NEW CYTOPLASMIC DYNEIN FUNCTIONS IN LIGHT OF THE DROSOPHILA *LABORC^{D}* MUTATION

Ph.D. THESIS

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PUBLICATIONS

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Current Biology **11**: 136–140 (2001). Impact factor: 8.733

II. Mónika Lippai, László Tirián, Imre Boros, József Mihály, Miklós Erdélyi, István Belecz, Endre Máthé, János Pósfai, Adam Nagy, Andor Udvardy, Efrosyni Paraskeva, Dirk Görlich and János Szabad

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Biokémia **22**: 53-60 (1998).

ABBREVIATIONS

AAA: ATP-ases associated with cellular activities

ATP: adenosin triphosphate

CNN: centrosomin

CP190: centrosomal protein of 190 kDa

Cdc: „cell division cycle" protein kinase

Cdk: ciklin dependent kinase

Dhc64C=dhc: cytoplasmic dynein heavy chain gene of Drosophila

dhc^{-}: loss-of-function dhc allele

DAPI: DNA staining dye

DM1a: mouse monoclonal anti β tubulin antibody

Dp+: duplication

EMS: ethyl methanesulfonate

FCS: fetal calf serum

FITC: fluorescein isotiocyanate

fs/Fs: recessive/dominant female sterile mutation

gnu: „giant nuclei" recessive female sterile mutation

HC: heavy chain

IC: intermediate chain

LIC: light intermediate chain

mel: recessive maternal effect lethal mutation

mRNA: „messenger" ribonucleic acid

MT: microtubule

MTOC: microtubule organizing cener

Ncd: nonclaret disjunctional

Nod: no distributive segregation

NuMA: nuclear protein that associates with the mitotic apparatus

PBS: phosphate buffered saline

png: „pan gu" recessive female sterile mutation

plu: „plutonium" recessive female sterile mutation

Ran: Ras-like nuclear protein

Ran BP1: Ran binding protein

Ras: Rous sarcoma protein

RCC1: regulator of chromatin condensation

TAAB: embedding medium for electron microscopy

WD: tryptophan, aspartic acid

Zw10: kinetochore protein

1. SUMMARY

Laborc^D is a dominant female sterile mutation of *Drosophila melanogaster*. *Laborc*^{D} identifies the Drosophila cytoplasmic dynein heavy chain (*Dhc64C*) gene. The Ph.D. dissertation is based on analysis of (i) the *Laborc*^{D}-associated mutant phenotypes and (ii) genetic characterization of *Laborc*^{D}. The Ph.D. dissertation presents in light of the *Laborc*^{D}-associated mutant phenotypes novel cytoplasmic dynein functions.

1. Centrosomes with incomplete centrioles assemble instantaneously and spontaneously upon egg activation in the unfertilized *Laborc*^{D} eggs. Since centrosomes never assemble in wild type unfertilized eggs we concluded involvement of the cytoplasmic dynein in a mechanism that prevents de novo assembly of centrosomes in wild type unfertilized eggs.
2. In fertilized *Laborc*^{D} eggs the first mitotic spindles contain multiple centrosomes prior to completion of the first cleavage division, a phenomenon never described in any other system previously. Presence of multiple centrosomes in spindle poles of the first mitotic division in the *Laborc*^{D} eggs is probably the consequence of precocious centrosome replication and separation. It most likely stems from elimination of a mechanism – in presence of the *Laborc*^{D}-encoded mutant molecules - that prevent centrosome replication prior to completion of mitosis and suggest exertion of the negative control through cytoplasmic dynein.
3. In fertilized *Laborc*^{D} eggs, the centrosomes detach from the nuclear envelope following the first cleavage division and the centrosome cycles proceed as in wild type, and while the centrosomes nucleate microtubule asters the chromosome cycles cease, nuclei degenerate. The mutant phenotype clearly shows involvement of cytoplasmic dynein in establishing connection between the centrosome and the chromosome cycles. Apparently the mutant centrosomes with *Laborc*^{D}-encoded heavy chains are not able to maintain the connection.
4. *Laborc*^{D} is a gain-of-function mutation of dominant negative type: the *Laborc*^{D}/+/+ females (with one mutant and two wild type alleles) produce a few offspring. Slight reduction of sterility of the *Laborc*^{D}/+/Dp+ females clearly shows participation of the *Laborc*^{D} encoded and the normal gene products in the same pathway and imply the dominant negative nature of *Laborc*^{D} i.e. the *Laborc*^{D}-encoded mutant gene product impede function of the normal gene product.
5. When paternally derived *Laborc*^{D} – like the *laborc*^{r} loss-of-function alleles – behaves as a recessive zygotic lethal mutation: the *Laborc*^{D}/– hemizygotes, just like the *laborc*^{r}/– ones perish at the beginning of larval life. Although the *Laborc*^{D} allele is expressed during zygotic development, when paternally derived, it does not disrupt development of the *Laborc*^{D}/+ females and males. *Laborc*^{D} causes difficulties only during the onset of embryogenesis in combination with a maternally provided partner present in the egg cytoplasm. Apparently *Laborc*^{D} identifies maternal function of an essential zygotic gene.

2. INTRODUCTION

Upon the commencement of embryogenesis, the egg cytoplasm must contain all the components essential for early embryogenesis. Since there is little if any zygotic gene expression during early embryogenesis the vast majority of the embryogenesis controlling factors are deposited into the egg cytoplasm during oogenesis by the female, and apparently their synthesis is under maternal control. The corresponding maternal genes engaged in the initiation of embryogenesis represent some of the *maternal-effect* genes. The exact mechanism of the initiation of embryogenesis is yet poorly understood. Many developmental biologists have focused their efforts on model systems in which embryonic development is easier to study than in mammals. The fruit fly *Drosophila melanogaster* is an excellent experimental organism for studying maternal effects. Following meiotic divisions, the first 13 mitotic cycles are nuclear divisions that occur synchronously in a shared cytoplasm. The cleavage cycles oscillate rapidly between the S and M phases without detectable gap phases. DNA replication and mitosis take only about 8-10 min during cycles 1-10, and gradually lenghten to 21 min by cycle 13. The speed of the cleavage cycles is facilitated by stockpiles of maternally provided materials. The zygotic genome is not transcribed before cycle 11, and transcription of most genes does not begin until cycle 14 (Orr-Weaver, 1994). Genetic dissection - gene identification and characterization through mutations - may be used to identify genes responsible for maternal effect. Genetic dissection implies first identification and understanding the function of single components and the subsequent reconstruction of the process. Mutations that block the initiation of embryogenesis are likely to identify the desired key components. Analysis of maternal effect lethal (*mel*) mutations, a special class of female sterile (*fs*) mutations, provided major contribution to our knowledge about the genes contributing to the regulation of embryogenesis in *Drosophila melanogaster* (Wieschaus, 1996). Analysis of the *mel* mutations promoted, among others, the understanding of cell differentiation along the anterior-posterior and the dorsal-ventral coordinates of the *Drosophila* embryo (Anderson and Nüsslein-Volhard, 1986; Nüsslein-Volhard et al., 1987). Females homozygous for a *mel* mutation deposit normal-looking and fertilized eggs. However, embryogenesis does not commence or leads to the formation of abnormal embryos.

The true maternal effect genes are rare. It is rather common that early embryogenesis is governed by genes that have both maternal and zygotic functions and, in fact about 90% of the *fs* mutations are weak (hypomorph) alleles of genes with essential zygotic functions (Schüpbach and Wieschaus, 1989). The complete loss-of-function (amorph) mutations in genes with both maternal and zygotic functions result in zygotic lethality and hence do not allow readily analysis of the maternal function, and go undetected in screens for *mel* mutations. Genes with both early

embryonic and zygotic functions may in principle be identified by dominant female sterile (*Fs*) mutations that disrupt the maternal-effect, however permit the zygotic functions, ie. females carrying an *Fs* mutation can develop to normal but sterile adults.

Although several features of embryonic cleavage cycle control have been revealed in Drosophila, a number of components and their functions remain to be elucidated. An example of the unknown mechanisms is the system that ensures coordinated regulation of the chromosome and the centrosome cycles during cleavage divisions. Knowing that most, if not all of the factors required during early embryogenesis are maternally provided, we isolated 75 dominant female sterile (*Fs*) mutations of *Drosophila melanogaster* hoping that a few will reveal new aspects of the commencement of embryogenesis. *Laborc*^{D} is one of the *Fs* mutations (Erdélyi and Szabad, 1989). As described below, *Laborc*^{D} identifies the cytoplasmic dynein heavy chain gene (Belecz et al., 2001). Cytoplasmic dynein, a minus-end-directed microtubule motor, has been implicated in a broad range of microtubule (MT) dependent activities during mitosis, meiosis and interphase (Karki and Holzbaur, 1999). In Drosophila and mammalian cells dynein is required for spindle formation and function. The formation of spindle poles in both centrosomal and acentrosomal spindles is driven by a common group of noncentrosomal accessory proteins including NuMA, cytoplasmic dynein, and dynactin (Gaglio et al., 1997). Dynein has been shown to play key role in centrosome separation (Vaisberg et al., 1993) and, in general, several findings underline the role of dynein in centrosome organization and function. Dynein appears to transport pericentriolar components to the centrosome during both interphase and mitosis. The dynein-transported molecules include dynactin, γ-tubulin and pericentrin during interphase, dynactin and NuMA during mitosis (Quintyne et al., 1999). Dynein is also necessary for attachment and migration of centrosomes along the nuclear envelope during interphase/prophase, as well as in the maintenance of centrosome attachment to mitotic spindle poles (Robinson et al., 1999). Cytoplasmic dynein functions at the kinetochor to coordinate chromosome separation and/or poleward movement at anaphase onset (Starr et al., 1998). During Drosophila oogenesis, cytoplasmic dynein is required to orient the cystocyte divisions and consequently oocyte determination (McGrail and Hays, 1997). During interphase, cytoplasmic dynein mediates movement of membranous vesicles, such as perinuclear positioning of the Golgi apparatus, ER to Golgi transport, and retrograde axonal transport (Karki and Holzbaur, 1999).

