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FEMALE MEIOSIS AND THE ONSET OF EMBRYOGENESIS IN *DROSOPHILA MELANOGASTER*

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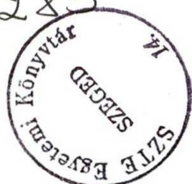

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1. PUBLICATIONS RELATED TO THE THESIS

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I. **Szabad, J., Máthé, E. and Puro, J.** (1995). *Horka*, a dominant mutation of *Drosophila*, induces nondisjunction and through paternal effect, chromosome loss and genetic mosaics. *Genetics* **139,** 1585-1599

II. **Erdélyi, M., Máthé, E. and Szabad, J.** (1997). Genetic and developmental analysis of mutant *Ketel* alleles that identify the *Drosophila* importin-β homologue. *Acta Biol. Hung.* **48,** 323-338

III. **Máthé, E., Boros, I., Jósvay, K., Kaijun L., Puro, J., Kaufman, T. C. and Szabad, J.** (1997). The *Tomaj* mutant alleles of *αTubulin67C* reveal a requirement for the encoded maternal specific tubulin isoform in the sperm aster, the cleavage spindle apparatus and neurogenesis during embryonic development in *Drosophila*. *J. Cell Sci.* (submitted)

IV. **Kuhfittig, S., Máthé, E., Szabad, J. and Reuter,G.** (1997). *Pitkin* a gain-of-function enhancer of position-effect variegation disrupts chromatin differentiation during oogenesis and embryogenesis in *Drosophila*. *J. Cell Sci* (submitted)

V. **Cserpán, I., Máthé, E., Patthy, A., Udvardy, A.** (1997). Characterization of *Drosophila* phosphorylation dependent nuclear localization signal binding protein. *Biochem. J.* (in press)

VI. **Lippai, M., Mihály, J., Erdélyi, M., Máthé, E., Puro, J., Belecz, I., Tirian, L., Posfai, J., Nagy, A., Udvardy, A., Boros, I. and Szabad, J.** (1997) A *Drosophila* importin-β homolgue encoded by the *Ketel* gene is essential for nuclear membrane assembly. *EMBO. J.* (submitted)

CONFERENCE ABSTRACTS

I. **Máthé, E., Jósvay, K. and Szabad, J.** (1994). Genetic and molecular analysis of the microtubule function in *Drosophila*. *Cell Biology International* **18(5),** 438

II. **Szabad, J., Erdélyi, M., Puro, J., Mihály, J., Lippai, M., Udvardy, A. and Máthé, E.** (1996). The genetics of nuclear protein import. *Hereditas* **124,** 297

POPULAR SCIENCE

Máthé Endre (1993). A sejt váza: állandóság és mozgás. *Élet és Tudomány* **3,** 67-69

2. INTRODUCTION

Drosophila melanogaster, like all the *holometabolous* insects, undergoes complete metamorphosis. The *Drosophila* embryo hatches from the egg into a larva. During the three larval instars, the larva feeds voraciously, growing through two series of molts. At the end of the final (3rd) larval instar, the larva stops feeding, surrounds itself with a pupal case and molts into a pupa. In the pupa, most of the larval tissues disintegrate and are reabsorbed, providing building blocks for subsequent organogenesis. The remaining larval tissues are specialized imaginal cells, which differentiate to form organs of the adult insect. The adults will form germ cells to provide basis for a continuation of generations.

This Ph.D. thesis deals with the female meiosis and the pronuclear stage of embryonic development of *Drosophila melanogaster*. The female meiosis is initiated during oocyte differentiation and arrests at metaphase I as the oocyte differentiation is completed in the ovaries. The first meiotic division proceeds when the egg is transferred through the oviduct into the uterus. The second meiotic division has been already completed and the pronuclear stage of embryonic development is initiated by the time the egg is laid. The pronuclear stage of development ends with the first embryonic mitosis (gonomeric division). The gonomeric division is followed by 12 rounds of rapid and synchronous cleavage cycles that lead to a syncytial embryo. After the cleavage cycles, the syncytial embryo undergoes cellularization, a blastoderm of about 5600 cells forms and the complex pattern of morphogenesis is initiated.

2.1. The development of *Drosophila* oocyte (egg chamber)

This paragraph provides a brief overview of the *Drosophila* oocyte development, considering the most important morphological features of oocyte determination and differentiation (reviewed by King, 1970; Spradling, 1993; Knowles and Cooley, 1994; Mahajan-Miklos and Cooley, 1994; Theurkauf, 1994).

Drosophila ovaries are composed of approximately 16 ovarioles, each of which contains an anterior germarium and a posterior vitellarium **(Fig. 1, Fig. 2)**.

Oogenesis begins in the germarium with the asymmetrical division of a germline stem cell into a daughter stem cell and a cytoblast. Subsequently, four consecutive mitotic divisions of the cytoblast give rise to a cyst of 16 germline cells (cystocytes).

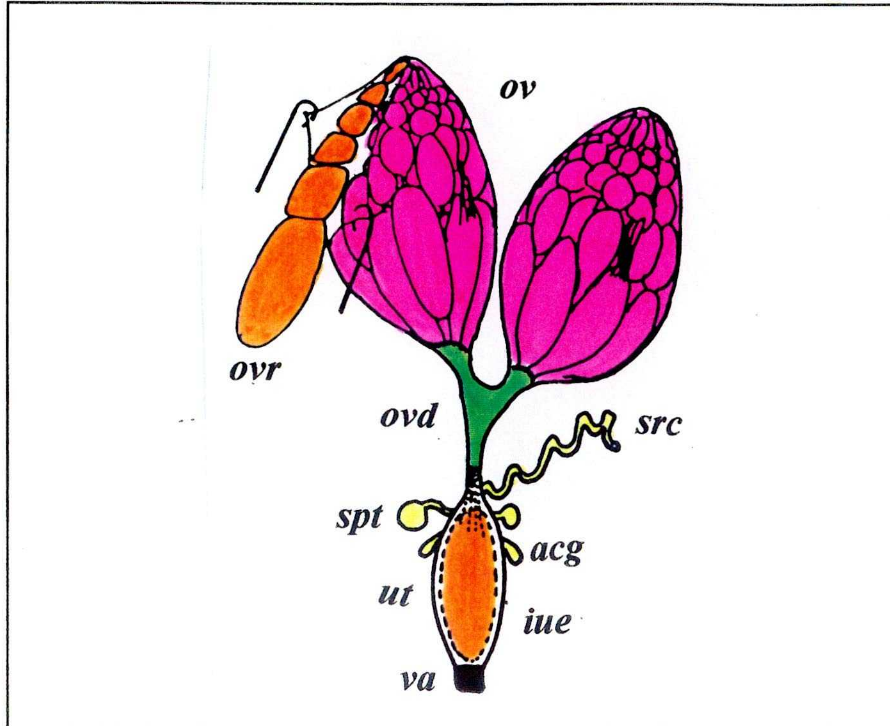

Fig. 1. The female reproductive organ of the adult *Drosophila*

ov - ovary; *ovr* - ovariole; *ovd* - oviduct; *ut* - uterus; *va* - vagina; *src* - seminal receptacule; *spt* - spermathecae; *acg* - accessory glands; iue - intrauterine egg.

Since cytokinesis is incomplete during the four mitotic divisions, the 16 germline cells remain connected by intracellular bridges also called ring canals. One cell from the 16 interconnected germline cell cyst differentiates into an oocyte, while the remaining 15 become nurse cells that synthesize the products (RNAs and proteins) required for oocyte development and early embryogenesis. The 16 interconnected germline cell cyst is enveloped by a monolayer of somatic follicle cells to form an egg chamber **(Fig. 2C)**. It is important to notice, that genome of developing oocyte is transcribed actively in germarial cysts (at the time of oocyte determination or oogenetic stage 1), but it becomes almost transcriptionally silent shortly after egg chambers leave the germarium **(Fig. 2A)**. Oocyte differentiation is hence supported by the nurse cells during vitellarial stages.

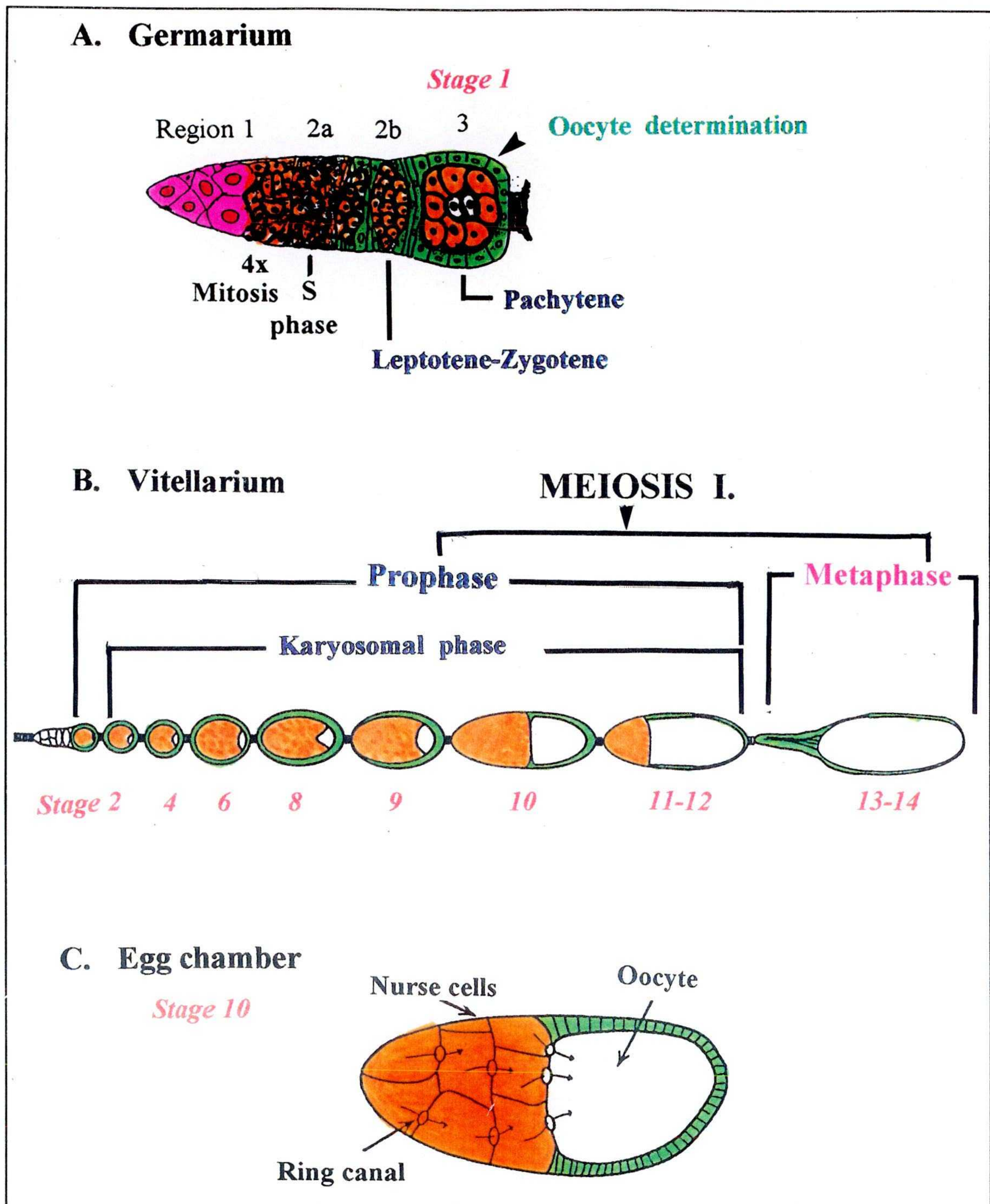

Fig. 2. Meiosis I and development of *Drosophila* oocyte / egg chamber

(A) The schematic representation of a germarium divided in three regions. The pink cells represent germline stem cells, the orange cells stand for clusters of germline cells (cystocytes) and the green cells are follicle cells. **(B)** Schematic representation of an ovariole, which consists of an anterior germarium and a posterior vitellarium. The ovariole contains egg chambers in increasing stages of growth and development from stage 1 to stage 14, which is the mature egg. **(C)** Schematic diagram of a stage 10 egg chamber in which the oocyte volume is approximately equal to that of the nurse cells. Arrows depict the flow of cytoplasm from the nurse cells into the oocyte through ring canals.

