine, M. F. Roussel, C. J. Sherr and A. T. Look (1992). Genomic organization, chromosomal localization, and independent expression of human cyclin D genes. Genomics *13:* 565-574.

Iversen, O. H., R. Bjerknes and F. Devik (1968). Kinetics of cell renewal, cell migration and cell loss in the hairless mouse dorsal epidermis. Cell. Kinet. *1:* 351.

Jones, P. H. and F. M. Watt (1993). Separation of human epidermal stem cells from transit amplifying cells on the basis of differences in integrin function and expression. Cell *73:* 713-724.

Jovett, T. (1986). Preparation of nucleic acids. *In* Drosophila: a practical approach. (Oxford, IRL Press Ltd.). pp. 275-286.

Karess, R. E., X. Chang, K. A. Edwards, S. Kulkarni, I. Aguilera and D. P. Kiehart (1991). The regulatory light chain of nonmuscle myosin is encoded by *spaghetti-squash*, a gene required for cytokinesis in Drosophila. Cell *65:* 1177-1189.

Karpen, G. H. and A. C. Spradling (1992). Analysis of subtelomeric heterochromatin in the *Drosophila* minichromosome Dp1187 by single P-element insertional mutagenesis. Genetics 132*:* 737-753.

Karsch-Mizrachi, I. and S. R. Haynes (1993). The Rb97D gene encodes a potential RNA-binding protein required for spermatogenesis in Drosophila. Nucl. Acids Res. *21:* 2229-2235.

Kassir, Y., D. Granot and G. Simchen (1988). *IME1*, a positive regulator gene of meiosis in *S. cerevisiae*. Cell *52:* 853-862.

Kassis, J. A. (1990). Spatial and temporal control elements of the Drosophila engrailed gene. Genes Dev. *4:* 433-443.

Kataoka, T., S. Powers, C. McGill, O. Fasano, J. Strathern, J. Broach and M. Wigler (1984). Genetic analysis of yeast *RAS1* and *RAS2* genes. Cell *37:* 437-445.

Keinanen, M., J. D. Griffin, C. D. Bloomfield, J. Machnicki and A. De La Chapelle (1988). Clonal chromosomal abnormalities showing multiple-cell lineage involvement in acute myeloid leukemia. N. Engl. J. Med. *318:* 1153-1159.

Kellerman, K. A., D. M. Mattson and A. I. Duncan (1990). Mutations affecting the stability of the fushi tarazu protein of Drosophila. Genes Dev. *4:* 1936-1950.

Kemphues, K. J., R. A. Raff, T. C. Kaufman and E. C. Raff (1979). Mutation in a structural gene for a ß-tubulin specific to testis in Drosophila melanogaster. Proc. Natl. Acad. Sci. *76:* 3991-3995.

Kemphues, K. J., E. C. Raff, R. A. Raff and T. C. Kaufman (1980). Mutation in a testis-specific ß-tubulin in Drosophila: analysis of its effects on meiosis and map location of the gene. Cell *21:* 445-451.

Kemphues, K. J., T. C. Kaufman, R. A. Raff and E. C. Raff (1982). The testis-specific β-tubulin subunit in *Drosophila melanogaster* has multiple functions in spermatogenesis. Cell *31:* 655-670.

Kiehart, D. P., I., Mabuchi and Inoué, S. (1982) Evidence that myosin does not contribute to force production in chromosome movement. J. Cell Biol. *94:* 165-178.

Kimble, J. and S. Ward (1988). Germ-line development and fertilization. *In* The nematode Caenorhabditis. (Cold Spring Harbor, NY: Cold Spring Harbor Press). pp. 191-213.

Kimble, J. E. and J. G. White (1981). On the control of germ cell development in *Caenorhabditis elegans*. Dev. Biol. *81:* 208-219.

Kimble, M., J. P. Incardona and E. C. Raff (1989). A variant β-tubulin isoform of *Drosophila melanogaster* (β3) is expressed primarily in tissues of mesodermal origin in embryos and pupae, and is utilized in populations of transient microtubules. Dev. Biol. *131:* 415-429.

King, R. C. (1970). Ovarian development in *Drosophila melanogaster*. (New York: Academic Press).

King, R. C. and S. F. Riley (1982). Ovarian pathologies generated by various alleles of the *otu* locus in *Drosophila melanogaster*. Dev. Genet. *3:* 69-89.

Klar, A. J. S. (1987). Differentiated parental DNA strands confer developmental asymmetry on daughter cells in fission yeast. Nature *326:* 466-470.

Klar, A. J. S. (1990). The developmental fate of fission yeast cells is determined by the pattern of inheritance of parental and grandparental DNA strands. EMBO J. *9:* 1407-1415.

Knoblich, J. A. and C. F. Lehner (1993). Synergistic action of Drosophila cyclins A and B during the G_2-M transition. EMBO J. *12:* 65-74.

Knoblich, J. A., K. Sauer, L. Jones, H. Richardson, R. Saint and C. F. Lehner (1994). Cyclin E controls S phase progression and its down-regulation during Drosophila embryogenesis is required for the arrest of cell proliferation. Cell *77:* 107-120.

Krek, W. and E. A. Nigg (1991). Mutations of $p34^{cdc2}$ phosphorylation sites induce premature mitotic events in HeLa cells: evidence for a double block to $p34^{cdc2}$ kinase activation in vertebrates. EMBO J. *10:* 3331-3341.