Cytoplasmic dynein is a multisubunit protein that contains two heavy chains and multiple intermediate, light intermediate and light chains. The cytoplasmic dynein complex works together with a second multiprotein complex, dynactin. Dynactin is required for most of the dynein mediated cellular activities, and is believed to function as an adapter complex that allows dynein to bind cargo (Schroer, 1996).

In this dissertation I report novel cytoplasmic dynein functions as revealed by mutant phenotypes associated with *Laborc*^{D}, a dominant female sterile mutation of Drosophila (Belecz et al., 2001). (1) In fertilized *Laborc*^{D} eggs, deposited by *Laborc*^{D}/+ females, multiple centrosomes appear at the spindle poles of the first cleavage division, indicating involvement of the cytoplasmic dynein in a mechanism that in wild type prevents centrosome replication prior to completion of mitosis. (2) The centrosomes detach from the cleavage nuclei following the initial cleavage divisions, replicate, separate and organize asters of MTs as in wild type, i.e. the chromosome and the centrosome cycles are uncoupled. While the centrosome cycles proceed as in wild type the few forming cleavage nuclei degenerate demonstrating that cytoplasmic dynein function is necessary for the coupling of the chromosome and centrosome cycles. (3) In unfertilized *Laborc*^{D} eggs, centrosomes with incomplete centrioles form without accompanying chromosome replication, showing involvement of cytoplasmic dynein in the mechanism that prevents de novo centrosome and centriole assembly in wild type unfertilized eggs.

3. MATERIALS AND METHODS

Strains

Laborc^{D} [= *Fs(3)Laborc*] is an EMS-induced dominant female sterile mutation (Erdélyi and Szabad, 1989). The *laborc*^{r} revertant (loss-of-function recessive) alleles were isolated following X-ray and EMS mutagenesis of the *Laborc*^{D} allele (Erdélyi and Szabad, 1989). The Drosophila cultures were kept on 25°C on standard cornmeal agar media.

Complementation analyses

Drosophila females heterozygous for the *laborc*^{r} revertant alleles and males heterozygous for loss-of-function alleles of the genes in the 64C cytological region [including mutant alleles of the *Dhc64C* (*dhc)* gene (Gepner et al., 1996)] were mated. In showing allelism between loss-of-function *dhc*$^{-}$ mutant and the *laborc*^{r} alleles we made use of the *P(dhc*$^{+}$*)*^{x}, an X-linked transgene with a genomic segment that contains the entire *dhc* gene (Gepner et al., 1996).

Preparation of whole-mount embryos for immunofluorescence

Embryos were collected for up to 3 h from wild type (Canton-S) and *Laborc*^{D}/+ females and dechorionated using a 50% bleach solution. Alternatively embryos were squeezed out from the uteri of the above females during CO_2 narcosis and dechorionated manually on a double face Scotch tape. After dechorionization, embryos were rinsed in distilled water and fixed in heptane/methanol (1:1). Heptane is used to permeabilize the vitelline membrane. After heptane/methanol treatment the embryos were transferred to fresh methanol and fixed for additional 30 min. Fixed embryos were rehydrated for 2x10-min periods in PBS containing 0.1% Triton X-100 (PBT). Before antibody labeling, embryos were blocked for 1 h at room temperature in PBT containing 10% fetal calf serum (FCS) (PBT-FCS). All antibodies were diluted in PBT-FCS with RNase (1 mg/ml) and incubations were performed at room temperature for up to 3h or at 4 °C for up to 18 h. After each antibody incubation, embryos were rinsed at 15–20-min intervals for 1-2 h in PBT-FCS at room temperature. Microtubules were labeled using the mouse DM1-a anti-β-tubulin monoclonal antibody (Sigma) diluted 1:400 and a FITC-conjugated goat anti–mouse secondary antibody (Jackson ImmunoResearch Labs) diluted 1:500. There was no taxol used to stabilize MTs. Centrosomes were labeled using the Rb188 rabbit polyclonal antibody against the CP190 centrosomal protein diluted 1:400 (Whitfield et al., 1988) and with the anti-centrosomin (CNN) rabbit polyclonal antibody diluted 1:400 (Heuer et al., 1995) and Texas red–conjugated goat anti–rabbit secondary antibody diluted 1:500 (Jackson ImmunoResearch Labs). DNA was stained with TOTO-3 (Molecular Probes). Optical sections

were collected with a Leica TCS NT and with a Zeiss LSM 410 confocal microscope. After labeling, embryos were mounted in Vectashield (Vector) or glycerol containing PBS and 1 mg/ml p-phenylenediamine.

Immunostaining of stage 14 oocytes

Ovaries were dissected in absolute methanol and transferred to a 10 ml plastic tube containing 2 ml of fresh methanol. About 10-20 single ovaries prepared in this way were sonicated with a sonifier B-12 from Branson Sonic Power Company fitted with a cone shaped probe of ~3-4 mm in diameter at the bottom. Sonication was applied in five cycles of 1sec each. Oocytes without chorion and vitelline membrane were transferred to fresh methanol and kept at room temperature for a further 2h (Tavosanis et al., 1997). Immunostaining was carried out as described above.

Electron microscopy

For transmission electron microscopy the embryos were dechorionated with bleach, transferred to heptane-glutaraldehyde, shaked for 1 min, then fixed in glutaraldehyde for 2 hours. After rinsing in 0,1 M cacodylate buffer (pH 7,2) the embryos were postfixed in 1% osmium tetroxide for 1 h and dehydrated in a graded series of ethanol and bulk stained in 1% uranyl acetate for 1h. After treatment with aceton the embryos were embedded in TAAB 812 and polimerized at 60 °C for 12h. Random and serial cut sections using a Leica ultramicrotome (Ultracut S) were collected on one hole copper grids and stained with uranyl acetate and lead citrate. Sections were analyzed and photographed with a Philips CM 10 electron microscope at 100kV.

4. RESULTS

THE GENETICS OF *LABORC^D*

Laborc^D is one of the EMS-induced dominant female sterile mutations (Erdélyi and Szabad, 1989). *Laborc^D* is fully penetrant and expressive (Erdélyi and Szabad, 1989). Remarkably the *Laborc^D*-associated defects are reduced in the *Laborc^D*/+/Dp+ females who carry an additional wild type copy of the *Laborc^D*-identified gene: offspring develop from about one percent of the eggs. Slight fertility of the *Laborc^D*/+/Dp+ females is a clear indication that *Laborc^D* is a gain-of-unction mutation and the mutant phenotypes are brought about by *Laborc^D*-encoded mutant protein molecules (Muller, 1932). The gain-of-function nature of *Laborc^D* is also confirmed by the fact that loss-of-function recessive *laborc^r* alleles can be generated through second mutagenesis of *Laborc^D* (Erdélyi and Szabad, 1989). Slight fertility of the *Laborc^D*/+/Dp+ females shows the dominant negative nature of *Laborc^D* and implies participation of the mutant and the normal gene products in the same pathway. *Laborc^D* was mapped to the left arm of the 3rd chromosome between the interval delineated by the *ru – h* recessive marker mutations, in the 61F7-8 and 66D11-12 cytological region (Erdélyi and Szabad, 1989). Of the duplications that cover different segments of the *ru – h* interval only the *Dp(3;3)BK7* duplication (mentioned above as *Dp+*) relieved the *Laborc^D*-associated sterility. *Dp(3;3)BK7* not only revealed the nature of *Laborc^D* but also showed that the *Laborc^D*-identified gene resides within the 64C-64D cytological region. We than carried out complementation analyses between the *laborc^r* alleles and representative mutant alleles of the genes that reside in the 64C-64D region (Lindsley and Zimm, 1992). The *laborc^r* alleles complement all the mutant alleles except the *dhc$^-$* mutant alleles of the cytoplasmic dynein heavy chain gene *Dhc64C* (*dhc*; Gepner et al., 1996) showing that *Laborc^D* is a gain-of-function allele of *dhc$^-$*. This conclusion is further supported by the fact that a *dhc$^+$* transgene overcomes lethality associated with the over twenty *laborc^r/dhc$^-$* combinations we tested: the *dhc$^+$*; *laborc^r/dhc$^-$* zygotes are fully viable and fertile. When paternally derived, *Laborc^D* behaves as the *laborc^r* (or the *dhc$^-$*) zygotic lethal mutant alleles: the *Laborc^D*/–, like the *laborc^r*/– or the *dhc$^-$*/– hemizygotes, die at the beginning of larval life, and the perishing larvae with different genotypes are indistinguishable. The former findings illustrate (i) zygotic requirement of the *dhc* gene and (ii) that the paternally derived *Laborc^D* allele does not function during the cellular stages of development.

OOGENESIS IN *LABORC^D* FEMALES

Oogenesis is largely normal in the *Laborc^D* females. A normal looking oocyte develops in all of the egg chambers. The meiotic divisions appear largely normal in the egg primordia. In a number of cases however defective first and second meiotic divisions appear. Despite the meiotic defects described below, meiosis is completed in the majority of the cases as revealed by the presence of the normal looking polar body nuclei in the *Laborc^D* eggs.

The first meiotic division

The first meiotic spindle forms in Drosophila in a centrosome independent way (Matthies et al., 1996). Microtubules are nucleated in the vicinity of chromatin. The first meiotic spindle is indistinguishable from wild type in the *Laborc^D* eggs deposited by the *Laborc^D*/+ females. The wild type first meiotic spindle has tapered poles without centrosomes, and contains highly compacted chromatin at the metaphase plate, with precociously separating fourth chromosomes (Fig. 1A). In a number of *Laborc^D* eggs the spindle poles are divergent suggesting involvement of cytoplasmic dynein in spindle pole focusing (Fig. 1C). In other cases, nondisjunction of the chromosomes take place (Fig. 1D), supporting that cytoplasmic dynein plays role in chromosome segregation.