The germarium has been divided into three regions based on morphology **(Fig. 2A)** (King, 1970; Mahowald and Strassheim, 1970). Germline stem cells reside in the anteriormost region (region 1), where they continuously divide (Wieschaus and Szabad, 1979). In region 2, sixteen germline cell cysts are present and are enveloped by migrating follicle cells to complete the formation of an egg chamber (Szabad and Hoffmann, 1989). The germarium has been further subdivided into two parts. In the anterior region (2a), two or more egg chambers may be positioned across the width of the germarium. In the posterior region (2b), a single egg chamber extends across the diameter of the germarium forming the shape of a lens. The most posterior germarial region (region 3) contains oogenetic stage 1 egg chambers that are ready to enter the vitellarium for further development.

Stage 1 egg chamber enters the vitellarium where it continues to develop as it moves posteriorly. The vitellarium contains egg chambers in various stages of development from oogenetic stage 2 to stage 14, which is the mature egg (King, 1970; Mahowald and Kambysellis, 1980). Oocyte volume gradually increases during stages 2-10a due to a slow cytoplasm streaming from the nurse cells and yolk uptake by the oocyte, which begins in stage 8. During stages 10b and 11, the rapid final phase of cytoplasm transport occurs, resulting in the rapid growth of the oocyte at the expense of nurse cells. Oocyte maturation is completed in stages 12-14 with the resorption of nurse cells and formation of vitelline membrane and chorion.

After the fourth mitotic divisions in the germarium, the 16 germline cells within a cyst enter premeiotic S phase **(Fig. 2A,B)**. Completion of the premeiotic S phase is followed by the initiation of meiosis I prophase (leptotene-zygotene) in all 16 germline cells within a cyst as determined by the presence of synaptonemal complexes and recombination nodules (Carpenter 1975, 1979, 1994). Synchrony of the meiosis I prophase is lost within the 16 germline cell cyst when two pro-oocytes enter pachytene and the other 14 cystocystes follow the nurse-cell pathway. Subsequently, only one of the two pro-oocytes, namely the determined oocyte will proceed to the karyosomal stage of meiosis I prophase, the other pro-oocyte will resign and join the nurse cell pathway. By the time the egg chamber exits the germarium, a karyosome forms in the oocyte

nucleus. The nurse cells grow rapidly without further divisions and their chromosomes go through several rounds of endoreplication and remain somatically paired.

To explain meiotic entry it is presumed that the meiosis promoting factor is initially present or produced by all 16 germline cells comprising a cyst. Shortly after the fourth mitotic division the meiosis promoting factor will move along the ring canals toward the pro-oocytes, which are both physically and topologically at the center of the cyst. As the meiosis promoting factor moves to the oocyte, the concentration of the meiosis promoting factor decreases in the remaining 15 cells of a cyst so they will turn to the nurse cell differentiation pathway (Spradling, 1993; Carpenter, 1994).

2.2. Female meiosis: hypothesis and achievements

Although combined genetic, molecular and cytological approaches helped a better understanding of *Drosophila* female meiosis, several important questions remain to be answered (Orr-Weaver, 1995).

Both EM and light microscopic studies (combined with Feulgen-Giemsa staining) have been conducted to characterize the exchange events during meiosis I in *Drosophila* females, including homologous pairing, synaptonemal complexes in germarial pro-oocytes and the chiasmata that hold homologs together until the metaphase I/anaphase I transition (Carpenter, 1975; 1979; Jang et al., 1995).

The karyosomal type of chromatin organization is a special characteristics of meiosis I prophase in *Drosophila* females, and leads to the absence of diplotene and diakinesis as it was revealed by Feulgen-Giemsa staining (Nokkala and Puro, 1976; Puro and Nokkala, 1977; Karpen et al., 1996). At present only the *proliferation disrupter (prod)* and *Damasa* genes of *Drosophila* seem to be involved in the karyosomal type of chromatin organization (Török et al., 1997; E. Máthé and J. Szabad unpublished results).

Cytological observations made on living or fixed mature oocytes permitted the characterization of some aspects of the meiosis I spindle formation. It is interesting that the microtubules first associate with the karyosome and then bundle to form a bipolar meiosis I spindle that lacks asters (Theurkauf and Hawley, 1992; Matthies et al., 1996; Riparbelli and Callaini, 1996). The bipolar nature of meiosis I spindle is not the result of

centrosomal microtubule organizing centers, since centrosomes degenerate during earlier stages of oogenesis (Carpenter, 1975; Theurkauf, 1994). The mechanisms of oocyte nuclear membrane disassembly, individualization of bivalents from karyosome, nucleation of microtubules during meiosis I and II spindle formation as well as the establishment of meiosis I and II spindle polarity are still poorly understood. Special efforts are made to identify gene products responsible for meiosis I and II spindle polarity. Despite the presence of DMAP190 centrosomal protein in the middle pole of meiosis II spindle, none of the known centrosomal proteins, including DMAP60, DMAP190 and γ-tubulin were found to be associated with the mature meiosis I spindle poles (Riparbelli and Callaini, 1996; J. Callaini personal communication).

Regarding genetic and molecular analysis of different meiotic and maternal-effect lethal mutants, several *Drosophila* genes have been identified with functions on female meiosis (Jang et al., 1995; McKim and Hawley, 1995). However, little is known about the regulatory mechanisms, such as the female meiotic spindle assembly checkpoint(s) and release of meiotic I metaphase arrest (Sagata, 1996).

The strictly maternally expressed *αTubulin67C* gene encodes for the α4-tubulin, which is needed for the proper organization and function of the female meiotic spindle (Matthews et al., 1993; Komma and Endow, 1997). The *non-claret disjunctional* (*ncd*) gene encodes for a kinesin-like motor molecule that is required for normal assembly of the female meiotic spindle and stabilization of this spindle during metaphase I arrest (Matthies et al., 1996). The characterization of *Drosophila* genes, like *altered disjunction* (*ald*), *Aberrant X segregation* (*Axb*), *meiotic-S51*, *meiotic-S332*, *no-distributive disjunctional* (*nod*), *orientation disruptor* (*ord*), *morewright* (*mwr*), whose functions are involved in the sister-chromatid arm or centromeric cohesion as well as the movements of chromosomes toward opposite spindle poles, has shed some light on certain aspects of chromosome segregation during female meiosis (Endow, 1993; Sawin and Endow, 1993; Miyazaki and Orr-Weaver, 1994; Afshar et al., 1995; Kerrebrock et al., 1995; Orr-Weaver, 1995; Fuller, 1995; Bickel et al., 1996; Bickel and Orr-Weaver, 1996; Rasooly, 1996). There seem to be two hypotheses available to explain the co-orientation or bipolar spindle attachment and segregation of the homologs and heterologs during meiosis I in *Drosophila* females.

According to the first hypothesis supported mostly by genetic data, three systems are involved. The first system proposes the requirement of recombination in proper homolog segregation, i.e. the chiasmata. The chiasmata stably hold the homologs together, ensuring the bipolar attachment of homologs to the meiosis I spindle (Hawley, 1988; Jang et al., 1995; Orr-Weaver, 1995). Interestingly, the fourth chromosomes, that do not participate in exchange, segregate faithfully during female meiosis, suggesting that additional mechanism(s) must exist to ensure the co-orientation and segregation of achiasmatic homologs and heterologs.

Based on theoretical grounds, R. Grell (1976) proposed that segregation of achiasmatic homologs and heterologs is mediated by a single distributive meiotic system, that is operated by a second phase of pairing, taking place after segregation of the chiasmatic homologs. During the second phase of pairing, called distributive pairing, the achiasmatic chromosomes form pairs according to their metaphase length. The distributive pairing was thought to be independent of homology. Instead of the distributive pairing system, based on genetic and cytological data, Hawley (1993) has proposed other two different systems. One system, requiring heterochromatic homology insures segregation of achiasmatic homologs, while the other system allows segregation of heterologs and depends on factors such as chromosome size, shape and availability (Hawley et al., 1992, 1993; Hawley and Theurkauf, 1993; Dernburg et al., 1996; Karpen et al., 1996).

The second hypothesis explains meiosis I co-orientation or bipolar spindle attachment and segregation of the homologs (chiasmatic and achiasmatic) and heterologs based on chromocentre function (Puro, 1991). Existence of the chromocentre was postulated by E. Novitski (1964), who proposed the chromocentre to ensure segregation of the achiasmatic homologs and heterologs instead Grell's distributive pairing system. The chromocentre was cytologically proven by Feulgen-Giemsa staining of *Drosophila* oocytes (Dövring and Sunner, 1979; Nokkala and Puro, 1976; Puro and Nokkala, 1977; Chubykin and Chadov, 1987). Based on the Feulgen-Giemsa staining, it appears that a single chromocentre is formed early in meiotic prophase by the pairing of pericentric heterochromatin of all chromosomes. Since diplotene-diakinesis stages of meiosis I prophase are missing, the chromocentre will preserve the pairing relations unchanged until prometaphase of meiosis I. The chromocentric spatial arrangement of centromeric

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regions will predetermine the proper co-orientation and segregation of all chromosomes during meiosis I. However, this hypothesis is waiting for more cytological and especially genetic data to be proven.

2.3. The pronuclear stage of embryonic development

Fertilization, and the concomitant centrosome restoration, formation of sperm aster, female and male pronuclei represent the introductory events to *Drosophila* embryogenesis (Foe et al., 1993). The genetic and immunocytological analysis of phenomena like the pronuclear reorganization of the paternal and maternal chromatin, replication of the chromatin inside the pronuclei and polar body nuclei, migration of daughter centrosomes along the pronuclei, sperm aster ensured female pronucleus specification-migration toward the male pronucleus will permit new inroads into the mystery of the onset of embryonic development.

Genetic characterization of the *Pitkin* gene suggests its requirement for proper reorganization of the pronuclear and cleavage chromatin (Kuhfittig et al., submitted). The maternally expressed γ-tubulin, centrosomin and probably the *Horka* gene product are responsible in part for the restoration and function of the first embryonic centrosome from the paternally inherited basal body (Zheng et al., 1991; Raff et al., 1993; Schatten, 1994; Sunkel et al., 1995; Szabad et al., 1995; Li and Kaufman, 1996). The KLP3A and NCD kinesin like proteins as well as the α-4tubulin are components of the sperm aster and are needed for the female pronucleus specification-migration (Komma and Endow, 1997; Williams et al., 1997; Máthé et al, submitted). The YA and p91 proteins seem to be involved in the formation and function of membranes and nuclear membrane associated phenomena of both pronuclei and cleavage nuclei (Lopez et al., 1994; Cserpán et al., submitted).

The pronuclear development culminates in the gonomeric division that ensures the fusion of female and male pronuclei. Since the early works by Huettner (1924) and Sonnenblick (1950) only Callaini and Riparbelli (1996) analyzed cytologically the gonomeric spindle organization and function. They showed that the paternal and maternal set of chromosomes do not intermingle in the gonomeric spindle because each chromosomal complement remains individually wrapped in its own nuclear membrane,

with only the poles completely open. Thus the paternal and maternal sets of chromosomes congregate in two separate gonomeric groups in the spindle at metaphase, and they remain thus grouped as they move to the spindle poles at anaphase. At present, genetic analysis has revealed only the *Ketel* gene product requirement for proper nuclear membrane formation at the time of completion of gonomeric division (Szabad et al., 1996; Lippai et al., in preparation).

3. AIMS

This thesis describes normal female meiosis and the onset of embryonic development in *Drosophila*, with emphasis on previously poorly documented aspects.

Female meiosis and the onset of embryonic development, including fertilization, pronuclei formation and gonomeric division, present several specific morphological features in *Drosophila melanogaster*. Phenomena like chromocentre and karyosome formation, meiotic chromosome condensation-decondensation, oocyte nuclear membrane disassembly, meiotic and gonomeric spindle organization, female and male pronuclei and sperm aster formation, pronuclear replication and centrosome restoration as revealed by cytological and indirect immunological methods combined with light and confocal microscopy are considered in this thesis.

Complementary data were collected (1) by a squash technique following Feulgen-Giemsa double staining and light microscopy (Puro and Nokkala, 1977), and (2) by new fixation and immunostaining methods combined with confocal microscopy. The immunostainings were performed using lamin, tubulin, prod and centrosome specific antibodies and DNA staining on whole-mount *Drosophila* egg chambers, eggs or embryos.

The main aim of this thesis is to serve as a reference for the phenotype analyses of various meiotic and maternal-effect lethal *Drosophila* mutants. The meiotic and maternal-effect lethal mutants serve as tools that allow us to perform the genetic and molecular dissection of the normal female meiosis and the onset of embryonic development in *Drosophila*.