Lasko, P. and M. Ashburner (1990). Posterior localization of vasa correlates with, but is not sufficient for, pole cell development. Genes Dev. *4:* 905 - 921.

Lehmann, R. and A. Ephrussi (1994). Germ plasm formation and germ cell determination in *Drosophila*. *In* Germ line Development. (Chichester, England: John Wiley & Sons). pp. 282-300.

Lehmann, R. and C. Nüsslein-Volhard (1986). Abdominal segmentation, pole cell formation, and embryonic polarity require the localized activity of *oskar*, a maternal gene in *Drosophila*. Cell *47:* 141-152.

Lehner, C. F. and P. H. O'Farrell (1989). Expression and function of Drosophila cyclin A during embryonic cell cycle progression. Cell *56:* 957-968.

Lehner, C. F. and P. H. O'Farrell (1990). The roles of *Drosophila* cyclins A and B in mitotic control. Cell *61:* 535-547.

Leith, J. T. and D. L. Dexter (1986). Mammalian tumor heterogeneity. (Boca Raton, FL: CRC Press).

LeMagueresse, B., C. Pineau, F. Guillou and B. Jegou (1988). Influence of germ cells upon transferrin secretion by rat Sertoli cells *in vitro*. J. Endocrinol. *118:* R13-R16.

Letsou, A., S. Alexander, K. Orth and S. A. Wasserman (1991). Genetic and molecular characterization of *tube,* a Drosophila gene maternally required for embryonic dorsoventral polarity. Proc. Natl. Acad. Sci. USA *88:* 810-814.

Letwin, K., L. Mizzen, B. Motro, Y. Ben-David, A. Bernstein and T. Pawson (1992). A mammalian dual specificity protein kinase, Nek1, is related to the NIMA cell cycle regulator and highly expressed in meiotic germ cells. EMBO J. *11:* 3521-3531.

Li, R. and A. W. Murray (1991). Feedback control of mitosis in budding yeast. Cell *66:* 519-531.

Liebrich, W. (1984). Premeiotic divisions of gonial cells are differently regulated in males and females of *Drosophila*. Intl. J. of Invert. Reprod. Dev. *7:* 369-371.

Lifschytz, E. (1978). Uncoupling of gonial and spermatocyte stages by means of conditional lethal mutations in *Drosophila melanogaster*. Dev. Biol. *66:* 571-578.

Lifschytz, E. (1987). The developmental program of spermiogenesis in Drosophila: a genetic analysis. Int. Rev. Cyt. *109:* 211-257.

Lifschytz, E. and D. Hareven (1977). Gene expression and the control of spermatid morphogenesis in *Drosophila melanogaster*. Dev. Biol. *58:* 276-294.

Lifschytz, E. and G. F. Meyer (1977). Characterization of male meiotic-sterile mutations in *Drosophila melanogaster*. Chromosoma *64:* 371-392.

Lifschytz, E. and N. Yakobovitz (1978). The role of X-linked lethal and viable male-sterile mutations in male gametogenesis of *Drosophila melanogaster*: genetic analysis. Mol. Gen. Genet. *161:* 275-284.

Lifschytz, E., D. Hareven, A. Azriel and H. Brodsly (1983). DNA clones and RNA transcripts of four lampbrush loops from the Y chromosome of Drosophila hydei. Cell *32:* 191-199.

Lin, H. and A. C. Spradling (1993). Germ line stem cell division and egg chamber development in transplanted *Drosophila* germaria. Dev. Biol. *159:* 140-152.

Lin, H., L. Yue and A. C. Spradling (1994). The *Drosophila* fusome, a germ line-specific organelle, contains membrane skeletal proteins and functions in cyst formation. Development *120:* 947-956.

Lindsley, D., L. Sandler, B. Baker, A. Carpenter, R. Denell, J. Hall, P. Jacobs, G. Miklos, B. Davis, R. Getthman, R. Hardy, A. Hessler, S. Miller, H. Nozawa, D. Parry and M. Gould-Somero (1972). Segmental aneuploidy and the genetic gross structure of the Drosophila genome. Genetics *71:* 157-184.

Lindsley, D. L. and K. T. Tokuyasu (1980). Spermatogenesis. *In* The genetics and development of Drosophila. (London: Academic Press; Vol. 2). pp. 225-294.

Lindsley, D. L. and G. G. Zimm (1992). The Genome of Drosophila. (San Diego: Academic Press).

Lord, B. I. and N. G. Testa (1988). The haematopoietic system. Structure and regulation. *In* Hematopoiesis: long-term effects of chemotherapy and radiation. (ed. N. G. Testa, R. P. Gale).

Lowe, S., T. Jacks, D. Housman and H. Ruley (1994). Abrogation of oncogene-associated apoptosis allows transformation of p53-deficient cells. Proc. Natl. Acad. Sci. USA *91:* 2026-2030.

Lowe, S. W. and H. E. Ruley (1993). Stabilization of the p53 tumor suppressor is induced by adenovirus E1A and accompanies apoptosis. Genes Dev. *7:* 535-545.

Lundgren, K., N. Walworth, R. Booher, M. Dembski, M. Kirschner and D. Beach (1991). mik1 and wee1 cooperate in the inhibitory tyrosine phosphorylation of cdc2. Cell *64:* 1111-1122.

Machesky, L. M. and T. D. Pollard (1993). Profilin as a potential mediator of membrane-cytoskeleton communication. Trends Cell Biol. *3:* 381-385.