The second meiotic division

The meiosis II spindle of Drosophila consist of two tandem spindles with anastral distal poles and an aster-associated spindle pole body between the central poles (Fig. 1B). The central spindle pole contains centrosomal proteins. The central spindle pole body of the second meiotic spindle in *Laborc^D* eggs is significantly larger than in wild type (Fig. 1E). Altough this phenotype is rare (2 out of 10), it indicates involvement of cytoplasmic dynein in assembly of the central spindle pole body of the second meiotic spindle. As in wild type, there are no centrosomes visible in the *Laborc^D* eggs in the meiotic divisions (Fig. 4D).

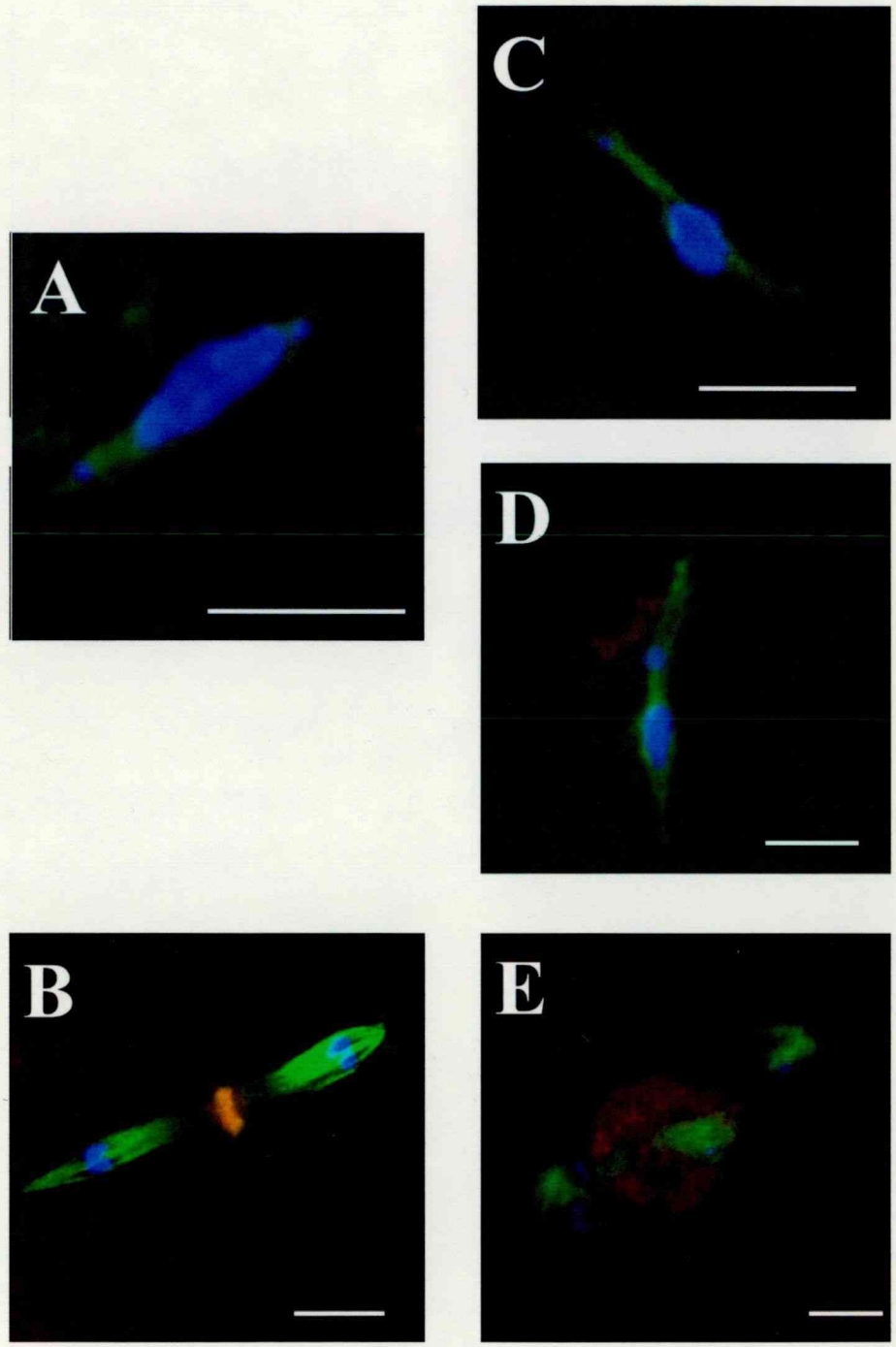


Figure 1. The first and the second meiotic spindles in wild type (A, B) and in *Laborc*^{D}eggs (C, D, E). The color codes are as follows: red = CP190, green = tubulin, blue = DNA. Scale bar = 10 μm.

CLEVEAGE CYCLES IN THE *LABORC^D* EGGS

The first cleavage mitotic spindles in *Laborc^D* eggs

The *Laborc^D*/+ females produce normal numbers of normal looking so-called *Laborc^D* eggs. In fertilized *Laborc^D* eggs the first cleavage mitosis forms as in wild type with one centrosome at each spindle pole (Fig. 2A). In wild type the centrosomes do not replicate prior to completion of mitosis. In fertilized *Laborc^D* eggs, however, the centrosomes begin to replicate prior to accomplishment of mitosis, as shown by presence of multiple centrosomes at the spindle poles (Fig. 2E). This observation probably refers to a mechanism that prevents centrosome replication prior to completion of mitosis. Cytoplasmic dyneins with *Laborc^D*-encoded mutant heavy chain ignore the mechanism.

Cleavage cycles in the *Laborc^D* eggs

Following the first and/or the second cleavage divisions, the centrosomes detach form the nuclear envelopes, replicate and the daughter centrosomes separate as in wild type (Fig. 2F, G). The usually four cleavage nuclei stop dividing. They remain deep down in the egg cytoplasm and eventually degenerate. Meanwhile the centrosome cycles proceed as in wild type and about two hours following fertilization the entire *Laborc^D* egg cortex is populated with free centrosomes (Fig. 2H). The centrosomes nucleate microtubule asters that appear slightly larger as compared to wild type. Detachment of the centrosomes from the nuclear envelopes shows involvement of cytoplasmic dynein in centrosome attachment to the nucleus as was described by Robinson et al., 1999. The uncoupled chromosome and the centrosome cycles in the *Laborc^D* eggs imply involvement of the cytoplasmic dynein in linking the chromosome and the centrosome cycles together.

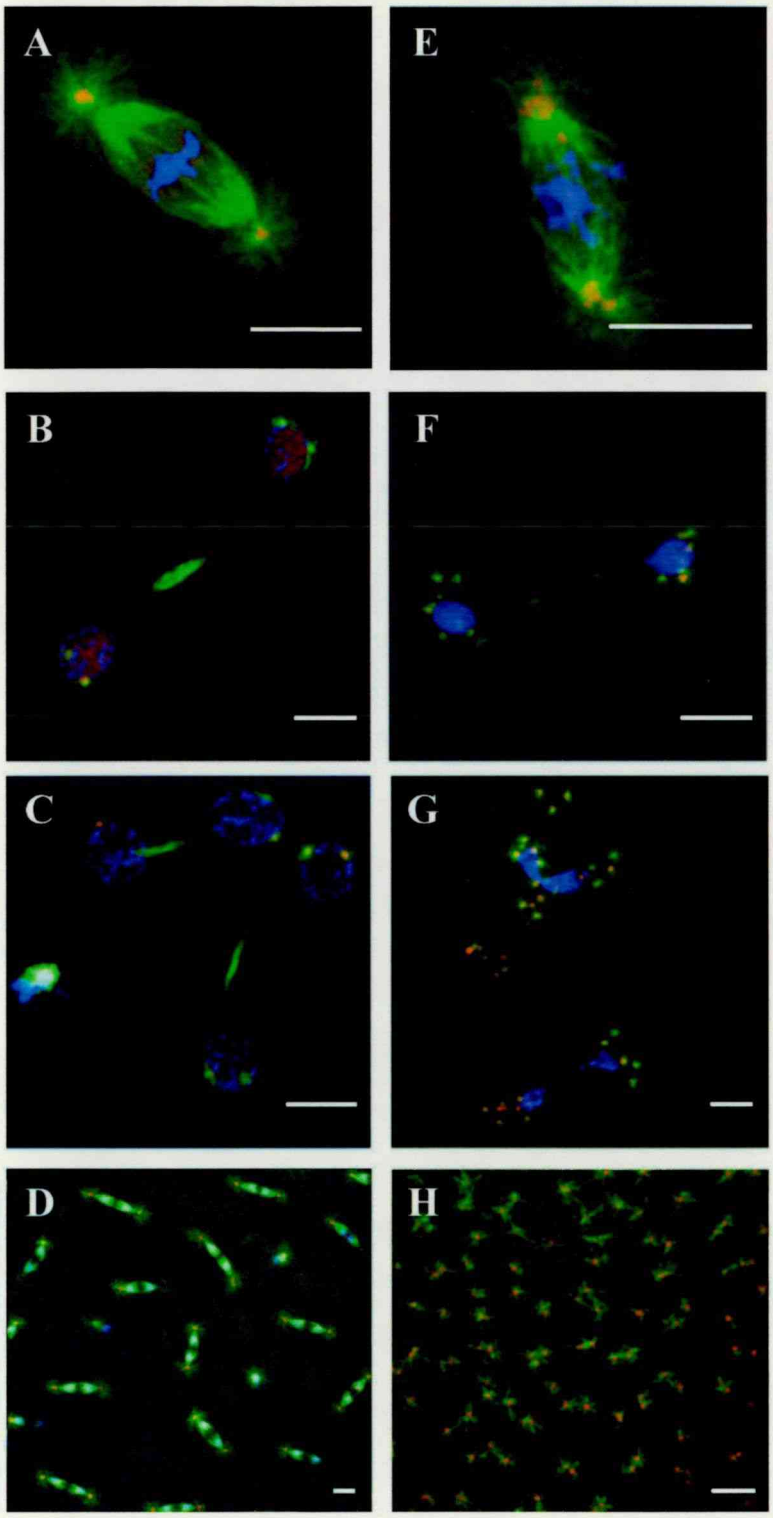


Figure 2. Initiation of cleavage cycles in fertilized wild type (A-D) and in *Laborc*^{D} eggs (E-H). In wild type, there is one centrosome at each spindle pole (A). Multiple centrosomes emerge at poles of the first cleavage spindle in the *Laborc*^{D} eggs (E). Wild type (B and C) and *Laborc*^{D} eggs (F and G) with two and four nuclei. While characteristic spindles appear in wild type eggs (D), CSs detach from the nuclei, populate and nucleate MT asters in cortex of the *Laborc*^{D} eggs. Color codes are as in Fig. 1. Scale bar = 10 μm.