4. MATERIALS AND METHODS

4.1. Fly strains and cultures

Drosophila strains were raised at 25°C on standard cornmeal agar media. Canton-S or Oregon-K wild-type strains were used. When cytologically distinguishable, mutant genotypes are given in figure legends. For symbols of marker mutations see Lindsley and Zimm (1992). All treatments of eggs and ovaries were carried out at room temperature unless otherwise stated.

4.2. Feulgen-Giemsa double staining of egg chambers and oocytes

Ovarioles were dissected either in Ringer's solution or in a hypotonic (75 mM) KCl solution, and transferred to fresh hypotonic KCl solution for 5 or 10 minutes, depending on whether germarial and vitellarial (stages 1-12) or mature oocytes (stages 13-14) were analyzed **(Fig. 1 and 2).**

Ovarioles containing egg chambers and mature oocytes were fixed for 2 hours in Carnoy's fixative, made of absolute ethanol, chloroform, and glacial acetic acid in a ratio of 6:3:1. The Carnoy's fixative is a rapidly penetrating fixative mixture, which coagulates proteins and chromatin, extracts lipids and many carbohydrate components are also preserved. Ovarioles were then transferred to absolute ethanol (for 2 hours), and rehydrated via 70% ethanol (overnight at 4°C), 50% ethanol (5 minutes), and 30% ethanol (3-5 minutes) to distilled water. After hydrolysis for 10-20 minutes in 1N HCl, the ovarioles were carried through the Feulgen procedure, including an 8-minute hydrolysis at 60°C, and a 4-5-minute staining with modified Schiff's reagent, containing 8 ml of 1N HCl, and 0.8 g of $K_2S_2O_5$ in 200 ml staining solution. The ovarioles were then transferred to distilled water. The mature oocytes and for younger stages egg chambers were transferred to a drop of water on a slide. The mature oocytes were cut to create anterior and posterior halves, while the younger egg chambers were whole-mount prepared. Excess water, chorion and the posterior halves of the mature oocytes were discarded and a drop of 45% acetic acid was added. Squashing of the mature oocytes and egg chambers was achieved by placing a coverslip over the drop and allowing a sheet of filter paper to absorb excess liquid. The slides were frozen on dry ice and the

coverslip was removed. Slides were dehydrated in absolute ethanol, immersed in glacial acetic acid for 20-30 seconds and air dried. Giemsa staining was carried out by a 30-minute treatment with 4% Giemsa (Merck No. 9204) solution in Sorensen's phosphate buffer, pH 6.8. Permanent slides were prepared after a rinse in distilled water, drying, and mounting in Entellan (Merck No. 7960).

4.3. Feulgen-Giemsa double staining of intrauterine eggs

Since meiosis was almost completed while the egg "traveled" in the oviduct and embryogenesis commenced right after completion of meiosis, eggs had to be collected from the uterus. Eggs were squeezed out from uteri by applying slight pressure on abdomens, and were immediately fixed in Carnoy's fixative (see above) that was modified by replacing the absolute ethanol by 80% ethanol. After 1-2 hours, roughly an equal volume of absolute ethanol was added and fixing was allowed to continue for several hours or overnight. Eggs were rehydrated, soaked in 1N HCl, and put through the Feulgen-Giemsa procedure as described above, except that a single egg with both halves on one slide was prepared.

4.4. Immunostaining of the egg chambers and oocytes

The non-coagulant fixatives like methanol, ethanol, acetone, formaldehyde and paraformaldehyde rapidly penetrate and convert the cytoplasm into insoluble gel and render the chromatin resistant to extraction by Tween-PBS. We used the following five **fixation procedures**, since they gave the best epitope presentation for the applied antibodies.

1. Ovaries were dissected in Ringer's solution or in 75 mM KCl and were left in 75 mM KCl solution for not more than 5 minutes. The ovaries were subsequently fixed for 2 hours in Carnoy's fixative (see above). A second fixation followed in absolute methanol for 15 minutes.

2. Ovaries were dissected directly in absolute methanol.

Following fixation procedures 1. and 2., the mature oocytes were dechorionized either in absolute methanol, using fine tungsten needles or in a 1:1 mixture of absolute methanol and commercial bleach (4% sodium hypochlorite). If younger egg chambers are analyzed

Dechorionization is not necessary. The dechorionized oocytes and/or egg chambers were washed twice in absolute methanol. During the next step, called permeabilization, the samples were treated for 5 minutes with a mixture of absolute methanol and heptane or acetone. Permeabilization is a crucial step because it allows antibodies to penetrate the fixed oocytes or egg chambers. Subsequently, the mature oocytes and egg chambers were washed twice in absolute methanol, and rehydrated in 70%, 50%, and 30% methanol solutions. The second rehydration (for 2 hours) was carried out in 10% fetal calf serum in Tween-PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 0.2% Tween 20, pH 7.5).

3. The ovaries were dissected in Tween-PBS and gathered in Eppendorf tubes containing ice cold Tween-PBS. The Tween-PBS was replaced with 300 μl PBS , 150 μl 4% or 10% paraformaldehyde in PBS and 550 μl heptane. The tube was shacked by hand for 1 minute. A second fixation was carried out with 500 μl PBS, 250 μl 4% or 10% paraformaldehyde in PBS for 20 minutes. The second fixative was removed and ovaries were washed twice in absolute methanol for 10 minutes. Then a third wash in absolute methanol was performed for 30 minutes. The ovaries were rehydrated in 70%, 50%, and 30% methanol solutions. The second rehydration (for 2 hours) was carried out in Tween-PBS, containing 10% fetal calf serum. In some cases, 4% or 8% formaldehyde was used instead of paraformaldehyde.

4. The ovaries were dissected in PBS and gathered in Eppendorf tubes containing PBS. The older egg chambers and mature oocytes were dechorionized in a 1:1 mixture of absolute methanol and commercial bleach (4% sodium hypochlorite). The dechorionized oocytes and egg chambers were washed twice in absolute methanol. Fixation followed in absolute methanol for 30 minutes. After fixation, the samples were treated for permeabilization for 5 minutes with a mixture of absolute methanol and acetone. Subsequently, the mature oocytes and egg chambers were washed twice in absolute methanol, and rehydrated in 70%, 50%, and 30% methanol solutions. A second rehydration was performed with Tween-PBS, containing 10% fetal calf serum for 2 hours.

5. The ovaries were dissected and fixed as it was described by Theurkauf and Hawley (1992). The fixative contains 8% formaldehyde and permits good antibody penetration for the mature oocytes.

Antibodies. After the second rehydration, the egg chambers and/or mature oocytes were incubated for 12-48 hours at 4^0C in the primary antibodies, and for 2-6 hours at room temperature in the secondary antibodies. YL1/2 rat monoclonal anti-αtubulin (Sera Labs Ltd.) and T47 mouse monoclonal anti-lamin antibodies (Paddy et al., 1991) were used to visualize meiotic or gonomeric spindles and the nuclear lamina of oocyte or pronuclei, respectively. The Prod antibody was used to visualize the pericentric heterochromatin of the oocyte chromosomes (Török et al., 1997). Fluorescein and Texas-red conjugated goat anti-rat, anti-mouse and anti-rabbit secondary antibodies were obtained from Jackson Immunoresearch Laboratories Inc. (Avondale, PA).

Specimen mounting. Before mounting, egg chambers and/or oocytes were washed three times in PBS. During the second PBS washing, the DNA was counter-stained for 5 minutes with 1 mg/ml propidium iodide. Specimen were mounted in a solution of 2.5% n-propyl gallate and 85% glycerol.

4.5. Immunostaining of intrauterine eggs

Eggs were collected either by dissection of female uteri in Ringer's solution or were forced out by treating females with CO_2 gas. Immunostaining of intrauterine eggs was carried out as described above, except that no KCl treatment was applied in the case of fixation procedure No. 1. The fixation procedure No. 2, based on absolute methanol, was preferred in most of the antibody stainings on intrauterine eggs.

4.6. Light and confocal microscopy

Permanent Feulgen-Giemsa stained preparations were analyzed and photographed using the Leitz Dialux 20 transillumination microscope and a Wild MPS automatic photo equipment. The Zeiss 410 confocal laser scanning microscope was used for collecting digital images of optical sections through egg chambers and eggs after fluorescence stainings. Image analysis was performed by the Carl Zeiss Microscope System LSM version 3.59 software.

6. RESULTS

6.1. The pachytene stage of meiosis I prophase

The pachytene stage is the earliest stage of meiosis I prophase that can be visualized by the Feulgen-Giemsa staining in the two pro-oocytes **(Fig. 2)**. The two pro-oocytes at the pachytene stage correspond to oogenetic stage 1-2 (King, 1970). Within the nuclei of the two pro-oocytes similar pachytene chromosomal threads were distinguished, while the nurse cell nuclei of the same cyst displayed more diffuse chromatin **(Fig. 3A)**.

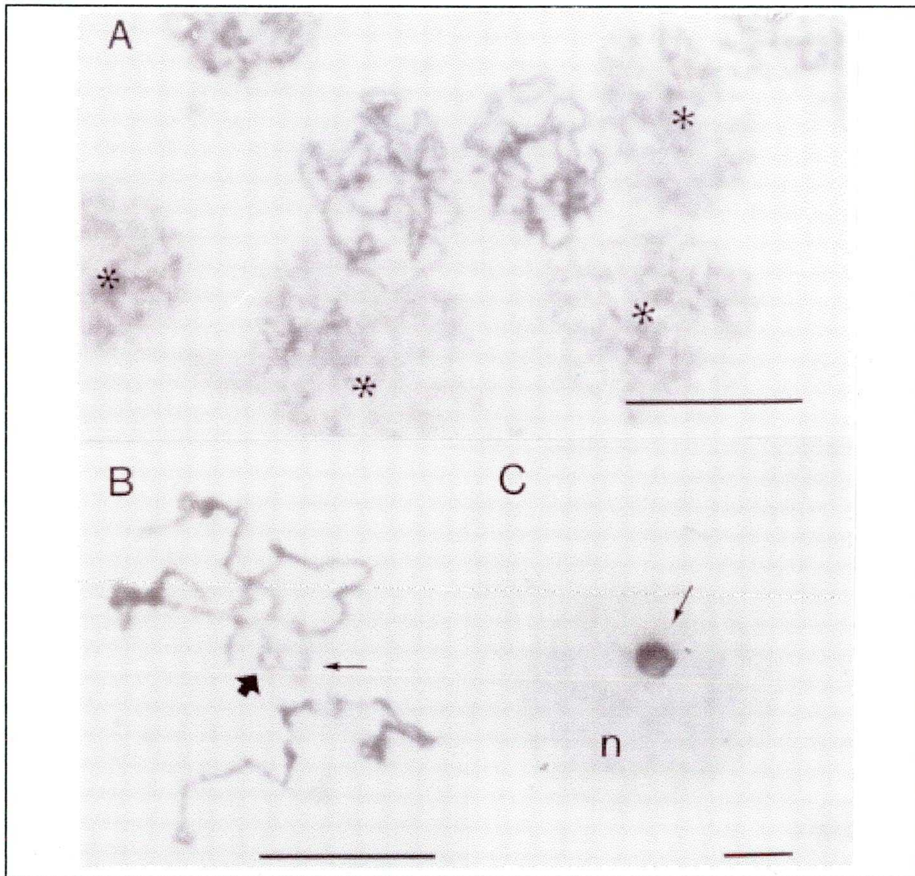

Fig. 3. The prophase of meiosis I, as revealed by Feulgen-Giemsa staining

(A) Pachytene threads corresponding to the two pro-oocyte nuclei and nurse cell nuclei (asterisks) that belong to the same cyst. **(B)** Centromeric regions of the synapsed chromosomes are interconnected by chromocentral fibbers (thin arrow). Five pairs of long arms and a loop corresponding to the fourth chromosomes (thick arrow) are apparent. **(C)** By stage 7, oocyte nucleus (n) with the chromosomes compressed into a karyosome (arrow). Bars = 10 μm. Figure reproduced with permission of Prof. J. Puro.

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Carpenter's EM observations (1975, 1979), namely the presence of synaptonemal complexes in the nuclei of pro-oocytes strongly supports the pachytene specific status of chromosomal threads in the germarial pro-oocytes.