Mahowald, A. P. (1962). Fine structure of pole cells and polar granules in *Drosophila melanogaster*. J. Exp. Zool. *151:* 201-215.

Mahowald, A. P. and G. Wei (1994). Sex determination of germ cells in *Drosophila*. *In* Germ line Development. (Chichester, England: John Wiley & Sons). pp. 193-209.

Malone, R. E. (1990). Dual regulation of meiosis in yeast. Cell *61:* 375-378.

Maniatis, T., E. F. Fritsch and J. Sambrook (1989). Molecular cloning: a laboratory manual. (Cold Spring Harbor, NY: Cold Spring Harbor Press; 2nd Edition).

Marsh, J. L. and E. Wieschaus (1978). Is sex determination in germ line and soma controlled by separate genetic mechanisms? Nature *272:* 249-251.

Marsh, L., A. M. Neiman and I. Herskowitz (1991). Signal transduction during pheromone response in yeast. Annu. Rev. Cell. Biol. *7:* 699-728.

Matsumoto, K., I. Uno and T. Ishikawa (1983). Initiation of meiosis in yeast mutants defective in adenylate cyclase and cyclic AMP-dependent protein kinase. Cell *32:* 417-423.

Maziarski, S. (1913). Sur la persistance des résidus fusoriaux pendant les nombreuses générations cellulaires au cours de l'ovogenèse de *Vespa vulgaris*. L. Arch. Zellforsch (Leipzig) *10:* 507-532.

McGuinness, M. P. and M. D. Griswold (1994). Interactions between Sertoli cells and germ cells in the testis. Sem. Dev. Biol. *5:* 61-66.

McKearin, D. and L. Christerson (1994). Molecular genetics of the early stages of germ cell differentiation during *Drosophila* oogenesis. *In* Germ line Development. (Chichester, England: John Wiley & Sons). pp. 210-222.

McKearin, D. M. and A. C. Spradling (1990). *bag-of-marbles*: a Drosophila gene required to initiate both male and female gametogenesis. Genes Dev. *4:* 2242-2251.

Meijer, L., D. Arion, R. Golsteyn, J. Pines, L. Brizuela, T. Hunt and D. Beach (1989). Cyclin is a component of the sea urchin egg M-phase specific histone H_1 kinase. EMBO J. *8:* 2275-2282.

Mello, C. C., B. W. Draper and J. R. Priess (1994). The maternal genes *apx-1* and *glp-1* and establishment of dorsal-ventral polarity in the early *C. elegans* embryo. Cell *77:* 95-106.

Mercer, W. E., M. T. Shields, M. Amin, G. J. Sauve, E. Appella, J. W. Romand and S. J. Ullrich (1990). Negative growth regulation in a glioblastoma tumor cell line that conditionally expresses human wild-type p53. Proc. Natl. Acad. Sci. USA *87:* 6166-6170.

Meyer, G. F. (1968). Spermiogenesis in normal and Y-deficient males of Drosophila melanogaster and *D. hydei.* (German). Z. Zellforsch. Mikrosk. Anat. *84:* 141-175.

Millar, J. B. A., C. H. McGowan, G. Lenaers, R. Jones and P. Russell (1991). $p80^{cdc25}$ mitotic inducer is the tyrosine phosphatase that activates $p34^{cdc2}$ kinase in fission yeast. EMBO J. *10:* 4301-4309.

Millar, J. B. A. and P. Russell (1992). The cdc25 M-phase inducer: an unconventional protein phosphatase. Cell *68:* 407-410.

Mintz, B. and E. S. Russel (1957). Gene-induced embryological modifications of primordial germ cells in the mouse. J. Exp. Zool. *134:* 207-237.

Mitchell, A. P. (1988). Two switches govern entry into meiosis in yeast. *In* Meiotic inhibition: molecular control of meiosis. (New York: Alan R. Liss, Inc). pp. 47-66.

Mitchell, A. P. and I. Herskowitz (1986). Activation of meiosis and sporulation by repression of the *RME1* product in yeast. Nature *319:* 738-742.

Mitchison, T. and J. Sedat (1983). Localization of antigenic determinants in whole Drosophila embryos. Dev. Biol. *99:* 261-264.

Miyata, H. and M. Miyata (1981). Modes of conjugation in homothallic cells of *Schizosaccharomyces pombe.* J. Gen. Appl. Microbiol. *27:* 365-371.

Mockrin, S. C. and E. D. Forn (1980). Acanthamoeba profilin interacts with G-actin to increase the rate of exchange of actin-bound adenosine 5'-triphosphate. Biochem. *19:* 5359-5362.

Monesi, V. (1962). Autoradiographic study of DNA synthesis and the cell cycle in spermatogonia and spermatocytes of mouse testis using tritiated thymidine. J. Cell. Biol. *14:* 1-18.

Mostofi, F. K. and I. A. Sesterhenn (1985). Pathology of germ cell tumors of testes. *In* Testicular Cancer. (New York: Alan R. Liss, Inc). pp. 1-34.

Motokura, T., T. Bloom, H. G. Kim, H. Jüppner, J. V. Ruderman, H. M. Kronenerg and A. Arnold (1991). A novel cyclin encoded by a *bcl*-linked candidate oncogene. Nature *350:* 512-515.

Muralidhar, M. G. and J. B. Thomas (1993). The Drosophila gene *bendless* encodes a neural protein related to ubiquitin conjugating enzymes. Neuron *11:* 253-266.