THE UNFERTILIZED *LABORC^D* EGGS

In Drosophila, cytoplasm of the wild type egg primordia contains a pool of dispersed centrosomal components without functional centrosomes. Upon egg activation, i.e. during egg transfer from the ovaries through the oviduct to the outer world, some of the centrosome componenets assemble and form the central spindle pole body of the second meiotic spindle, irrespectively of fertilization. Four haploid nuclei form in a few minutes, and one of the nuclei will become the female pronucleus in the fertilized eggs, the other three will form the polar bodies. In unfertilized eggs all the four meiotic products will form polar body nuclei. Centrosomes of the embryos form when the sperm-derived centrioles recruit centrosome components form the egg cytoplasm. Centrosomes do not form in the unfertilized wild type eggs and development does not proceed beyond the four haploid nuclei stage (Foe et al., 1993). Contrary to the wild type eggs, in unfertilized *Laborc^D* eggs the egg cytoplasm was full with centrosomes as revealed by the presence of the CP190 and the CNN centrosomal proteins. The centrosomes nucleated small MT asters (Fig. 3A). However, unlike centrosomes in the fertilized *Laborc^D* eggs (Fig. 3B), the centrosomes were slightly reduced in size and nucleated only small asters. It is important to note that there were no other nuclei than the four haploid products of the two meiotic divisions in the unfertilized *Laborc^D* eggs.

We also noticed in a small number (1%) of the fertilized *Laborc^D* eggs two separate groups of multiple centrosomes of both fertilized and unfertilized types (Fig. 3C.). While the anterior centrosome group might have originated form the sperm basal body, centrosomes in the another group might have assembled as in the unfertilized eggs.

The formation of centrosomes in the unfertilized *Laborc^D* eggs clearly shows that dynein is involved in the mechanism that prevents de novo centrosome assembly in the unfertilized wild type eggs.

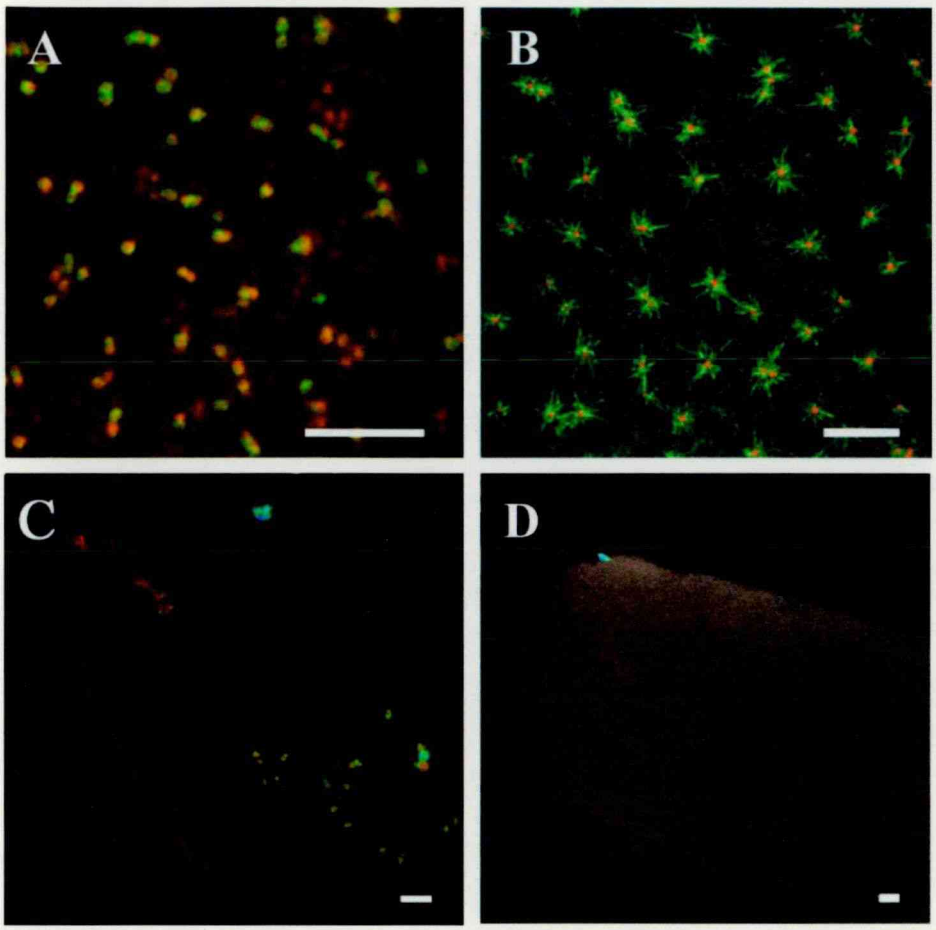


Figure 3. (A) Rudimentary centrosomes assemble in unfertilized *Laborc*^{D} eggs and nucleate small MT asters. (B) Normal looking free centrosomes and MT asters populate cortex of the fertilized *Laborc*^{D} eggs. (C) Both types of centrosomes and asters appear in about one percent of the fertilized *Laborc*^{D} eggs. (For wild type control see Fig. 2D.) (D) There is no indication of centrosome formation in mature *Laborc*^{D} oocytes as shown by the absence of assembly of the CP190 and the CNN centrosomal proteins. The first meiotic spindle is shown on top left part of the figure. The color codes are as follows: red = CP190 (CNN gives identical staining), green = tubulin, blue = DNA. Scale bar = 10 μm.

Ultrastructural analysis of the unfertilized *Laborc*^{D} eggs

To find out whether or not centrosomes in the unfertilized *Laborc*^{D} eggs contained centrioles we carried out an ultrastructural analysis. Apparently incomplete centrioles form, and their number appears similar to the number of the centrosomes detected by immunofluorescence. However the centrioles are rudimentary: the so-called carthweel structure in the middle of the centriole appears normal in size and organization and the MT doublets are missing (Fig. 4B).

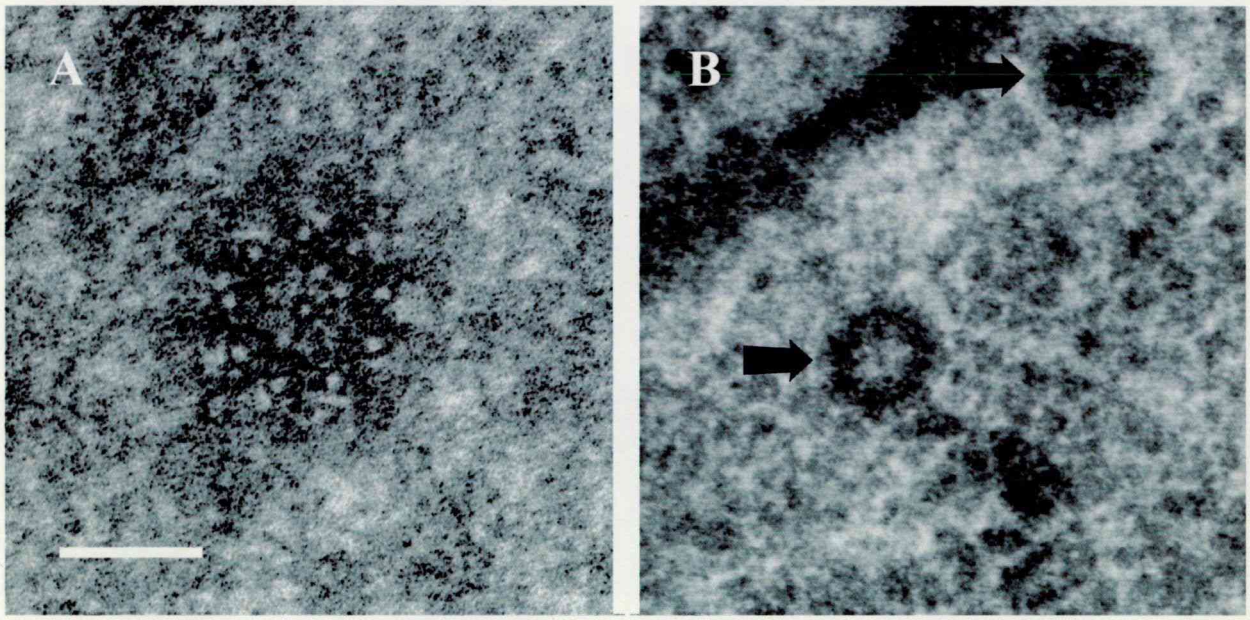


Figure 4. EM photographs of centriole cross-sections. (A) Wild-type cleavage embryo. (B) Rudimentary centrioles (arrows) in unfertilized *Laborc*^{D} eggs. Note the lack of centriole MTs. Scale bar = 100 nm.