The Feulgen-Giemsa staining revealed a single chromocentre in the nuclei of pro-oocytes during oogenetic stage 1-2, and in the oocyte nucleus up to oogenetic stage 12. It is likely that the chromocentre binds together the centromere and pericentric heterochromatin of the three major pairs of chromosomes **(Fig. 3B).** Position of the fourth chromosome pair was not clear during oogenetic stages 1-2, but squash preparations made of stage 3 oocytes reveal a short loop of paired threads at the chromocentre, evidently representing the fourth pair of chromosomes.

5.2. The karyosomal stage of meiosis I prophase

The presence of karyosomal stage instead of diplotene-diakinesis stages known e.g. in humans, is another main characteristics of meiosis I prophase in *Drosophila* females. The karyosomal stage corresponds to oogenetic stages 2-12 **(Fig. 2)**.

The Feulgen-Giemsa staining revealed that during oogenetic stages 2-6, arms of the pachytene chromosomes started to coil and eventually assembled into a tight package around the chromocentre(s). This structure is called the karyosome. During oogenetic stages 7-8, the karyosome organization becomes very compact its diameter varies between 4-7 μm **(Fig. 3C)**. In oogenetic stage 9, the karyosome becomes looser with five desynapsed pairs of homologous arms emanating from the chromocentre. The homologous arms are connected at one or more sites, presumably representing chiasmata (data not shown). In oogenetic stages 10-12, the compact structure of the karyosome is restored **(Fig. 5A,B)**.

It is likely that the chromocentre and karyosome of *Drosophila* oocyte requires specific chromatin components. The Prod protein is the only known chromosomal protein that presents stage specific distribution in the female germline (E. Máthé and J. Szabad unpublished results). Török et al. (1997) have found that the Prod protein is a permanent component of pericentromeric α-heterochromatin of all mitotic chromosomes but is absent from the chromocentre of larval polytene chromosomes in which the α-

heterochromatin is underrepresented. Immunostainings revealed no differences on the chromatin organization of nurse cells and oocyte nuclei during oogenetic stage 1-2 since all chromosomes showed Prod staining (data not shown).

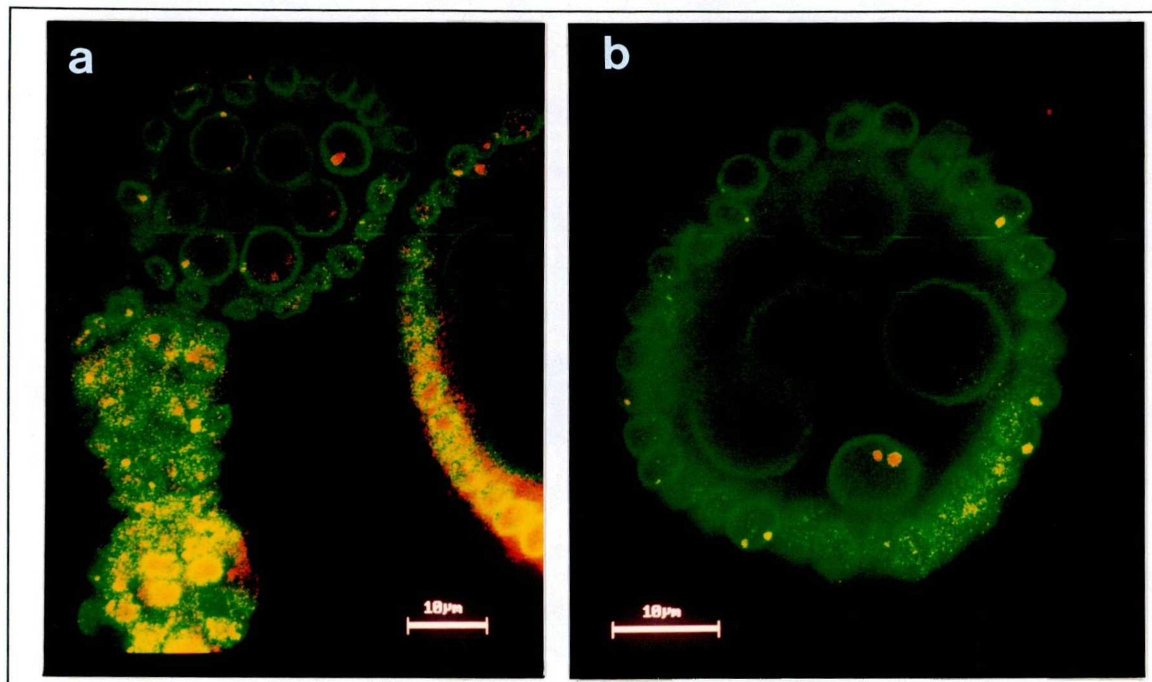

Fig. 4. Location of the Prod protein in the female germline

(A) Oocyte nucleus (stage 3-4), displaying a single Prod signal that identify the chromocentre. **(B)** Oocyte nucleus (stage 4-5) with splited chromocentre. Note the absence of Prod staining from the nurse cell nuclei. Merged pictures of confocal sections, lamin (green) and Prod (red). Overlaying red and green signals result in orange.

From oogenetic stage 3-4 to 12, one or two nearby Prod signals were apparent in the oocyte nucleus, while the nurse cell nuclei did not show Prod staining **(Fig. 4)**. It is likely that the presence of one or two nearby Prod signals in the oocyte nucleus can be explained by the pairing of pericentric heterochromatin of all chromosomes, that leads to chromocentre and karyosome formation. The absence of Prod staining from the polytenic nurse cells is expected since the α-heterochromatin is underrepresented in the chromocentre of salivary gland polytene chromosomes.

5.3. Prometaphase I: oocyte nuclear membrane disassembly

According to the above comparative cytological study and based on the suggestions of Puro and Nokkala (1977), the oogenetic stage 13 corresponds to the prometaphase of meiosis I and can be subdivided into four substages, viz. A, B, C and D.

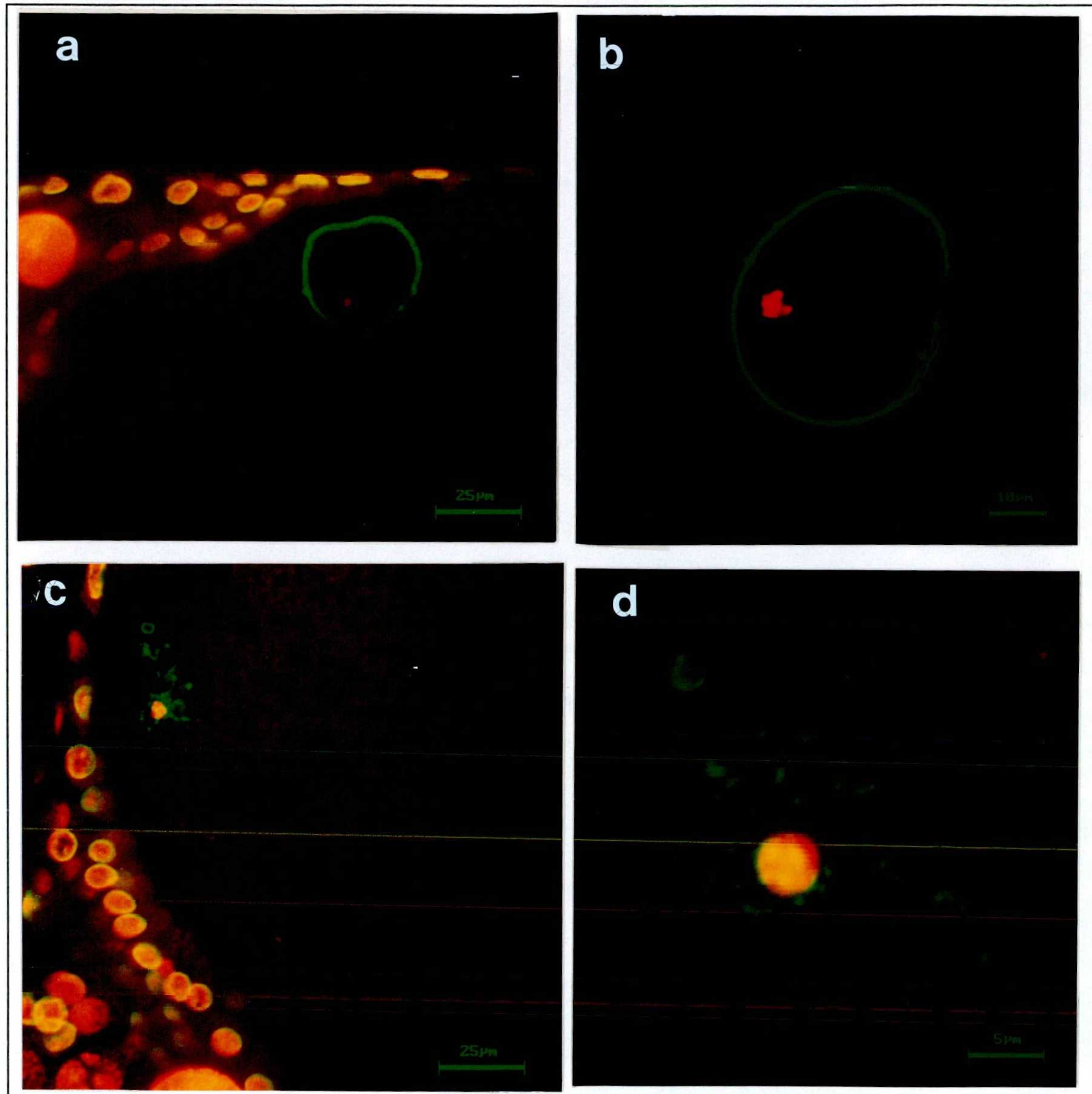

Fig. 5. Immunostaining of stage 13 oocytes.

Intact oocyte nucleus **(A, B)** and a nucleus immediately after breakdown of the nuclear membrane **(C, D)** as revealed by double labeling with anti-lamin antibody (green) and propidium iodide (red). The nuclear lamin, lining the nuclear envelope, becomes dispersed in the cytoplasm during disassembly of the nuclear membrane. Overlaying red and green signals result in orange.

At oogenetic stage 13A, the oocyte nuclear membrane is still present as revealed by anti-lamin antibody staining **(Fig. 5A,B)**. Although, the karyosome is still an assembly of condensed chromatin, however, occasionally loops of chromosomes protrude. Three dimensional reconstruction of confocal pictures of oocyte nuclei stained for lamin and DNA clearly shows that the karyosome is always juxtaposed to the nuclear lamina.

The nuclear membrane of the oocyte nucleus breaks down during oogenetic stage 13B, demarcating the beginning of the meiotic I prometaphase. **(Fig. 5C,D)**. It seems that the nuclear membrane disassembly is achieved by vesiculation but we can not exclude the possibility of intralumenal fusion, tubulation or the combination of these mechanisms.

5.5. Prometaphase I: spindle formation and individualization of bivalents

An envelope-like array of microtubules appears around the karyosome after disassembly of the oocyte nuclear membrane during oogenetic stage 13B **(Fig. 6A)**. Bivalents started to individualize within the microtubule envelope and chromosome arms extend from the karyosome.

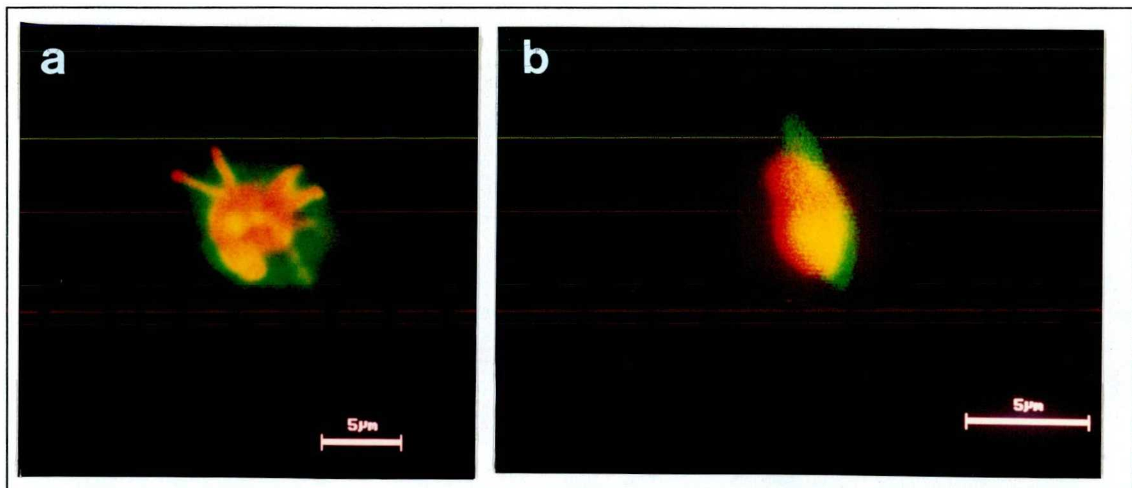

Fig. 6. Meiosis I spindle organization as revealed by immunostaining.