Murray, A. (1992). Creative blocks: cell-cycle checkpoints and feedback controls. Nature *359:* 599-604.

Murray, A. W., M. J. Solomon and M. W. Kirschner (1989). The role of cyclin synthesis and degradation in the control of maturation promoting factor activity. Nature *339:* 280-286.

Nath, V. (1965) Animal Gametes (Male). (Bombay: Asia Publishing House)

Norbury, C., J. Blow and P. Nurse (1991). Regulatory phosphorylation of the $p34^{cdc2}$ protein kinase in vertebrates. EMBO J. *10:* 3321-3329.

Nurse, P. (1990). Universal control mechanism regulating onset of M-phase. Nature *344:* 503-507.

Nurse, P. and P. Thuriaux (1980). Regulatory genes controlling mitosis in the fission yeast. Nature *292:* 558-560.

Nurse, P., P. Thuriaux and K. Nasmyth (1976). Genetic control of the cell division cycle in the fission yeast *Schizosaccharomyces pombe*. Mol. Gen. Genet. *146:* 167-178.

O'Kane, C. J. and W. J. Gehring (1987). Detection *in situ* of genomic determinants in whole *Drosophila* embryos. Proc. Natl. Acad. Sci. USA *84:* 9123-9127.

Oakberg, E. F. (1956). A description of spermiogenesis in the mouse and its use in analysis of the cycle of the seminiferous epithelium and germ cell renewal. Am. J. Anat. *99:* 391-413.

Ogawa, M. (1993). Differentiation and proliferation of hematopoietic stem cells. Blood *81:* 2844-2853.

Okada, M., I. H. Leinman and H. A. Schneiderman (1974). Restoration of fertility in sterilized *Drosophila* eggs by transplantation of polar cytoplasm. Dev. Biol. *37:* 43-54.

Oliver, B., Y. K. Kim and B. S. Baker (1993). *Sex-lethal*, master and slave: a hierarchy of germ-line sex determination in *Drosophila*. Development *119:* 897-908.

Oliver, B., D. Pauli and A. P. Mahowald (1990). Genetic evidence that the *ovo* locus is involved in Drosophila germ line sex determination. Genetics *125:* 535-50. [erratum appears in Genetics *126:* 477].

Oliver, B., N. Perrimon and A. P. Mahowald (1988). Genetic evidence that the *sans-fille* locus is involved in *Drosophila* sex-determination. Genetics *120:* 159-171.

Olivieri, G. and A. Olivieri (1965). Autoradiographic study of nucleic acid synthesis during spermatogenesis in *Drosophila melanogaster*. Mut. Res. *2:* 366-380.

Osmani, A. H., S. L. McGuire and S. A. Osmani (1991). Parallel activation of the NIMA and $p34^{cdc2}$ cell cycle-regulated protein kinases is required to initiate mitosis in A. nidulans. Cell *67:* 283-291.

Pagano, M., R. Pepperkok, F. Verde, W. Ansorge and G. Draetta (1992). Cyclin A is required at two points in the human cell cycle. EMBO J. *11:* 961-971.

Pardee, A. B. (1989). G1 events and regulation of cell proliferation. Science *246:* 603-608.

Parker, L. L., S. Atherton-Fessler and H. Piwnica-Worms (1992). $p107^{wee1}$ is a dual-specificity kinase that phosphorylates $p34^{cdc2}$ on tyrosine 15. Proc. Natl. Acad. Sci. USA *89:* 2917-21.

Patel, N. H., P. M. Snow and C. S. Goodman (1987). Characterization and cloning of Fasciclin III: a glycoprotein expressed on a subset of neurons and axon pathways in Drosophila. Cell *48:* 975-988.

Pauli, D. and A. P. Mahowald (1990). Germ-line sex-determination in *Drosophila melanogaster*. Trends Genet. *6:* 259-264.

Pauli, D., B. Oliver and A. P. Mahowald (1993). The role of the *ovarian tumor* locus in *Drosophila melanogaster* germ line sex determination. Development *119:* 123-134.

Peacock, W. J, G. L. G. Miklos and D.J. Goodchild (1975). Sex chromosome meiotic drive systems in Drosophila melanogaster. I. Abnormal spermatid development in males with a heterochromoatin-deficient X chromosome (sc^4 sc^8). Genetics *79:* 613-634.

Peifer, M., S. Orsulic, L. Pai and J. Loureiro (1993a). A model system for cell adhesion and signal transduction in *Drosophila*. Development (*Suppl.*)*:* 163-176.

Peifer, M., S. Orsulic, D. Sweeton and E. Wieschaus (1993b). A role for the *Drosophila* segment polarity gene *armadillo* in cell adhesion and cytoskeletal integrity during oogenesis. Development *118:* 1191-1207.

Pelliniemi, L. J., K. Fröjdman and J. Paranko (1993). Embryological and prenatal development and function of Sertoli cells. *In* The Sertoli Cell. (Clearwater, FL: Cache River Press). pp. 87-113.

Perrimon, N., L. Engstrom and A. P. Mahowald (1989). Zygotic lethal with specific maternal eefect phenotypes in Drosophila melanogaster. I. Loci on the X chromosome. Genetics *121:* 333 - 352.

Perrimon, N., D. Mohler, L. Engstrom and A. P. Mahowald (1986). *X*-linked female-sterile loci in *Drosophila melanogaster.* Genetics *113:* 695-712.