5. DISCUSSION

LABORC^D IS A DOMINANT NEGATIVE MUTATION IN THE *dhc* GENE

Laborc^D was mapped to the left arm of the third chromosome (Erdélyi and Szabad, 1989). Duplication and deficiency mapping located *Laborc^D* to the 64C cytological region. The *laborc^r* loss-of-function revertant alleles are zygotic lethal mutations and were generated through second mutagenesis of the *Laborc^D* allele (Erdélyi and Szabad, 1989). To find out whether *Laborc^D* and the *laborc^r* alleles identify an already known gene, we carried out complementation analyses with lethal alleles of genes in the 64C region. Since the *laborc^r* alleles did not complement the *dhc$^-$* mutations, that identify the *Dhc64C* cytoplasmic dynein heavy chain gene (Gepner et al., 1996) we concluded that *Laborc^D* was an allele of that gene. This conclusion is further supported by the finding that the *P(dhc$^+$)^X* transgene (Gepner et al., 1996) overcomes lethality of the *laborc^r/dhc$^-$* combinations.

When combined with tandem duplications that include the 64C region, about 1% of the *Laborc^D/+/Dp+* females produced a few offspring. This result clearly shows that *Laborc^D* is a gain-of-function mutation i.e. the *Laborc^D*-related defects are brought about by mutant gene product. It furthermore implies that *Laborc^D* is a dominant negative mutation and implies that the *Laborc^D*-encoded mutant and the normal gene products participate in the same process.

Maternal function of the Laborc protein

Interestingly the *Laborc^D* mutation does not interfere with viability, oogenesis and male fertility. The toxic effect of the *Laborc^D*-encoded mutant protein is manifested only in the embryos. Because *Laborc^D* was induced with EMS, it is most likely a point mutation, which does not affect expression of the *Laborc^D* allele, similarly to the *Ketel^D* mutation (Lippai et al. 2000; Tirián et al., 2000). *Laborc^D* thus identifies maternal gene function of a zygotic gene: the egg cytoplasm – the maternal dowry – of the wild type females contains dynein heavy chain protein to match the unique needs of the cleavage stages of embryogenesis during which there is little if any zygotic gene expression (Foe et al., 1993). The 13 cleavage cycles last only 8-10 minutes each and require special factors to accomplish the fastest known eukaryotic cycles in the absence of zygotic gene expression.

When paternally derived, *Laborc^D* behaves as the *laborc^r* (or the *dhc$^-$*) zygotic lethal alleles: the *Laborc^D/–*, like the *laborc^r/–* or the *dhc$^-$/–* hemizygotes, die at the beginning of larval life and the different types of perishing larvae are indistinguishable. The former findings illustrate (i) zygotic requirement of the *Dhc64C* gene and (ii) that the paternally derived *Laborc^D* allele does

not function during the cellular stages of development. In fact *Laborc*^{D} is most likely expressed in cells of the zygotes as the normal *Dhc64C* gene (Hays et al., 1994; Li et al., 1994). Nonetheless *Laborc*^{D} does not possess toxic effects since the *Laborc*^{D}/+ females and males are viable and the males are fertile. The most likely explanation for the restricted action of *Laborc*^{D} during the cleavage cycles is that *Laborc*^{D} identifies maternal function of the zygotically essential *Dhc64C* gene and the deleterious effects are brought about following interaction of the *Laborc*^{D}-encoded mutant protein with a maternally provided partner present only in the egg cytoplasm. Similar phenomenon was first described for the *Ketel*^{D} alleles (Lippai et al., 2000; Tirián et al., 2000).

Although the mutant molecule might well be present in the somatic cells, it can not exert any toxic effects in the absence of the appropriate partner. The mutant *Laborc*^{D} protein is "simply" nonfunctional without its partner, explaining the recessive loss-of-function phenotype in the *Laborc*^{D}/-zygotes. While functional dynein molecules form, the cytoplasmic dynein heavy chains associate with light, light-intermediate and intermediate chain subunits. When in action, cytoplasmic dyneins have been known to associate with cargoes, MTs, the dynactin complexes and molecules that regulate dynein functions (Karki and Holzbaur, 1999). We propose that association of the *Laborc*^{D} encoded mutant cytoplasmic dynein heavy chain molecules with a maternally provided dynein component-„designed" for embryogenesis-leads to the formation of mutant cytoplasmic dynein and to defects characteristic of the *Laborc*^{D} females. As described in the coming chapters, there are plenty of candidates since the cytoplasmic dynein complexes are composed from different types of molecules. The above mentioned maternally provided partner awaits to be identified. In summary, the *Laborc*^{D} alleles identify the maternal function of a both maternally and zygotically required gene function.

THE MOTOR PROTEINS

Dyneins, kinesins and myosins represent the three major classes of molecular motors that move along cytoskeletal elements (Fig. 5). Dyneins move toward the minus end of the MTs, and most kinesins move toward the plus end of the MTs (Belecz and Szabad, 1998). Myosins move along the actin microfilaments. The kinesins and myosins have a motor domain that contains both ATPase and filament-binding sites attached to an α-helical lever arm and neck region. Structural analysis of the kinesin and myosin heads revealed striking similarity in the motor domains of the kinesin and myosin enzymes (Kull et al., 1996; Rayment et al., 1993). The similarity implies that the general mechanisms by which the motor proteins generate force while move along their respective filament systems, are related at a fundamental mechanism and raised the possibility that all cytoskeletal motors function similarly. However, one thorn remained in this rather

comforting picture of molecular motor conformity, and that is dynein. Unlike in myosins and kinesins, the microtubule-binding domain is located far from the ATP-hydrolysis site in dyneins (Gee et al., 1997; Koonce, 1997). The *in-vitro* microtubule-translocation parameters of dyneins also are quite different from those of kinesins (Wang et al., 1995). Recent studies indicate that the dynein motor unit is constructed around a series of AAA domains (ATPases associated with cellular activities, Neuwald et al., 1999) suggesting that dyneins are fundamentally distinct from kinesins and myosins at both the structural and mechanistic levels (Fig. 6B).

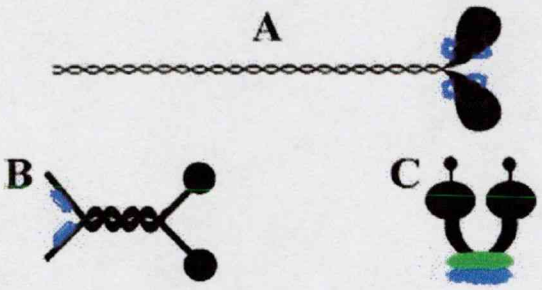


Figure 5. Schematic representation of the motor proteins. (A) myosin, (B) kinesin, (C) dynein. Heavy chains: black, light chains: blue, intermediate and light intermediate chains: green.

THE DYNEIN SUBUNITS

Heavy chains

Dyneins are massive molecular motor complexes of 1-2 MDa that generate force towards the minus end of the microtubules. Three subfamilies of dynein heavy chains (HCs) have been identified: the axonemal (i) inner and (ii) outer arm dynein heavy chains and the (iii) cytoplasmic dynein heavy chains. The cytoplasmic dynein heavy chains include two isoforms: HC 1a, an ubiquitously expressed isoform and isoform HC 1b, which appears to be more functionally restricted (Criswell et al., 1996; Pazour et al., 1999). The heavy chains are the force-producing subunits that interact with microtubules and hydrolyze ATP (Fig. 6A). Molecular weigtht of the HCs are ~530 kDa. The overall architecture of the dynein particle is illustrated in Fig. 6A. Cytoplasmic dyneins appear to be homodimers of HCs with identical motor properties. Each HC comprises an N-terminal domain of ~160 kDa which forms the stem of the structure and interacts with additional HCs and other components. In vitro binding studies have identified a ~200 amino acid residue segment within this region that can mediate both HC-HC dimerization and HC-intermediate chain (IC) interactions (King, 2000). The C-terminal segments of the HCs form the ~350 kDa globular head domains. In the head domain there is a highly conserved central section of ~100 kDa that contains four nucleotide binding motifs (P1-P4) while the P1 motif marks the ATP hydrolytic site, role of the other nucleotide binding motifs are unclear. The microtubule-binding domain is located at the tip of a slender stalk emanating from the head (Koonce, 1997). The stalk is composed from two coiled coil segments. Sequence analyses of dyneins revealed only four putative nucleotide-binding motifs (P1-P4) within the central region of the HC

(Gibbons et al., 1991). However, more recent studies clearly indicate that the dynein motor also contains six AAA domains plus an unrelated seventh unit at the C terminus (Fig. 6B; Neuwald et al., 1999). The first four AAA units correspond to the previously identified motifs. The fifth and sixth AAA domains had not been noted originally, because they lack intact consensus P-loop motifs. The coiled coil and microtubule-binding region that protrudes from the main head (Koonce, 1997) is located between the fourth and fifth AAA domains. Three-dimensional reconstructions of the dynein motor domain from high-resolution negative stain images reveal a 13.5 nm diameter spheroidal structure composed of seven globular subdomains arranged around a central cavity (Samso et al., 1998, Fig. 6B).

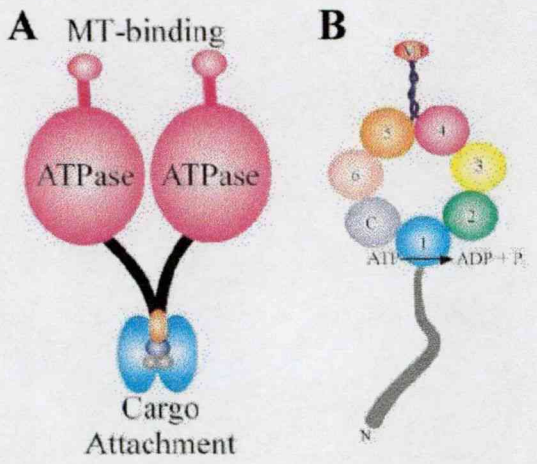


Figure 6. Organization of the dynein motor domain. (A) Generic model of a dynein particle. The C-terminal portion of each HC forms a globular head containing the ATPase sites and has a small stalk-like structure that terminates in a microtubule-binding globular unit. The base of the dynein particle consists of the N-terminal regions of the HCs and a series of accessory proteins that function in cargo binding and might also have regulatory roles. (B) Model for the organization of an individual HC illustrating the heptameric structure of the head. Note that almost the entire globular motor unit consists of the six AAA domains, an unrelated C-terminal subdomain and the coiled-coil microtubule-binding region. (Adapted from King, 2000).