(A) Karyosome at the beginning of individualization of bivalents, stained for DNA (red) and tubulin (green), after hypotonic pretreatment and Carnoy fixation. **(B)** Short meiosis I spindle at prometaphase, after methanol fixation, without hypotonic pretreatment. Merged confocal pictures, overlaying red and green signals result in orange.

Three dimensional reconstruction of confocal sections through the karyosome revealed that the microtubules interact with the individualizing bivalents, associating with the entire surface of the karyosome **(Fig. 6A)**. Thus, the chromosome-microtubule interactions are not limited to the chromocentre or centromere, but extend along the entire chromosomal arms.

During oogenetic stage 13C (mid prometaphase), the envelope-like microtubule array transforms into a very short bipolar spindle **(Fig. 6B)**. The karyosomal organization is resolved into individual bivalents inside the short spindle and the chromocentre disassembles. It seems that the bipolar of meiosis I spindle is already established when the bivalents detach from the chromocentre **(Fig. 7B)**. It is likely that the poleward acting forces are not acting yet, because the fourth chromosomes are still lying close to each other near the midzone of the small spindle **(Fig. 7B)**.

During stage 13 D (late prometaphase) the very short spindle is transformed into a long tapered spindle **(Fig. 7C,D)**. The poleward acting forces must be switched on at this stage since the achiasmatic fourth chromosomes are arranged between the metaphase plate and spindle poles, while the chiasmatic chromosomes are situated at the metaphase plate.

5.5. Meiosis I metaphase

Full metaphase I is reached at the end of oogenetic stage 13D **(Fig. 7D)**, and meiosis I remains arrested at metaphase through oogenetic stage 14, until the egg leaves the ovary and enters, passes through the oviduct (Sonnenblick, 1950).

In general, the Carnoy's and methanol fixatives used for immunostaining on whole-mounts and for the Feulgen-Giemsa staining on squash preparations preserves well the morphology of chromosomes, but removes substantial fraction of chromosomal proteins (Gatti et al., 1994). However, in case of meiosis I spindle, the Carnoy and methanol fixations resulted in poorly defined chromosomes **(Fig. 6B)**. The individual bivalents appear only after an appropriate expansion of the meiosis I spindle and slight chromatin decondensation in hypotonic KCl solution **(Fig. 7)**.

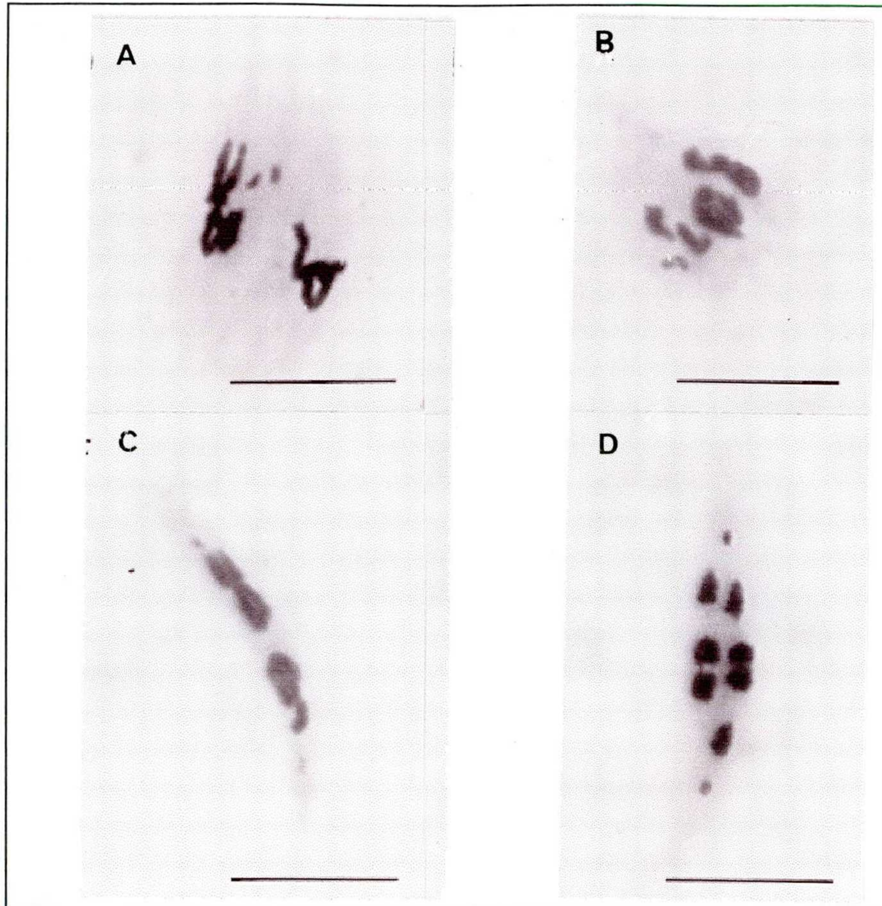

Fig. 7. Prometaphase and metaphase of meiosis I revealed by Feulgen-Giemsa staining
Eggs were pretreated with hypotonic KCl for 5-10 minutes, squashed and Feulgen-Giemsa stained. **(A)** Disintegration of the chromocentre is followed by rapid individualization of bivalents in eggs of *XXY* females. Homologues lie parallel to each other and are still intimately paired. The *X* chromosomes at 1 o'clock and the extra *Y* chromosome at 11 o'clock. The 4th chromosome pair (arrow) is adjacent to the *Y* chromosome. **(B)** Mid-prometaphase is defined by the fourth chromosomes still lying close to each other near the equator of the short spindle. The *X* chromosomes at 11 o'clock share a chiasma. **(C, D)** In late prometaphase in eggs of *XXY* females, the 4th and other achiasmatic chromosome pairs, if present, move towards the poles. In present instance, the univalent *X* chromosomes (upper pole) segregate from a univalent *Y* (lower pole), resulting in secondary nondisjunction of the *X* chromosomes. The chiasmatic bivalents oscillate around the equator until stable metaphase is reached late in stage 13. Bars = 10 μm. The figure was reproduced with the permission of Prof. J. Puro.

5.6. Completion of meiosis I and meiosis II

Meiosis I metaphase arrest is released and the spindle activated when the egg leaves the ovary and passes through the oviduct into the uterus (Sonnenblick, 1950; Mahowald et

al., 1983). During metaphase the meiosis I spindle is parallel to the egg surface, but after metaphase release is oriented perpendicular to the egg surface, with one pole pointing to the dorsal egg cortex.

Spindle activation includes (1) release of chiasmata as the consequence of abolition of homologous pairing, (2) release of sister-chromatid cohesion along the chromosomal arms, and (3) lengthening of the spindle midzone. At the same time, (4) the chromosomes are moved close to the spindle poles **(Fig. 8A)**. At the end of meiosis I, the chromosomes reach the poles, and remain condensed, and telophase nuclei do not form.

While anaphase I is still in progress, a single sperm enters every egg in the uterus **(Fig. 8)**. In Feulgen-Giemsa squash preparations, the fertilized and unfertilized eggs can be distinguished by the presence or absence of the 1.7-1.9 mm long sperm tail. The sperm head remains relatively condensed and possesses a characteristic hook-shape until the end of second meiotic division **(Fig. 8B)**.

Both anaphase I and all the subsequent meiotic stages proceeds identically in unfertilized and fertilized eggs, indicating that meiosis metaphase I release and spindle activation are independent of fertilization.

Meiosis II is governed by a unique type of spindle apparatus that appears to be an extension of the meiosis I spindle. During anaphase I, the midzone of the meiosis I spindle starts to differentiate and accumulate microtubules **(Fig. 8A)**. Subsequently, the meiosis I spindle is gradually transformed into a pair of meiosis II twin spindles, sharing a common pole also called middle pole **(Fig. 8B)**. The microtubules nucleated by the middle pole are organized like an aster as evidenced from anti-tubulin immunostainings **(Fig. 8C)**. The orientation of meiosis II spindle apparatus is perpendicular to the longitudinal axis of the egg.

Despite absence of the centrioles, the middle pole seems to function as a common microtubule organizing center (MTOC) for the twin spindles. Centrosomal proteins were detected inside the middle pole. When fertilized intrauterine eggs were stained with the Rb-188 antibody specific for the DMAP190 centrosomal component of *Drosophila* embryo (Whitfield et al., 1995), a stage specific staining pattern appeared as reported by Riparbelli and Callaini (1996). Besides the DMAP190, other MTOC specific

proteins are also localized inside the middle pole of meiosis II spindle like the γ-tubulin (J. Callaini, personal communication).

During the metaphase of meiosis II, kinetochores of the bichromatidic chromosomes are reoriented with respect to the new poles (metaphase II orientation), and subsequently segregation of daughter chromatids take place (anaphase II).

Fig. 8. The meiotic spindles and sperm as revealed by Feulgen-Giemsa and immunostainings **(A)** Meiosis I anaphase. Note the position of the chromosomes and the accumulation of microtubules (arrow) in the spindle midzone. **(B)** Meiosis II metaphase. **(D)** The sperm head remains condensed until the end of the meiotic divisions. Feulgen-Giemsa double staining and the pictures were reproduced with the permission of Prof. J. Puro. Bars = 10 μm. **(E)** Confocal picture of a meiosis II spindle stained for tubulin.

5.7. Formation of female and male pronuclei

Four haploid nuclei, called polar body nuclei form in the egg, upon completion of the female meiosis. Inside the polar body nuclei the chromosomes start to decondense simultaneously **(Fig. 9A,B)**.

In fertilized eggs, the sperm head expands and becomes spherical concomitant with the four female meiotic products. During the postmeiotic interphase also called the pronuclear stage of development, the sperm head develops into the male pronucleus, and the innermost of the four female polar body nuclei becomes female pronucleus. The size of the female and male pronuclei and polar body nuclei gradually increases up to about 10-15 μm in diameter **(Fig. 9C,D)**. It is assumed that chromatin reorganization and replication takes place in the four female meiotic products and sperm head during pronuclear stage of development. However, the direct evidences for the chromatin reorganization and replication of pronuclei and polar body nuclei are still missing.

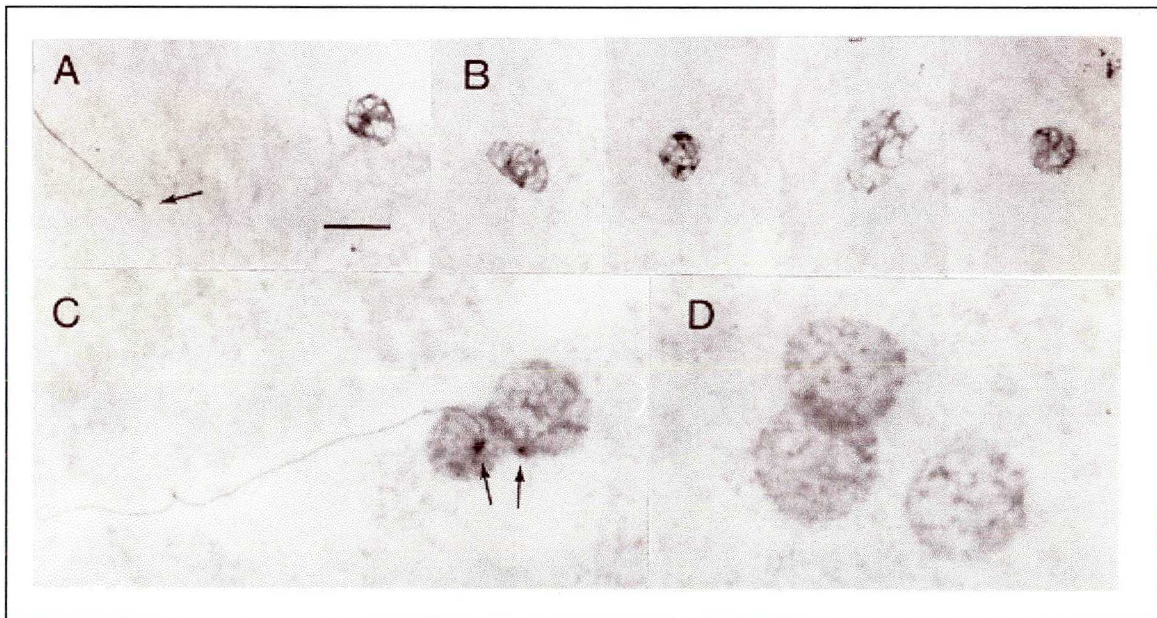

Fig. 9. The male and female pronuclei as revealed by Feulgen-Giemsa staining

After completion of meiosis, chromatin in the sperm nucleus **(A)** and in the four female meiotic products **(B)** begin to decondense. The arrow indicates the proximal part of sperm tail where the basal body is located. Both female and male pronuclei **(C)** and polar body nuclei **(D)** increase in size and approach each other. Note the sperm tail associated and replicated-separated centrosome (arrows) on C. Bar = 10 μm. The figure was reproduced with the permission of Prof. J. Puro.