Peter, M., A. Gartner, J. Horecka, G. Ammerer and I. Herskowitz (1993). FAR1 links the signal transduction pathway to the cell cycle machinery in yeast. Cell *73:* 747-760.

Pines, J. and T. Hunter (1989). Isolation of a human cyclin cDNA: Evidence for cyclin mRNA and protein regulation in the cell cycle and for interaction with $p34^{cdc2}$. Cell *58:* 833-846.

Pimpinelli, S. and P. Ripoll (1986). Nonrandom segregation of centromeres following mitotic recombination in Drosophila melanogaster. Proc. Natl. Acad. Sci. USA *83:* 3900-3903.

Pollard, T. D. and J. Cooper (1986). Actin and actin-binding proteins. Annu. Rev. Biochem. *55:* 987-1035.

Pondaven, P., L. Meijer and D. Beach (1990). Activation of M-phase-specific histone H1 kinase by modification of the phosphorylation of its $p34^{cdc2}$ and cyclin components. Genes Dev. *4:* 9-17.

Posada, J., J. Sanghera, S. Pelech, R. Aebersold and J. A. Cooper (1991). Tyrosine phosphorylation and activation of homologous protein kinases during oocyte maturation and mitogenic activation of fibroblasts. Mol. Cell. Biol. *11:* 2517-2528.

Potten, C. S. and T. D. Allen (1975). The fine structure and cell kinetics of mouse epidermis after wounding. J. Cell Sci. *17:* 413-447.

Potten, C. S. and J. H. Hendry (1973). Clonogenic cells and stem cell in epidermis. Int. J. Radiat. Biol. *24:* 537-540.

Potten, C. S. and R. J. Morris (1988). Epithelial stem cell in vivo. J. Cell. Sci. (*Suppl.*) *10:* 45-62.

Price, J. V., R. J. Clifford and T. Schüpbach (1989). The maternal ventralizing locus *torpedo* is allelic to *faint little ball,* an embryonic lethal and encodes the Drosophila EGF receptor homolog. Cell *56:* 1085-1092.

Quinn, L. S., H. Holtzer and M. Nameroff (1985). Generation of chick skeletal muscle cells in groups of 16 from stem cells. Nature *313:* 692-694.

Raff, M. C., E. R. Abney and S. J. Fok (1985). Reconstitution of a developmental clock in vitro: a critical role for astrocytes in the timing of oligodendrocyte differentiation. Cell *42:* 61-9.

Raff, M. C., L. E. Lillien, W. D. Richardson, J. F. Burne and M. D. Noble (1988). Platelet-derived growth factor from astrocytes drives the clock that times oligodendrocyte development in culture. Nature *333:* 562-565.

Reed, S. I. (1991). Pheromone signaling pathways in yeast. Curr. Opin. Genet. Dev. *1:* 391-396.

Reed, S. I. (1992). The role of p34 kinase in the G1 to S-phase transition. Annu. Rev. Cell Biol. *8:* 529-561.

Regan, C. L. and M. T. Fuller (1990). Interacting genes that affect microtubule function in Drosophila melanogaster: two classes of mutation revert the failure to complement between *hay*^{nc2} and mutations in tubulin genes. Genetics *125:* 77-90.

Rheinwald, J. G. and H. Green (1975a). Formation of a keratinizing epithelium in culture by a cloned cell line derived from a teratoma. Cell *6:* 317-330.

Rheinwald, J. G. and H. Green (1975b). Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. Cell *6:* 331-343.

Rhyu, M. S., L. Y. Jan and Y. N. Jan (1994). Asymmetric distribution of numb protein during division of the sensory organ precursor cell confers distinct fates to daughter cells. Cell *76:* 477-491.

Robertson, H. M., C. R. Preston, R. W. Phillis, D. M. Johnson-Schlitz, W. K. Benz and W. R. Engels (1988). A stable genomic source of P element transposase in *Drosophila melanogaster*. Genetics *118:* 461-470.

Ronchi, E., J. Treisman, N. Dostani, G. Struhl and C. Desplan (1993). Down-regulation of the Drosophila morphogen bicoid by the torso receptor-mediated signal transduction cascade. Cell *74:* 347-355.

Roosen-Runge, E. C. (1977). The Process of Spermatogenesis in Animals. (London: University Press).

Rosenthal, N. (1989). Muscle cell differentiation. Curr. Opin. Cell. Biol. *1:* 1094-1101.

Roth, S., D. Stein and C. Nusslein-Volhard (1989). A gradient of nuclear localization of the dorsal protein determines dorsoventral pattern in the Drosophila embryo. Cell *59:* 1189-1202.

Rungger-Brändle, E. (1976). Spermatogenesis in larval testes of *Drosophila hydei*, cultured in vivo. Roux's Arch. Dev. Biol. *180:* 79-91.

Rushlow, C. A., K. Han, J. L. Manley and M. Levine (1989). The graded distribution of the dorsal morphogen is initiated by selective nuclear transport in Drosophila. Cell *59:* 1165-1177.

Russell, L. D. Sertoli-germ cell interrelations: a review. Gamete Res. *3:* 179-202.

Russell, P. and P. Nurse (1986). cdc 25^{+} functions as an inducer in the mitotic control of fission yeast. Cell *45:* 145-153.

Russell, P. and P. Nurse (1987). Negative regulation of mitosis by wee1+, a gene encoding a protein kinase homolog. Cell *49:* 559-67.