Intermediate chains

Cytoplasmic dyneins contain two ICs of ~70-80 kDa which are are located at the base of the soluble dynein particle. The ICs are members of the WD-repeat (tryptophan, aspartic acid) protein family. ICs apparently act directly in the attachment of the dynein motor to its cargo. Cytoplasmic dynein IC composition is complex and molecular and biochemical studies have revealed a plethora of isoforms derived from differential phosphorylation and alternative splicing (King, 2000). The IC isoforms are differentially expressed during development of specific cell types (King, 2000). Although functional significance of the IC isoform diversity remains unknown, one obvious possibility is that different versions of ICs are capable of interacting either with different proteins or with the same protein but with varying affinities. ICs interact directly with the p150 Glued component of dynactin (Karki and Holzbaur, 1995). The dynactin complex is an additional multimeric structure that acts as an activator of dynein-based motility (King and Schroer, 2000; see below).

Light intermediate chains

The 50-60 kDa light intermediate chain (LIC) dynein proteins contain an ATP-binding motif and are distantly related to ABC transporters (Hughes et al., 1995). However, there is as yet no direct evidence that they actually bind nucleotide and their precise location within the dynein particle has yet to be resolved. The dynactin independent interaction between pericentrin and cytoplasmic dynein is specifically mediated by LIC (Purohit et al., 1999)

Light chains

1. *Dynein LC8*

LC8 belongs to a group of very highly conserved proteins present in distantly related organisms as mammals, nematodes and plants. LC8 protein is a stoichiometric component of both brain cytoplasmic dynein and myosin (King, 2000). The LC8 protein is present in many, seemingly unrelated enzyme systems. Drosophila partial loss-of-function alleles lead to morphogenetic defects in bristle and wing development, female sterility and also cause alterations in axonal guidance during development. Total loss-of-function alleles result in embryonic lethality through the induction of apoptotic pathways (Phillis et al., 1996). The LC8 associated phenotypes were originally interpreted as being caused by defects in cytoplasmic dynein; however, it now seems more likely that certain of the defects derive from the disruption of other enzyme activities.

2. *The Tctex1 dynein light chain family*

The diverse group of Tctex1 dynein light chains was first identified in cytoplasmic dynein (King, 2000). Examination of the current databases revealed several additional members of the family including the rp3 protein that is also a demonstrable component of cytoplasmic dynein (King, 2000). The Tctex1 and rp3 light chains are differentially regulated in both a developmental and tissue-specific manner. LCs may play a role in the binding of particular cargoes or in differentially regulating some essential aspect of dynein function (King, 2000).

3. *The roadblock/LC7 light chain family*

The most recent class of dynein LC reported contains closely related homologues that are present in both flagellar and cytoplasmic dyneins (King, 2000). Mutations in the Drosophila *roadblock* gene lead to defects in axonal transport and mitosis suggesting that members of the roadblock/LC7 light chain family play essential roles in dynein function.

DYNACTIN

Cytoplasmic dynein works in conjunction with a second multiprotein complex, dynactin (Fig. 7). Dynactin is generally believed to function as an adapter that allows dynein to bind cargo.

Dynactin consist of 11 different subunits (Schroer, 1996; Allan, 2000). Dynactin has two distinct structural domains, an actin like miniflament backbone and a flexible projecting sidearm. Dynein binds the dynactin sidearm subunit, $p150^{Glued}$. The distal end of the $p150^{Glued}$ sidearm also contains a pair of MT binding sites. Transient MT binding by dinactin allows the dynein motor to move more processively (King and Schroer, 2000).

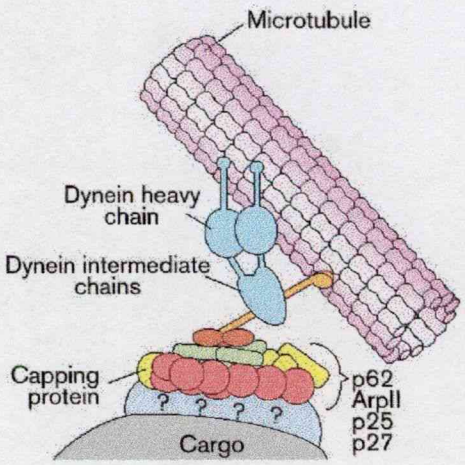


Figure 7. Model of the dynactin complex and its proposed interaction with microtubules and cytoplasmic dynein (light blue). Dynactin subunits are: 8 or 9 ×Arp1 (actin-related protein 1; pink); 4 or 5 ×dynamitin (green); 2 ×p150 Glued (orange); 2 × p24 (brown); one each of p62, conventional actin, Arp11, capping protein α and ß , p27 and p25 (yellow). ? indicates unknown cargo attacment factors. (Adapted from Allan, 2000)

REGULATION OF DYNEIN MOTOR FUNCTIONS

Dynein motor functions are under exquisite and precise control both in the cytoplasm and the flagellum. How might dynein-based motor activity be controlled? A number of possibilities for which there is some evidence exist including the following: direct phosphorylation/dephosphorylation of the HCs, phosphorylation of ICs and LCs that result in an alteration in motor activity and ligand-induced conformational changes in LCs directly associated with the HCs (King, 2000).

Association of motors with specific cargoes

Mechanisms that account for the targeting of dyneins and kinesins to cargoes may differ. For kinesins, the multiple cellular functions are provided at least in part by multiple kinesin-related heavy chain polypeptides and associated light chains (reviewed by Goldstein, 1993; Moore and Endow, 1996). Sequence differences outside of the motor domain of the kinesin heavy chains contribute to the targeting of distinct kinesins to specific functions, either directly or by association with other proteins. For example, in *Drosophila*, the kinesin-like protein Nod contains a DNA-binding motif in the nonmotor domain that localizes it to chromosomes during female meiosis (Ashfar et al., 1995). The mechanisms that target the dyneins to their cargoes are less clear. The intermediate, light-intermediate, and light chain subunits are located in a position

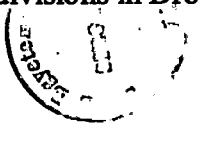
to interact with other cellular components; their assembly and regulation may mediate the targeting of cytoplasmic dyneins to specific cargoes (see review by King, 2000). The different subunits of dynactin are also believed to function as an adapter that allows dynein to bind different cargoes.

CYTOPLASMIC DYNEIN FUNCTIONS

Cytoplasmic dyneins perform a broad range of cellular functions, including chromosome segregation, spindle formation, nuclear migration, Golgi positioning, retrograde membrane transport, and functioning in the endocytic pathway (Karki and Holzbaur, 1999). Only those functions are discussed below, which are important for interpretation of the *Laborc*^{D} phenotype. During the meiotic divisions *Laborc*^{D} results in unfocused spindle poles, chromosome nondisjunction, and over-assembly of the central spindle pole body of the second meiotic spindle in a number of cases. During the cleavage divisions *Laborc*^{D} uncouples the chromosome and the centrosome cycles and leads to centrosome migration, attachment and (probably) centrosome replication defects.

Centrosome separation during interphase

For proper CS separation, centrosomes must move (tightly associated with the nucleus) until they are diametrically opposed on the nucleus. One possible mechanism for centrosome separation is that plus end directed kinesins exert pushing forces on overlapping microtubules emanating from the two centrosomes. In the second mechanism, pulling forces generated by minus end directed motors acting on astral microtubules in front of the moving centrosomes. Dynein could generate such pulling forces by being anchored in the cytoplasm or at the cell cortex. Alternatively pulling forces may result from dynein molecules anchored on the nucleus (Fig. 8). The latter model is attractive because it explains both how centrosomes separate and how they remain tightly associated with the nucleus. In the latter scenario, the minus ends of astral microtubules, along with the centrosome, are pulled when they encounter anchored cytoplasmic dynein on the nucleus. Longer astral microtubules encounter more anchored motors, and thus experience a stronger pulling force than shorter ones. After centrosome duplication, microtubules extending away from the centrosomes along the nucleus are long, whereas those projecting the other centrosome are short. Thus, lenght dependent forces may ensure that daughter centrosomes move away from each other until such pulling forces are balanced, which occurs, when they are diametrically opposed. It has been shown recently, that cytoplasmic dynein is required for centrosome attachment to the nucleus and centrosome migration along the nuclear envelope during the embryonic cleveage divisions in Drosophila (Robinson et al., 1999).



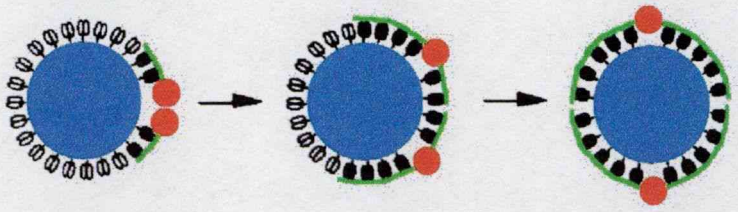


Figure 8. Possible model of dynein-dependent separation of daughter centrosomes. Chromatin: blue, centrosomes: red, MTs: green. Cytoplasmic dynein molecules that interact with astral microtubules are shown in dark shading, others in light shading.