The sperm tail-associated centrosome is reorganized during a phenomenon called centrosome restoration (Schatten, 1994). The restored centrosome replicates and separates by the time the pronuclei reach their final size. One of the separated centrosomes will remain in contact with the sperm tail **(Fig. 9C, 10C)**. It is obvious that, like in most species the centrosome or at least the centrosome replicating element is paternally inherited in *Drosophila*.

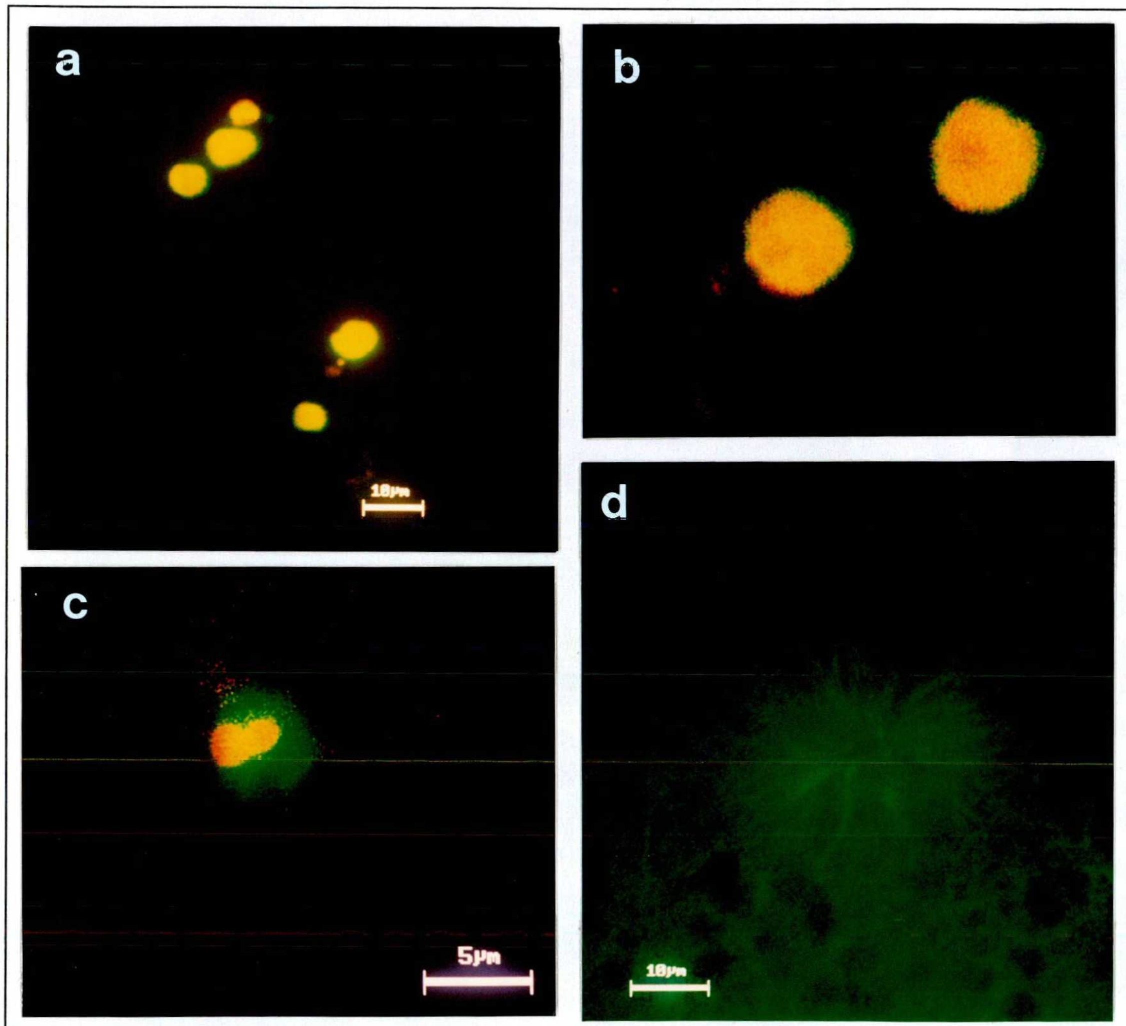

Fig. 10. The pronuclei and sperm aster as revealed by immunostaining

(A) Female and male pronuclei (middle) and the polar body nuclei stained for lamin (green) and DNA (red). **(B)** An enlarged female and male pronuclei stained for lamin (green) and DNA (red). Note the uniform DNA staining, showing the decondensed chromatin. **(C)** Small sperm aster stained for tubulin (green) and centrosome (yellow). The centrosome is replicated and started to separate. **(D)** The prominent sperm aster stained for tubulin (green). Merged confocal pictures, overlaying red and green signals result in orange.

Due to the microtubule nucleation property of the centrosome, a sperm aster forms around the proximal end of the sperm tail. The sperm aster is formed right after the sperm entry to the egg. The sperm aster remains small (ϕ 7 μm) until meiosis II anaphase and becomes so-called prominent sperm aster as meiosis II is completed. The sperm aster was readily visualized by anti-tubulin staining on whole-mount preparations **(Fig. 10C,D)**.

By the end of pronuclear stage, the innermost nucleus in the row of four polar body nuclei is singled out as a female pronucleus by the prominent sperm aster, as it is suggested based on the dominant gain-of-function *Tomaj* alleles-associated phenotypes (Máthé et al., submitted). Along the microtubules of sperm aster, the female pronucleus migrates from the meiotic region of the egg toward the male pronucleus. As a consequence of female pronucleus specification-migration, the female and male pronuclei are arranged side by side eventually, in the gonomeric region of the egg **(Figs. 9C, 10A)**.

5.8. Polar bodies in fertilized eggs

Of the four female meiotic products in an egg, the three outer ones become polar body nuclei. They develop synchronously with the female and male pronuclei until an arrest at a stage, corresponding to metaphase of the gonomeric division.

The condensation of polar body chromosomes does not occur without previous chromatin replication, since the timing of these phenomena is monitored by cell cycle checkpoints. Evidence for the occurrence of chromatin replication during the pronuclear stage of embryonic development came from the observation that arrested polar body chromosomes were bichromatidic **(Fig. 11)**. This conclusion was also confirmed by the bichromatidic chromosomes of the polar bodies in unfertilized eggs **(Fig. 12B)**.

Rabinowitz (1941) described the history of the polar body nuclei up to their degeneration during the blastoderm stage. The following description based on immunostainings corroborate his major findings with the exception of those concerning the fusion timing of polar bodies. According to Rabinowitz, of the three polar bodies the two posteriorly located groups fuse (diplo-fusion) during the second embryonic cleavage

cycle. This group then migrates toward the third polar body and fuses with it during the sixth embryonic cleavage cycle.

Fig. 11. Polar bodies in fertilized eggs as revealed by immunostaining.

Notes: The merged picture of a single (left) and two fused polar bodies (right), stained for DNA (red) and tubulin (green). Overlaying red and green signals result in orange.

While the possibility that groups of arrested polar body chromosomes do migrate can not be excluded, the three fused polar bodies are observed fairly frequently also in embryos during the second and third embryonic cleavage cycles. A possible explanation for the diplo-fusion can be that during gonomeric prometaphase, when the nuclear membranes of the pronuclei and polar body nuclei break down, the condensed chromosomes of the two non-sister polar body nuclei (those flanking the middle pole) are captured by microtubules radiating from the middle pole and are brought into a unipolar metaphase configuration **(Fig. 11)**. Chromosomes of the third polar body chromosomes are included if the outermost polar body nucleus has migrated close to the others and gets within reach of the middle pole microtubules. If not, the outermost polar body nucleus is captured by microtubules nucleated by the former outermost pole of the meiosis.

5.9. The gonomeric division

The term "gonomery", coined by Haeker in 1895 (Rieger et al., 1991), refers to a separate grouping of maternal and paternal chromosomes during the first mitotic or cleavage division of embryonic development. Huettner (1924), and later Sonnenblick (1950), reported an occurrence of gonomery in *Drosophila melanogaster*. The following description, based on immunostainings confirms Sonnenblick's major findings.

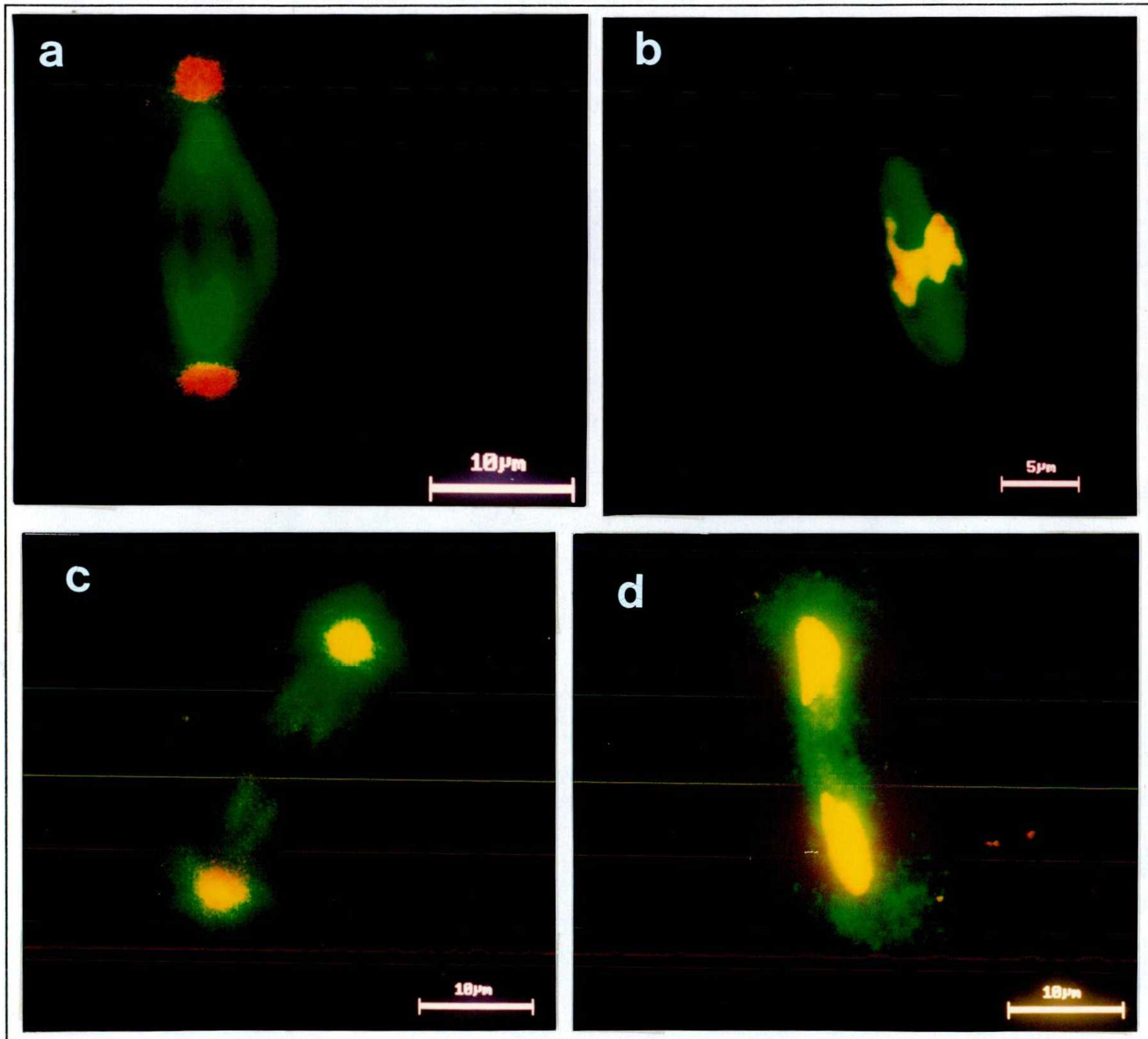

Fig. 12. The gonomeric division as revealed by immunostaining

(A) Gonomeric spindle at metaphase stained for centrosomin (yellow) and tubulin (green). **(B)** Gonomeric spindle at metaphase stained for DNA (reddish orange) and tubulin (green). **(C)** Gonomeric spindle at anaphase stained for centrosomin (yellow) and tubulin (green). Note the spindle elongation and the aster microtubules at anaphase. **(D)** Gonomeric spindle at telophase stained for DNA (red-yellow) and tubulin (green). Note the rapid decondensation of paternal and maternal chromosomes at telophase. Merged confocal pictures, overlaying red and green signals result in orange-yellow.