Salz, H. K. (1992). The genetic analysis of *snf*: a *Drosophila* sex determination gene required for activation of *sex-lethal* in both the germ line and the soma. Genetics *130:* 547-554.

Salz, H. K., T. W. Cline and P. Schedl (1987). Functional changes associated with structural alterations induced by mobilization of a P-element inserted in the *Sex-lethal* gene of *Drosophila*. Genetics *117:* 221-231.

Saxton, W. M., J. Hicks, L. S. B. Goldstein and E. C. Raff (1991). Kinesin heavy chain is essential for viability and neuromuscular functions in Drosophila, but mutants show no defects in mitosis. Cell *64:* 1093-1102.

Schäfer, M., R. Kuhn, F. Bosse and U. Schäfer (1990). A conserved element in the leader mediates post-meiotic translation as well as cytoplasmic polyadenylation of a Drosophila spermatocyte mRNA. EMBO J. *9:* 4519-4525.

Schejter, E. D. and B. Shilo (1989). The Drosophila EGF receptor homolog (DER) gene is allelic to *faint little ball* a locus essential for embryonic development. Cell *56:* 1093-1104.

Schild, D. and B. Byers (1978). Meiotic effects of DNA defective cell division cycle mutations of *Saccharomyces cerevisiae*. Chromosoma *70:* 109-130.

Schubiger, M. and J. Palka (1987). Changing spatial patterns of DNA replication in the developing wing of *Drosophila*. Dev. Biol. *123:* 145-153.

Schüpbach, T. (1982). Autosomal mutations that interfere with sex determination in somatic cells of *Drosophila* have no direct effect on the germ line. Dev. Biol. *89:* 117-127.

Schüpbach, T. (1985). Normal female germ cell differentiation requires the female X chromosome to autosome ratio and expression of *Sex-lethal* in *Drosophila melanogaster*. Genetics *109:* 529-548.

Schüpbach, T. (1987). Germ line and soma cooperate during oogenesis to establish the dorso-ventral pattern of egg shell and embryo in *Drosophila melanogaster*. Cell *49:* 699-707.

Schüpbach, T. and E. Wieschaus (1989). Female sterile mutations on the second chromosome of *Drosophila melanogaster*. I. Maternal effect mutations. Genetics *121:* 101-117.

Schüpbach, T. and E. Wieschaus (1991). Female sterile mutations on the second chromosome of *Drosophila melanogaster*. II. Mutations blocking oogenesis or altering egg morphology. Genetics *129:* 1119-1136.

Schüpbach, T., R. J. Clifford, L. J. Manseau and J. V. Price (1991). Dorso-ventral signalling processes in *Drosophila* oogenesis. Symp. Soc. Dev. Biol. *49:* 163-174.

Schüpbach, T., E. Wieschaus and R. Nöthiger (1978). A study of the female germ line in mosaic Drosophila. Roux's Arch. EntwMech. Org. *184:* 41-56.

Schuster, E. O. and B. Byers (1989). Pachytene arrest and other meiotic effects of the Start mutations in *Saccharomyces cerevisiae*. Genetics *123:* 29-43.

Shellenbarger, D. L. and D. P. Cross (1979). Genetic dissection of fertility in *Drosophila* males: properties of temperature-sensitive lethal-temperature-sensitive male-sterile mutations. Dev. Biol. *71:* 308-322.

Sherr, C. J. (1994). G1 phase progression: cycling on cue. Cell *79:* 551-555.

Shoup, J. R. (1967). Spermiogenesis in wild type and in a male sterility mutant of Drosophila melanogaster. J. Cell Biol. *32:* 663-675.

Siegfried, E., T.-B. Chou and N. Perrimon (1992). *wingless* signaling acts through *zeste-white* 3, the Drosophila homolog of glycogen synthase kinase-3, to regulate *engrailed* and establish cell fate. Cell *71:* 1167-1179.

Skaer, H. (1989). Cell division in Malpighian tubule development in Drosophila melanogaster is regulated by a single tip cell. Nature *342*: 566-569.

Skaer, H. (1992). Cell proliferation and rearrangement in the development of the Malpighian tubules of the hemipteran, *Rhodnius prolixus*. Dev. Biol. *150:* 372-380.

Skaer, H. and A. Martinez-Arias (1992). The *wingless* product is required for cell proliferation in the Malpighian tuble anlage of *Drosophila melanogaster*. Development *116:* 745-754.

Smith, H. E. and A. P. Mitchell (1989). A transcriptional cascade governs entry into meiosis in *Saccharomyces cerevisiae*. Mol. Cell. Biol. *9:* 2142-2152.

Solomon, M. J., M. Glotzer, T. H. Lee, M. Philippe and M. W. Kirschner (1990). Cyclin activation of $p34^{cdc2}$. Cell *63:* 1013-1024.

Sonnenblick, B. P. (1941). Germ cell movements and sex differentiation of the gonads in the *Drosophila* embryo. Proc. Natl. Acad. Sci. USA *26:* 373-381.

Spradling, A. C. (1993). Developmental genetics of oogenesis. *In* The Development of Drosophila melanogaster. (Cold Spring Harbor, NY: Cold Spring Harbor Press). pp. 1-70.

Steward, R. (1989). Relocalization of the dorsal protein from the cytoplasm to the nucleus correlates with its function. Cell *59:* 1179-1188.

Standart, N., J. Minshull, J. Pines and T. Hunt (1987). Cyclin synthesis, modification and destruction during meiotic maturation of the starfish oocyte. Dev. Biol. *124:* 248-258.