In the *Laborc*^{D} eggs following the first or the second cleavage divisions, the centrosomes detach from the nuclear envelopes showing involvement of cytoplasmic dynein in centrosome anchorage to the nucleus (Belecz et al., 2001; Fig. 3E, F). The detached centrosomes continue replication, nucleate MT asters and start migrating to the egg cortex. By the time the centrosomes reach the egg cortex, they nucleate enormous microtubule asters and about two hours following fertilization the entire cortex of the *Laborc*^{D} egg cytoplasm contains free centrosomes (Fig. 3G, H).

The size of the free centrosomes is normal and they appear to be fully functional because all of them nucleate MT asters. The usually four cleavage nuclei stop dividing. They remain deep down in the egg cytoplasm and degenerate eventually (Fig. 3F). In DAPI stained preparations, fragmented chromatin of the degenerating nuclei appear as giant polyploid nuclei as reported earlier (Erdélyi and Szabad, 1989). It is unclear how *Laborc*^{D} leads to the cessation of the chromosome cycle. Apparently the chromosome and the centrosome cycles are uncoupled in the *Laborc*^{D} eggs implying an involvement of the cytoplasmic dynein in linking the two types of cycles together.

As in wild type fertilized eggs, the blocks on chromosome and centrosome cycles are removed in fertilized eggs of females homozygous for either of the female sterile mutations *plu*, *png* or *gnu* (Fig. 9). In the *plu* and *png* eggs the centrosome cycles cease and only a few asters appear adjacent to the large polyploid nuclei (Shamanski and Orr-Weaver, 1991). The *plutonium* gene encodes a small ankyrin repeat protein with a direct role in coupling S and M phases during cleavage divisions and *pan gu* gene function is required for *plutonium* activities (Elfring et al., 1997). In fertilized *gnu* eggs, however, both cycles proceed and while large polyploid nuclei form inside the egg cytoplasm centrosomes populate the entire egg cortex (Freeman et al., 1986). Evidently harmony of the chromosome and the centrosome cycles is disrupted in the *gnu* eggs. Molecular function of the *giant nuclei* gene is not known. The chromosome and the centrosome cycles can also be uncoupled by aphidicolin (Raff and Glover, 1989).

Laborc^{D} is a new and unique addition to the above mutations. In fertilized *Laborc*^{D} eggs the chromosome replication is released, however while it comes to a standstill after usually two rounds of replication the centrosome cycles proceed as in wild type. Since *Laborc*^{D} is a gain of

function allele of the *Dhc64C* gene, the above observations illustrate involvement of cytoplasmic dynein in establishing harmony of the centrosome and chromosome cycles. Analysis of eggs of double mutant females should reveal the genetic hierarchy among the above genes (Glover, 1991).

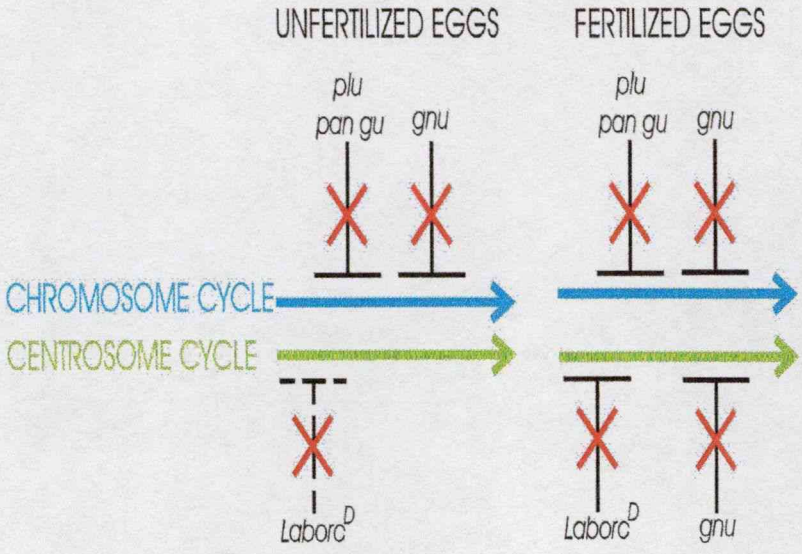


Figure 9. Mutations that eliminate the block on chromosome and/or centrosome cycles in unfertilized or in fertilized eggs leading to the formation of polyploid nuclei and/or free centrosomes that nucleate MT asters. (Dashed line represents the formation of rudimentary centrosomes and MT asters.)

Centrosome separation during cell division

Cytoplasmic dyneins are essential during the separation of the centrosomes in prophase. It appears that by metaphase dynein activity may no longer be required to maintain the separation of the spindle poles: the spindle apparatus, that is composed from bundles of kinetochore microtubules and the overlapping polar microtubules originating from the opposite spindle poles, have already stabilized the spacing of the centrosomes (Vaisberg et al., 1993).

There are several ways in which a minus end directed microtubule motors achieve centrosome separation (Fig. 10). One model for centrosome separation resembles to some extent the situation in axonemes, where ciliary dyneins act between parallel doublet microtubules. If a dynein molecule is temporarily attached to a fixed point on one microtubule while it slides along a microtubule from the other pole, the two closely spaced centrosomes will be forced apart. Such sliding would lead to a gathering of microtubules into the space between the centrosomes, forming a central spindle (Fig. 10A). Other microtubule motors, like the plus end directed kinesins, might then bind to the interdigitating microtubules stabilizing the spindle and further separating the poles (Fig. 10B). Another mechanism for spindle pole separation during anaphase B is when dyneins (anchored to the cell membrane) exert a pulling force on the poles via the astral microtubules (Fig. 10C). The first cleavage spindle in *Laborc*^{D} eggs is shorter normal (Fig 2E) providing *in vivo* evidence for the role of cytoplasmic dynein in centrosome separation during the cleavage divisions.

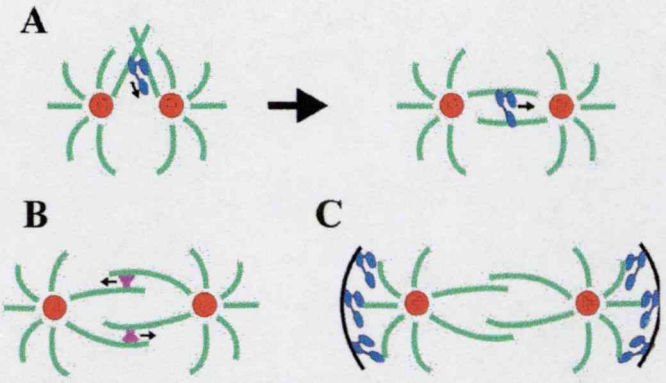


Figure 10. Cytoplasmic dynein's possible roles in daughter centrosome separation during cell division. Dyneins: blue, kinesins: lilac, MTs: green, and centrosomes: red.

Chromosome segregation and the role of dynein at the kinetochre

Cytoplasmic dynein is the only known kinetochore assiciated protein capable of driving chromosome movement towards the centrosomes. However dynein functions at the kinetochores are ambiguous. Immunolocalization of dynein to the kinetochores of tissue culture cells, combined with the analysis of kinetochore microtubule polarity, first suggested a potential role of dynein in providing the force for chromosome movements along the mitotic spindle (Pfarr et al., 1990; Steuer et al., 1990). Functional evidence that supports such a role for dynein is limited to the recent observation in *Tetrahymena* that micronuclear chromosomes fail to segregate in cell lines in which the cytoplasmic dynein gene, *DYH1*, is knocked out (Lee et al., 1999). More recent reports of dynein and dynactin dynamics at the kinetochore suggest that both types of molecules may function to mediate microtubule binding at the kinetochore (Starr et al., 1998; Walczak et al., 1998). However the bulk of the dynein leaves the kinetochore very early in mitosis, soon after the kinetochores begin to attach to microtubules (King et al., 2000). The possible functions of the dynein fraction that left the kinetochores is therefore limited to the initial attachment and movement of chromosomes and/or to a role in monitoring the attachment state of kinetochores. The remaining dynein molecules at the kinetochore may be sufficient for pulling the chromosomes during anaphase to the opposite poles. There are two kinetochore proteins, ZW10 and Rod, which are essential to localize dynein to the kinetochore (Starr et al., 1998). Model for the ZW10/Rod-dependent targeting of dynein to the kinetochore is shown in Fig. 11.

In summary, dynein at the kinetochore can not be uniquely required for chromosome microtubule attachments or movements before anaphase onset. Dynein might participate in the checkpoint mechanisms that sense bipolar tension across the centromere, delaying anaphase onset until all the chromosomes are properly aligned on the metaphase plate. Alternatively, dynein might be required at the kinetochore to supplement and/or coordinate other microtubule motors in moving chromosomes to the poles during anaphase (Sharp et al., 2000). During the

first meiotic division in *Laborc*^{D} eggs in a number of cases nondisjunction of the chromosomes take place (Fig. 1D), supporting that cytoplasmic dynein plays some role in chromosome segregation.