After the pronuclear stage of development that must include a chromatin replication, the chromatin of pronuclei and polar body nuclei start to condense. The chromatin condensation occurs in synchronized fashion in both female and male pronuclei and polar body nuclei.

During the gonomeric division, two spindles are organized by the pair of centrosomes **(Fig. 12A)**. The two spindles are separated by derivatives of nuclear membranes comparable to the spindle envelopes in later embryonic mitoses (Stafstrom and Staehelin, 1984; Callaini and Riparbelli, 1996).

At the onset of the gonomeric anaphase, the spindle elongates and sister chromatids of both paternal and maternal chromosomes are still separated **(Fig. 12B, C)**. However, the paternal and maternal chromatids moving to the same pole, reach the spindle poles in common gonomeric groups at telophase. At the end of gonomeric telophase, both maternal and paternal chromosomes became wrapped in a common nuclear membrane, and the paternal and maternal chromosomes rapidly decondense, forming the first cleavage nuclei **(Fig. 12D)**. Thus, at the onset of the *Drosophila* embryonic development, the fusion of the paternal and maternal chromosomes is ensured by the unique type of gonomeric spindle.

5.9. Polar bodies in unfertilized eggs

All four female meiotic products remain in a clear cytoplasmic island, the meiotic region of the egg, in the unfertilized eggs. As in fertilized eggs, the pronuclear stage is followed by the initiation of the gonomeric cycle, including condensation of chromosomes and breakdown of the nuclear membranes. However, since there was no centrosome present to organize sperm aster and to specify the female pronucleus, all four female meiotic products behave like polar bodies and as a rule, they fuse. Triplo- or tetra-fusion of polar body nuclei is common.

The triplo-fusion of polar body nuclei was observed in at about 95% of the examined unfertilized wild-type eggs **(Fig. 12A,B)**. This triplo-fusion is mediated by microtubules that are most likely nucleated by the former middle pole of meiosis II spindle. Tetra-fusion occurred in the remaining 5% of the unfertilized wild-type eggs

(Fig. 12C). In some rare instances a barrel-shaped, non-functional spindle, lacking centrosomes forms from the four polar body nuclei **(Fig. 12D)**. This barrel-shaped spindle is always arrested at metaphase, since the chromosomes are located at the equatorial region. Pycnotic and fragmenting chromosomes are usually seen in such barrel-shaped spindles that can be considered typical apoptotic syndromes of chromatin degeneration.

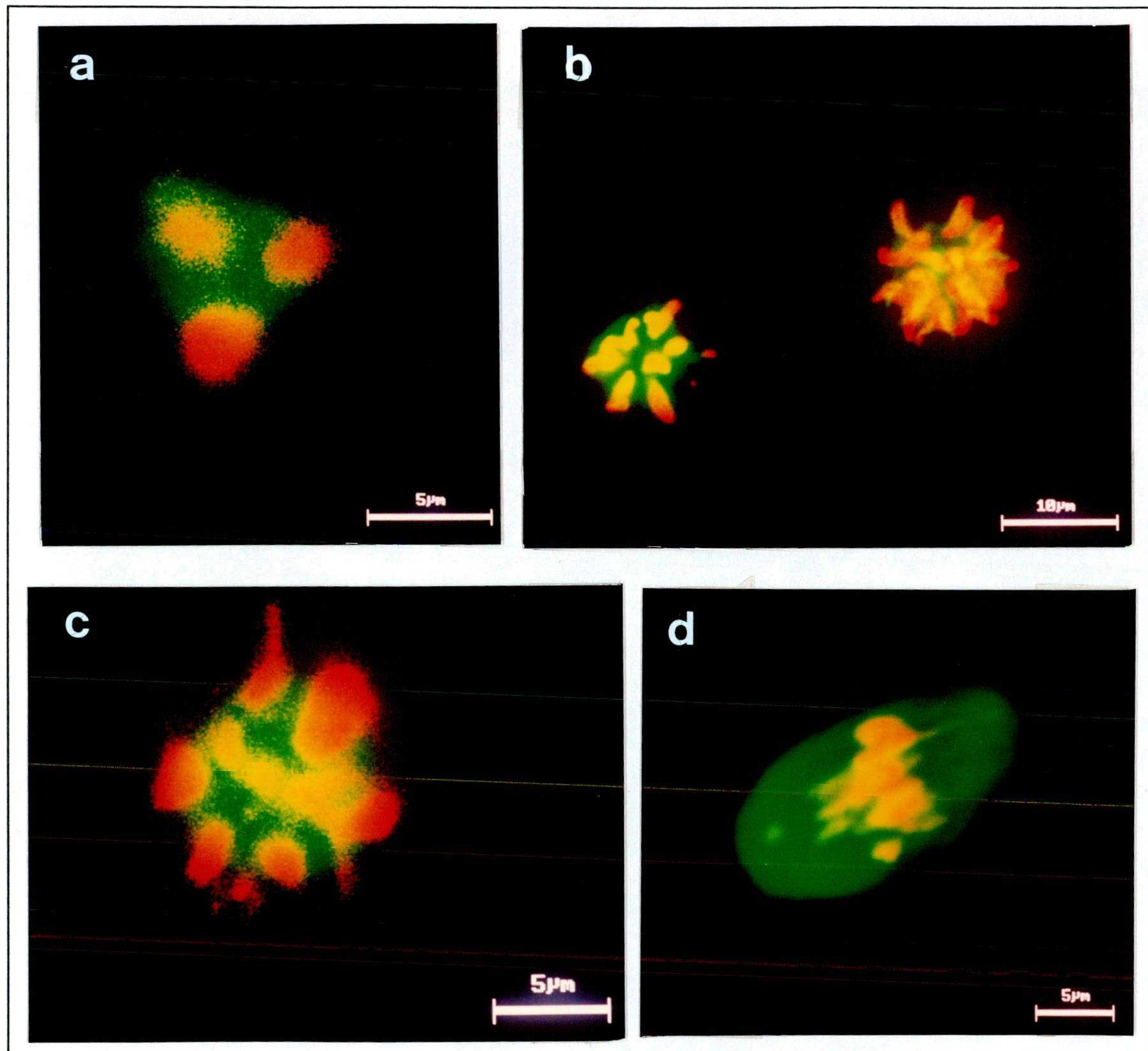

Fig. 13. Polar bodies in unfertilized eggs, as revealed by immunostaining
(A) Triplo-fusion of polar body nuclei. **(B)** Single polar body with bichromatidic chromosomes and a triplo polar body resulted from the triplo-fusion of tree polar body nuclei. **(C)** Tetra polar body resulted from the fusion of four polar body nuclei. **(D)** Giant barrel-shaped spindle, resulted from the fusion of the four polar body nuclei. Merged confocal pictures, tubulin is green and DNA is red. Overlaying red and green signals result in orange-yellow.

6. DISCUSSION and FUTURE PERSPECTIVES

The cytological investigation of female meiosis and the onset of embryonic development of *Drosophila melanogaster* is presented in this Ph.D. thesis. Given the descriptive nature of this comparative cytological study, many of the results were already discussed in the previous **RESULTS** chapter. Therefore in the following only some of the most important aspects and/or questions will be pointed out.

6.1. How does chromocentre form?

In *Drosophila,* the chromocentral arrangement of the pericentric heterochromatin of all chromosomes occurs typically in polyploid cells of differentiated tissues (salivary glands, fat-body, nurse cells etc.), suggesting that chromocentre formation is related to an endoreplication specific cell cycle regulation. Lilly and Spradling (1996) have shown that CYCLIN E controls endoreplication and during such modified cell cycles the checkpoint ensuring S-phase completion is absent. However, endoreplication is repressed in the oocyte nucleus and an unknown meiosis controlling cell cycle mechanism is turned on. Initially, the meiotic control is not confined to one cell inasmuch as two cystocytes start assembling structures specific for pachytene (Schmekel et al., 1993), **(Fig. 1, Fig. 3A)**. As a result of oocyte determination, only one of these two pro-oocytes will finally continue on the meiotic pathway (Spradling, 1993). Genes involved in the initiation and/or control of female meiosis as well as the underlying molecular mechanism remain to be elucidated. It is generally accepted that function of the *egalitarian* gene of *Drosophila* is necessary for the inhibition of meiotic entry in nurse cells and for meiotic maintenance in the oocyte, while the *encore* gene is needed to represses the endoreplication cycle in oocytes (Carpenter, 1994; Hawkins et al., 1996).

The time of chromocentre formation in oocytes of *Drosophila* is not known precisely **(Fig. 1)**. The pericentric heterochromatin of all chromosomes appear to be well separated in the germarial cysts up to the pachytene that corresponds to oogenetic stage 1 (Carpenter, 1975; 1979). The chromocentre is already present in Feulgen-Giemsa stained stage 1-2 oocytes, binding together the centromeric region of all three major

chromosome pairs. The small fourth chromosome pair seems to bind the chromocentre at oogenetic stage 3 **(Fig. 3)**.

The presence of a single or split chromocentre throughout the karyosomal stage of meiosis I prophase until the beginning of meiosis I prometaphase, is also suggested by Dernburg et al. (1996). Based on fluorescence in situ hybridization of pericentric heterochromatin DNA sequences they concluded that the close proximity of pericentric heterochromatin of all four chromosomes may lead to a chromocentral type of chromatin organization.

Our confocal studies on whole-mount oocytes stained with Prod- and lamin-antibodies and for DNA provided remarkably clear images, and allowed the 3-dimensional reconstruction of oocyte nuclei. The Prod protein is located in pericentric α-heterochromatin of *Drosophila* (Török et al., 1997), and therefore represent an excellent marker to monitor the formation of chromocentre in oocyte. During oogenetic stages 1-2, the centromeres of all chromosomes are slightly or seriously apart from each other in both oocyte and nurse cell nuclei. From oogenetic stage 3 up to stage 12, the pericentromeric heterochromatin of all chromosomes fuse, forming a single or split chromocentre, since one or two nearby Prod-signals is commonly seen in the oocyte nucleus **(Fig. 4)**. Interestingly, there are no PROD-signals in nurse cell nuclei after stages 3-5, suggesting the polytenic nature of nurse cell chromosomes similarly to salivary gland polytene chromosomes. The anti-PROD immunostaining suggests that modifications affecting the pericentric α-heterochromatin take place on the chromatin level of both oocytes and nurse cells after oocyte determination and during stages 3-5. It would be also interesting to find out the other components that mediate the reorganization of pericentric heterochromatin and lead to a single or split chromocentre formation in oocyte nucleus.

Taken together, the single chromocentre predicted by Novitski (1964) is not the predominant karyosomal structure throughout the meiosis I prophase, but one should point out that the single or split chromocentre forms through pairing of pericentric heterochromatin of all chromosomes. It seems that the most appropriate function of the chromocentre is to bring all chromosomes in close vicinity, a precondition of karyosome formation. It is obvious that the chromocentre can not be considered to represent the

fundamental basis for chromosome co-orientation and segregation during meiosis I in *Drosophila* females.

6.2. Karyosome and Meiosis I prophase

Drosophila melanogaster, like all dipteran insects, has a meroistic type of ovary, characterized by the presence of nutritive nurse cells, whose function is related to differentiation of the oocyte during vitellogenesis (Richards and Davies, 1977; Spradling, 1993), **(Fig. 1)**.