Stanley, H. P., J. T. Bowman, L. J. Romrell, S. C. Reed and R. F. Wilkinson (1972). Fine structure of normal spermatid differentiation in *Drosophila melanogaster*. J. Ultrastruc. Res. *31:* 433-466.

Steele, F. and J. E. O'Tousa (1990). Rhodopsin activation causes retinal degeneration in Drosophila *rdgc* mutant. Neuron *4:* 883-890.

Steinmann-Zwicky, M. (1988). Sex determination in Drosophila: the X-chromosomal gene *liz* is required for *Sxl* activity. EMBO J. *7:* 3889-3898.

Steinmann-Zwicky, M. (1992). Sex determination of Drosophila germ cells. Sem. Dev. Biol. *3:* 341-347.

Steinmann-Zwicky, M., H. Schmid and R. Nöthiger (1989). Cell autonomous and inductive signals can determine the sex of the germ line of *Drosophila* by regulating the gene *Sxl*. Cell *57:* 157-166.

Stern, B., G. Ried, N. J. Clegg, T. A. Grigliatti and C. F. Lehner (1993). Genetic analysis of the *Drosophila cdc2* homolog. Development *117:* 219-232.

Strausfeld, U., J. C. Labbé, D. Fesquet, J. C. Cavadore, A. Picard, K. Sadhu, P. Russell and M. Dorée (1991). Dephosphorylation and activation of a $p34^{cdc2}$/cyclin B complex *in vitro* by human CDC25 protein. Nature *351:* 242-245.

Strome, S., C. Garvin, J. Paulsen, E. Capowski, P. Martin and M. Beanan (1994). Specification and development of the germ line in *Caenorhabditis elegans*. *In* Germ line Development. (Chichester, John Wiley & Sons). pp. 31-45.

Surti, U., L. Hoffner, A. Chakravarti and R. Ferrell (1990). Genetics and biology of human ovarian teratomas. I. Cytogenetic analysis and mechanism of origin. Am. J. Hum. Genet. *47:* 635-643.

Tatchell, K., L. Robinson and M. Breitenbach (1985). *RAS2* of *Saccharomyces cerevisiae* is required for gluconeogenic growth and proper response to nutrient limitation. Proc. Natl. Acad. Sci. USA *82:* 3785-3789.

Tates, A. D. (1971). Cytodifferentiation during spermatogenesis in Drosophila melanogaster: An electron microscope study. Ph.D. thesis (Leiden: Rijksuniversiteit).

Tax, F. E., J. J. Yeargers and J. H. Thomas (1994). Sequence of *C. elegans lag-2* reveals a cell-signalling domain shared with *Delta* and *Serrate* of *Drosophila*. Nature *368:* 150-154.

Telfer, W. H. (1975). Development and physiology of the oocyte-nurse cell syncytium. Adv. Insect Physiol. *11:* 223-319.

Temple, S. and M. C. Raff (1986). Clonal analysis of oligodendrocyte development in culture. Evidence for a developmental clock that counts cell divisions. Cell *44:* 773-779.

Theriot, J. A. and T. J. Mitchison (1993). The three faces of profilin. Cell *75:* 835-838.

Thomas, B. J., D. A. Gunning, J. Cho and S. L. Zipursky (1994). Cell cycle progression in the developing *Drosophila* eye: *roughex* encodes a novel protein required for the establishment of G1. Cell *77:* 1003-1014.

Till, E. and E. A. McCulloch (1961). A direct measurement of radiation sensitivity of normal mouse bone marrow cells. Mut. Res. *14:* 213-222.

Toda, T., S. Cameron, S. P., M. Zoller and M. Wigler (1987). Three different genes in *S. cerevisiae* encode the catalytic subunits of the cAMP-dependent protein kinase. Cell *50:* 277-287.

Toda, T., S. Cameron, P. Sass, M. Zoller, J. D. Scott, B. McMullen, M. Hurwitz, E. G. Krebs and M. Wigler (1987). Cloning and characterization of *BCY1*, a locus encoding the regulatory subunit of the cAMP-dependent protein kinase in *Saccharomyces cerevisiae*. Mol. Cell. Biol. *7:* 1371-1377.

Toda, T., I. Uno, T. Ishikawa, S. Powers, T. Kataoka, D. Broek, S. Cameron, J. Broach, K. Matsumoto and M. Wigler (1985). In yeast, *RAS* proteins are controlling elements of adenylate cyclase. Cell *40:* 27-36.

Tokuyasu, K. T. (1974a). Dynamics of spermiogenesis in *Drosophila melanogaster*. III. Relation between axoneme and mitochondrial derivatives. Exp. Cell Res. *84:* 239-250.

Tokuyasu, K. T. (1974b). Dynamics of spermiogenesis in *Drosophila melanogaster*. IV. Nuclear transformation. J. Ultrastruct. Res. *48:* 284-303.

Tokuyasu, K. T. (1974c). Spoke heads in sperm tail of *Drosophila melanogaster*. J. Cell Biol. *63:* 334-337.

Tokuyasu, K. T. (1975a). Dynamics of spermiogenesis in *Drosophila melanogaster*. V. Head tail alignment. J. Ultrastruct. Res. *50:* 117-129.

Tokuyasu, K. T. (1975b). Dynamics of spermiogenesis in *Drosophila melanogaster*. VI. Significance of "onion" nebenkern formation. J. Ultrastruct. Res. *53:* 93-112.