The karyosome is another characteristic of meiosis I prophase in insects with meroistic ovaries. In meroistic ovaries, the chromosomes are packed in a dense chromatin structure called karyosome after pachytene **(Fig. 3C, Fig. 5)**. The karyosome is transcriptionally almost completely inactive (Bier et al., 1967; 1969). As a consequence of karyosome formation, the diplotene and diakinesis stages of meiosis I prophase are absent. The karyosome is morphologically distinct from the diplotene and diakinesis specific meiotic chromosomes. Functionally, the karyosome may be defined as a "resting" stage, preceding condensation of chromosomes during meiosis I prometaphase.

Karyosome does not form in mammalian or amphibian ovaries, or in insects with a panoistic type of ovaries (with no nutritive cells) where RNAs, lipids, glycogen, and yolk proteins are mainly synthesized by the oocyte itself (Richards and Davies, 1977). The increased transcriptional level in panoistic oocyte nuclei correlates with the occurrence of a diffuse or loose ('lamp-brush') chromosome organization between pachytene and late diplotene or diakinesis.

During the karyosomal stage of *Drosophila* female meiosis, the size of oocyte nucleus, like in most insects with meroistic ovaries increases tremendously. Just before the breakdown of the nuclear membrane, volume of the nucleus is two to three hundred times larger than that of the karyosome **(Fig. 3, Fig. 5)**. In Neuropterans, the difference is even greater than in *Drosophila* (S. Nokkala, unpublished observation). Bringing all chromosomes together in such huge nuclei, is only possible by interchromosomal organization. It seems likely that in *Drosophila* females, the chromocentre and

karyosome ensure interchromosomal organization. Other mechanisms also exist, e.g., in *Sphinx ligustri* (Sphingidae, Lepidoptera) where all 28 bivalents associate nonhomologously via telomeres during early vitellogenesis and form a branched chain of chromatin, packed in a karyosome until the bivalents are individualized during prometaphase and metaphase of meiosis I (Nokkala, 1987).

What is the biological importance of bringing all chromosomes together in the *Drosophila* oocyte's nucleus by chromocentre-karyosome? A speculative answer is an efficient silencing of the oocyte genome and the assembly of a functional meiosis I spindle.

6.3. Assembly of meiosis I spindle

Meiosis I prophase is completed when the nuclear membrane breaks down and individualization of the bivalents takes place inside the developing meiosis I spindle. The assembly of meiosis I spindle is a step-wise process (microtubule envelope, short spindle, tapered-long spindle), and the observed morphological modifications of meiosis I spindle are considered to be direct consequences of spindle polarity establishment.

An envelope-like array of microtubules, lacking defined poles, differentiates around the karyosome after the breakdown of oocyte nuclear membrane **(Fig. 6)**. The chromosomal control of meiosis I spindle formation was proposed to explain formation of the microtubule envelope around the karyosome (Theurkauf and Hawley, 1992; Orr-Weawer, 1995). The chromosomal control of spindle formation is not limited to *Drosophila* oocytes since it is also specific for several centrosomes-MTOC missing meiotic systems (for review see McKim and Hawley, 1995).

Our 3-dimensional confocal reconstruction of the microtubule envelope, surrounding the karyosome and individualizing chromosomes suggest that the chromosomal arms associate along their entire length with microtubules **(Fig. 6**, yellow signal**)**. It seems that the microtubule-chromosome interaction is mediated by the NOD chromokinesin-like protein that positions and holds chromosomes in the developing meiosis I spindle (Theurkauf and Hawley, 1992; Komma and Endow, 1997). The NOD protein is spread along the length of chromosomal arms in *Drosophila* oocytes and

contains functional domains for both DNA and microtubule binding (Zhang et al., 1990; Afshar et al., 1995).

The fact that none of the known centrosomal proteins (DMAP60, DMAP190, γ-tubulin) bind the karyosome-chromosomes and the poles of short and tapered meiosis I spindles (J. Callaini personal communication), raise two important questions (1) the nucleation of microtubules that is still waiting to be answered, and (2) the establishment of meiosis I spindle polarity.

It was proposed that the polarity of meiosis I spindle is established by kinesin-like microtubule motor molecules (McDonald et al, 1990; Hatsumi and Endow, 1992; Afshar et al., 1995). The NCD kinesin-like protein appears to be essential for forming and stabilizing the meiosis I spindle, possibly by its capability to bundle microtubules (Matthies et al., 1996), **(Fig. 7)**.

Inside a mature meiosis I spindle, the chiasmatic bivalents find a stable arrangement in the equator due to a pulling force applied to each pair of kinetochores of a bivalent. Achiasmatic chromosomes, like the fourth chromosomes, are free to move closer to the poles **(Fig. 7C,D)**, until halted at the position determined by the limited space between microtubules in the tapered meiosis I spindle.

Although two hypotheses are competing, none of them can explain accurately the co-orientation (bipolar attachment) and segregation of the chiasmatic and nonchiasmatic homologs and heterologs during female meiosis I (for review see Orr-Weaver, 1995). It is likely that further studies are needed on the roles of chromocentre-karyosome, chiasmata and tapered meiosis I spindle to elucidate the enigmatic chromosomal segregation in the acentriolar meiosis I spindle of *Drosophila* females.

6.4. Meiosis I metaphase arrest

It has been shown recently, that in vertebrates the meiotic metaphase arrest is exerted by MOS protein-kinase (encoded by the *c-mos* proto-oncogene), (reviewed by Sagata, 1996). MOS functions as a cytostatic factor, which stabilizes the M-phase promoting factor (MPF), resulting in an arrest at meiosis II metaphase. If the cyclin degradation

mechanism inactivates the MPF, the meiosis II metaphase arrest will be released and meiosis is completed irrespective of fertilization.

The meiosis I metaphase arrest system in invertebrates having arrest is not as well known. One hypothesis suggests that accessory proteins that are present in eggs only at meiosis I are required for the MOS action to cause arrest at meiosis I metaphase (Sagata, 1996).

McKim and Hawley (1995) have proposed that the tension on kinetochore of the chiasmatic chromosomes is the arrest factor in *Drosophila* oocytes at meiosis I metaphase. Anaphase I proceeds only after the release of sister chromatid cohesion distal to the chiasmata. Puro's (1991) observations on recombination defective *Drosophila* mutants lacking all chiasmata suggest that chiasma can not alone account for the meiosis I metaphase arrest. In these recombination defective mutants, all pairs of homologous chromosomes segregate precociously in metaphase and move towards the poles until are arrested in an anaphase-like configuration. Thus, beside chiasma-based kinetochore tension further unknown factors and mechanisms must exist to exert meiosis I metaphase arrest.

6.5. Assembly of the twin spindles of meiosis II

Meiosis II occurs in *Drosophila* females as a direct continuation of anaphase I without decondensation of chromosomes between the two meiotic divisions **(Fig. 8)**.

The anaphase I spindle transforms into twin metaphase II spindles that share a common pole, also called middle pole (Riparbelli and Callaini, 1996). Transformation of meiosis I spindle into meiosis II spindle includes differentiation of the midzone of anaphase I spindle to form the middle pole in between the two anaphase I groups of chromosomes. The middle pole must nucleate new microtubules that have reversed polarity, as compared to the microtubules flanking the midzone of anaphase I spindle. Accordingly, immunostaings have shown that centrosomal proteins like DMAP60, DMAP190 and γ-tubulin were accumulated in the middle pole (Riparbelli and Callaini, 1996; J. Callaini unpublished results). However, no centrosomal proteins are present at the distal poles of the twin meiosis II spindles, and therefore the structure and function of

the distal poles remain still obscure, regarding microtubule nucleation, spindle polarity and chromosome movement.

6.6. Initiation of embryonic development

Our results concerning fertilization and gonomeric division substantiate the general image depicted diagrammatically by Foe et al. (1993). Specifically, I verified the paternal origin of the embryonic centrosome, the synchronous development of female and male pronuclei and three polar body nuclei, and the mechanism of the pronuclear fusion via gonomeric division. I have also confirmed Doane's observations (1960), that following the release of metaphase I arrest, meiosis proceeds to completion irrespective of fertilization.

In unfertilized eggs, all four polar body nuclei remain in close vicinity to each other, indicating that the sperm generated sperm aster is required for proper specification-migration of the presumptive female pronucleus. The first embryonic cell cycle is initiated in the four meiotic products without any contribution from the sperm. During postmeiotic interphase, that corresponds to the first embryonic cycle in fertilized eggs, the four polar body nuclei enter S-phase. Evidences for the postmeiotic DNA synthesis came from the presence of bichromatidic polar body chromosomes disposed into a unipolar metaphase configuration **(Fig. 13B)**. Thus the initiation of DNA synthesis in polar body nuclei is an immediate consequence of chromatin decondensation with no check-point between female meiosis and the S-phase of the first embryonic cycle. Therefore the post-meiotic interphase appears to be similar to all subsequent interphases during early embryogenesis (Glover, 1991; Foe et al., 1993). However, without paternal contribution in unfertilized eggs, the development is arrested at the metaphase of the first embryonic cycle, suggesting the existence of a gonomeric spindle assembly check-point, probably signaling lack of bipolar spindle (Wolf, 1996).

In fertilized eggs, the centrosome has a conductor role in co-ordination of early developmental events (Schatten, 1994). Results presented in this thesis support the concept of embryonic centrosome restoration. The centrosome restoration takes place during a short period of time between fertilization and completion of female meiosis. The

new centrosome contains the basal body of the sperm and recruits maternally provided centrosomal proteins, like the γ-tubulin, DMAP60 (J. Callaini personal communication), DMAP190 and centrosomin **(Fig. 13A)**.

The centrosome has two primary functions in the completion of fertilization: (1) organizing a sperm aster that effects specification-migration of the female pronucleus, and (2) organizing a bipolar double spindle to carry out the gonomeric division. Both events are under maternal control and can be blocked by mutations. Dominant, gain-of-function mutations in the *Tomaj* gene, that encodes for the α4-tubulin, lead to an abnormal sperm aster structure and function and as a result the female and male pronuclei never approach each other. Thus the maternally provided normal α4-tubulin containing microtubules are important components of the sperm aster (Máthé et al., in press). Absence of the maternally provided kinesin-like protein KLP3A (due to loss-of-function mutations) prevents migration of the female pronucleus but allows formation of a bipolar spindle (even though non-functional) for the haploid male pronucleus (Williams et al., 1997). It was proposed that the KLP3A acts as a motor molecule that mediates the movement of female pronucleus along the microtubules of the sperm aster.

The *Ketel* and *Ya* are the only known genes that have been known to be involved in the gonomeric division. Recessive, loss-of-function *Ya* mutations have been described that block gonomeric spindle formation (Lin and Wolfner, 1991; Lopez et al., 1994; Erdélyi et. al, 1997). Dominant, gain-of-function *Ketel* mutations allow a nonfunctional gonomeric spindle formation, so the gonomeric division is never completed (Lippai et al, in preparation). It is likely that normal functions of both *Ya* and *Ketel* genes are related to the nuclear membrane structure and/or function, suggesting the importance of a functional nuclear membrane for the organization of the gonomeric spindle.

6.7. The genetic dissection

In our attempt to understand female meiosis and the onset of embryonic development in *Drosophila*, besides the immunocytological strategy we make use of the genetic dissection. In general, by genetic dissection any structure or phenomenon can be picked

apart or dissected, by discovering which genes influence it. Additionally, genetic dissection provides a means to characterize proteins that would be difficult to purify using biochemical methods. Each mutant gene (allele), that produces an abnormality (mutant phenotype) identifies a component in the normal (wild-type) process and by interrelating all the genetically controlled components a larger picture can be assembled.

We described previously a collection of dominant female sterile mutations (*Fs*), and based on their phenotypes it may be assumed that *Fs*-identified genes encode important and/or specific components required for female meiosis and the onset of embryonic development in *Drosophila* (Erdélyi and Szabad, 1989; Szabad et al., 1989). Some of the *Fs*-identified genes like *Tomaj* and *Ketel* were already cloned, the sequence of the encoded proteins deduced and specific antibodies prepared (Máthé et al., 1994; Szabad et al, 1996; Erdélyi et al., in press; Máthé et al., submitted; Lippai et al, submitted). Other *Fs*-identified genes like *Horka, Damasa* are on way to be cloned, while the *Kompolt* gene is genetically characterized (Szabad et al., 1995). We believe that genetic dissection of the *Fs*-identified *Drosophila* genes combined with confocal immunocytochemistry and time-lapse confocal microscopy on living oocytes and embryos will let us to elucidate some of the fascinating events of female meiosis and early embryonic development.

MOTTO: NON RECUSO LABOREM...

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