Tokuyasu, K. T., W. J. Peacock and R. W. Hardy (1972a). Dynamics of spermiogenesis in *Drosophila melanogaster*. I. Individualization process. Z. Zelforsch. *124:* 479-506.

Tokuyasu, K. T., W. J. Peacock and R. W. Hardy (1972b). Dynamics of spermiogenesis in *Drosophila melanogaster*. II. Coiling process. Z. Zellforsch. *127:* 492-525.

Uemura, T., S. Shephard, L. Ackerman, L. Y. Jan and Y. N. Jan (1989). *numb*, a gene required in determination of cell fate during sensory organ formation in Drosophila embryos. Cell *58:* 349-360.

Verheyen, E. M. and L. Cooley (1994). Profilin mutations disrupt multiple actin-dependent processes during *Drosophila* development. Development *120:* 717-728.

Vogt, P. and W. Hennig (1986a). Molecular structure of the lampbrush loops nooses of the Y chromosome of Drosophila hydei. I. The Y chromosome-specific repetitive DNA sequence family ay1 is dispersed in the loop DNA. Chromosoma *94:* 449-458.

Vogt, P. and W. Henning (1986b). Molecular structure of the lampbrush loop nooses of the Y chromosome of Drosophila hydei. II. DNA sequences with homologues to multiple genomic locations are major constituents of the loop. Chromosoma *94:* 459-467.

Vojtek, A., B. Haarer, J. Field, J. Gerst, T. D. Pollard, S. Brown and M. Wigler (1991). Evidence for a functional link between profilin and CAP in the yeast *S. cerevisiae*. Cell *66:* 497-505.

Warn, R. M., H. O. Gutzeit, S. L. and A. Warn (1985). F-actin rings are associated with the ring canals of the Drosophila egg chamber. Exp. Cell Res. *157:* 355-363.

Warrior, R. (1994). Primordial germ cell migration and the assembly of the *Drosophila* embryonic gonad. Dev. Biol. *166:* 180-94.

Watt, F. M. and H. Green (1982). Stratification and terminal differentiation of cultured epidermal cells. Nature *295:* 434-436.

Wei, G., B. Oliver, D. Pauli and A. P. Mahowald (1994). Evidence for transformation of germ line cells in ovarian tumor mutants of *Drosophila*. Dev. Biol. *161:* 318-320.

Weinberg, R. A. (1991). Tumor suppressor genes. Science *254:* 1138-1146.

Welsh, M. K., M. E. Ireland and G. J. Treisman (1985). Stimulation of rat Sertoli cell adenylate cyclase by germ cells *in vitro*. Biol. Reprod. *33:* 1050-1056.

Western, H. and D. F. Bainton (1979). Association of alkaline phosphatase positive reticulum cells in bone marrow with granulocytic precursors. J. Exp. Med. *150:* 919-937.

White-Cooper, H., L. Alphey and D. M. Glover (1993). The cdc25 homologue *twine* is required for only some aspects of the entry into meiosis in *Drosophila*. J. Cell Sci. *106:* 1035-1044.

Whiteley, M., P. D. Noguchi, S. M. Sensabaugh, W. F. Odeuwald and J. A. Kassis (1992). The *Drosophila* gene *escargot* encodes a zinc finger motif found in *snail*-related genes. Mech. Dev. *36:* 117-127.

Whitfield, W. G. F., C. Gonzalez, E. Sanchez-Herrero and D. M. Glover (1989). Transcripts of one of two Drosophila cyclin genes become localized in pole cells during embryogenesis. Nature *338:* 337-340.

Whithers, H. R. (1967). Recovery and repopulation *in vivo* by mouse skin epithelial cells during fractionated irradiation. Rad. Res. *32:* 227-239.

Wieschaus, E. and J. Szabad (1979). The development and function of the female germ line in Drosophila melanogaster: a cell lineage study. Dev. Biol. *68:* 29-46.

Wilson, C., R. K. Pearson, H. J. Bellen, C. J. O'Kane, U. Grossniklaus and W. J. Gehring (1989). P-element-mediated enhancer detection: an efficient method for isolating and characterizing developmentally regulated genes in Drosophila. Genes Dev. *3:* 1301-1313.

Wood, W. B. and J. King (1979) Genetic control of complex bacteriophage assembly. *In* Comprehensive virology. (Plenum; vol. 13). pp. 581-633.

Xiong, Y., J. Menninger, D. Beach and D. C. Ward (1992). Molecular cloning and chromosomal mapping of CCND genes encoding human D-type cyclins. Genomics *13:* 575-584.

Xiong, Y., H. Zhang and D. Beach (1993). Subunit rearrangement of the cyclin-dependent kinases is associated with cellular transformation. Genes Dev. *7:* 1572-1583.

Xu, T. and G. M. Rubin (1993). Analysis of genetic mosaics in developing and adult Drosophila tissues. Development *117:* 1223-1237.

Yochem, J., K. Weston and I. Greenwald (1989). The *Caenorhabditis elegans lin-12* gene encodes a transmembrane protein with overall similarity to *Drosophila Notch.* Nature *335:* 547-550.

Zindy, F., E. Lamas, X. Chenivesse, J. Sobczak, J. Wang, D. Fesquet, B. Henglein and C. Bréchot (1992). Cyclin A is required in S phase in normal epithelial cells. Biochem. Biophy. Res. Commun. *182:* 1144-1154.

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