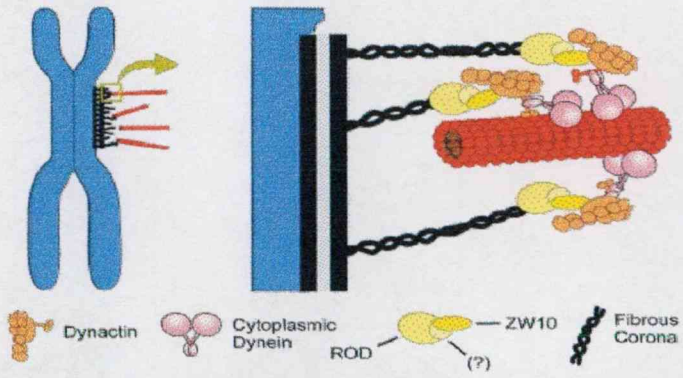


Figure. 11. A complex containing ZW10 and Rod proteins (two kinetochore proteins), as well as potential unknown additional components (?), is associated with the fibrous corona of the prometaphase kinetochore. Direct interactions between ZW10 and the p50 subunit of the dynactin complex then bring dynactin to the kinetochore. Dynactin in turn recruits cytoplasmic dynein to the kinetochore, providing one possible contact between the kinetochore and microtubules. (Adapted from Starr et al., 1998)

Recruitment of centrosome proteins

There is now good evidence for microtubule-dependent (Kuriyama, 1982; Balczon et al., 1999; this manuscript) and microtubule-independent (Moritz et al., 1998; Khodjakov and Rieder, 1999) mechanisms for the recruitment of proteins into the centrosomes. Dynein-mediated and passive diffusion mechanisms represent parallel pathways for centrosome assembly. It is possible that one pathway predominates over the other in certain biological systems or at different stages of the cell cycle. In embryonic systems, for example, high levels of centrosome proteins (Gard et al., 1990) may be sufficient to drive the initial stages of microtubule-independent recruitment onto centrioles, although dynein-mediated transport becomes a major contributor at later times. Dynein has been shown to transport pericentriolar components to the centrosome during both interphase and mitosis. The dynein-transported molecules include dynactin, γ-tubulin and pericentrin during interphase, dynactin and NuMA during mitosis (Quintyne et al., 1999). The *Laborc*^{D} mutation revealed cytoplasmic dynein dependent transport of centrosomal components during the assembly of the central spindle pole body of the second meiotic spindle, and in unfertilized eggs, after the completion of meiosis.

The Laborc^{D} *mutation leads to enlargement of the central spindle pole body of the second meiotic spindle*

The meiosis II spindle of Drosophila oocytes is distinctive in structure, consisting of two tandem spindles with anastral distal poles and an aster-associated spindle pole body between the central poles (Fig. 1B). Assembly of the anastral/astral meiosis II spindle occurs by reorganization of the

meiosis I spindle, without breakdown of the meiosis I spindle (Endow and Komma, 1998). The unusual disk- or ring-shaped central spindle pole body forms de novo in the center of the elongated meiosis I spindle, followed by formation of the central spindle poles. γ-Tubulin transiently localizes to the central spindle pole body, implying that the body acts as a microtubule nucleating center for assembly of the central poles. Localization of γ-tubulin to the meiosis II spindle is dependent on the kinesin like minus end directed microtubule motor protein, Nonclaret disjunctional (Ncd; Endow and Komma, 1998). The central spindle pole body of the second meiotic spindle in *Laborc*^{D} eggs is significantly larger than in wild type (Fig. 1E). Altough this phenotype is rare (2 out of 10), it indicates the redundant involvement of cytoplasmic dynein in the assembly of the central spindle pole body of the second meiotic spindle, and suggests that the *Laborc*^{D} encoded protein transports more than usual CP190 into the central spindle pole body. We suppose, that the *Laborc*^{D} encoded protein cannot respond properly to a negative regulatory signal which results in enlargement of the central spindle pole body. The negative regulatory signal may prevent dynein molecules to assemble more than usual centrosomal components to the central spindle pole body. Because after oocyte activation translation of maternally provided mRNA-s commences the above mentioned negative regulatory signal must already be present at the time when the second meiotic division progresses.

The tandemly oriented second meiotic spindles detach from the central spindle pole body showing that dynein (probably with other minus end directed MT motors, such as Ncd) is required to anchor the second meiotic spindle poles to the central spindle pole body. Roles for cytoplasmic dynein in the assembly of the central spindle pole body and in the attachment of the inner spindle poles to the central spindle pole body has not been shown in Drosophila previously. In addition to the pericentriolar components dynactin, γ-tubulin, pericentrin, dynactin and NuMA which have been previously shown to be transported by dynein to the MTOC-s, our results suggest that CP190 is also transported actively by cytoplasmic dynein to the central spindle pole body of the second meiotic spindle.

In unfertilized Laborc^{D} *eggs centrosomes with incomplete centrioles assemble*

During the development of fertilized eggs, centrosome inheritance must be precisely controled because if both gametes contribute functional centrosomes, the zygote will have an abnormal spindle. In Drosophila, as in most animal species, egg cells do not carry centrosomes that are lost during oogenesis (Schatten, 1994). Most centrosome/centriole components are maternally supplied and are dispersed in the egg cytoplasm (Schatten, 1994). Eggs are activated while they travel form the ovaries through the oviduct: the two meiotic divisions are completed and

translation of the maternally provided mRNAs commences (Foe et al., 1993). In absence of fertilization a thus far unknown mechanism prevents both chromosome replication and centrosome formation.

Centrosomes of the embryos derive from the sperm that introduces during fertilization a centriole pair (Foe et al., 1993). The centriole replicates and, as generally believed, recruits centrosome components from the egg cytoplasm to form functional centrosomes.

In *Drosophila*, some of the centrosome components assemble upon egg activation and form the central spindle pole body of the second meiotic spindles, irrespectively of fertilization (Puro, 1991; Riparbelli et al., 1997). However, centrosomes never form in the unfertilized wild type eggs and development does not proceed beyond the four haploid nuclei stage (Foe et al., 1993). Contrary to wild type, centrosomes assemble in cytoplasm of the unfertilized *Laborc*^{D} eggs (Fig. 3A). The centrosomes nucleate small asters of MTs. The fact that there are no centrosomes in the mature *Laborc*^{D} oocytes (Fig. 3D) and a few minutes later (after the completion of meiosis) there are lots of centrosomes in cytoplasm of the newly deposited unfertilized *Laborc*^{D} eggs shows that centrosomes did not multiply in an exponential fashion - through repeated replication and separation - as in fertilized wild type and *Laborc*^{D} eggs but the rather assembled instantaneously shortly after egg deposition.

Examination of unfertilized *Laborc*^{D} eggs revealed a previously unknown mechanism that prevents de novo assembly of centrosomes in wild type unfertilized eggs after oocyte activation. Because *Laborc*^{D} identifies the cytoplasmic dynein heavy chain gene, we conclude involvement of the cytoplasmic dynein in the above mechanism. Our hypothesis is that the *Laborc*^{D} encoded proteins cannot respond to a yet unknown negative regulatory signal and assemble centrosomal proteins into centrosomes.

Both rudimentary, characteristic for the unfertilized *Laborc*^{D} eggs, and normal centrosomes appear only in about 1 percent of the fertilized *Laborc*^{D} eggs (Fig. 3C), suggesting that fertilization changes egg cytoplasm chemistry such that centrosome and MT components do not assemble spontaneously but rather „await" for the nucleating activities of the sperm-derived centrosomes.

To find out whether or not centrosomes in the unfertilized *Laborc*^{D} eggs contained centrioles we carried out an ultrastructural analysis. Embryonic centrioles posess nine doublet MTs, together with an internal structure called the cartwheel (Debec et al., 1999, Fig. 4A). In the unfertilized *Laborc*^{D} eggs apparently incomplete centrioles form, and their number appears similar to the number of the centrosomes detected by immunofluorescence (Fig. 4B). However the centrioles are rudimentary: the central carthweel of the centriole appears normal in size and

organization and the MT doublets are missing. The presence of rudimentary centrioles suggest the role of cytoplasmic dynein in centriole assembly.

There are a few Drosophila genes known that are engaged in preventing chromosome replication in the unfertilized wild type eggs (Fig. 9). Replication begins when the block is removed as in eggs of virgin females homozygous for either of the maternal effect lethal mutations *plutonium* (*plu*), *pan gu* (*png*) or *giant nuclei* (*gnu*) (Shamanski and Orr-Weaver, 1991; Freeman and Glover, 1987). However, the block on the centrosome cycles is not released in unfertilized eggs of the above mutant females. The mechanism of replication block release is not clear. $Laborc^{D}$ is the first mutation in Drosophila that eliminates the block imposed on centrosome assembly. Assembly of the centrosome constituent proteins CP190 and CNN showed that centrosomes form in the unfertilized $Laborc^{D}$ eggs without releasing the block on chromosome replication. The formation of centrosomes suggests that dynein is involved in the mechanism that prevents de novo centrosome assembly in unfertilized wild type Drosophila eggs. The assembled centrosomes, however, possess unique features. (i) They assemble instantaneously and not through successive rounds of the centrosome cycles. The process is most likely related to the $Laborc^{D}$-encoded mutant dynein molecules that can not respond to a centrosome-assembly-prevention signal and hence organize centrosomes. Nature of the signal is unknown. The complex nature of dynein composition and function provides a wide array of possibilities. Understanding the molecular nature of $Laborc^{D}$ may shed light on the interacting partner(s). (ii) The centrioles are incomplete and only the central core region, the so called cartwheel forms, suggesting requirement of the sperm introduced components in centriole MT doublet formation. (iii) The assembled centrosomes organize asters of MTs. However, the MT asters are reduced in size and their rudimentary appearance may be related to the incomplete centrioles.

Dynein in spindle assembly

The general view that centrosomal microtubule organizing centers are essential features of spindle assembly and organization has been questioned recenly. Several examples of meiotic spindles, as well as early embryonic mitotic spindles in animals, have been known to exist without centrosomes, displaying a spindle morphology that is more reminiscent of a barrel shape and lacking astral microtubules. Also, many plant cells are devoid of morphologically recognizable centrosomes. The concept of centrosome-free spindle pole formation depending on the action of microtubule motors was directly demonstrated (Heald et al., 1996). The establishment of spindle bipolarity without centrosomes involves two independent mechanisms (Fig. 12/2). The first is sorting of microtubules into a bipolar axial array, which may be achieved

by plus end-directed, multimeric motors that can promote anti-parallel microtubule sliding and axial alignment. Candidates for such an activity are the tetrameric motors of the BimC kinesin family (Kashina et al., 1996). The second is the bundling of the oriented microtubules into poles, involving the minus end-directed, microtubule motor cytoplasmic dynein. Live observations of meiotic spindle formation in Drosophila oocytes (Matthies et al., 1996) have revealed that the spindles form by an "inside-out" mechanism in which microtubules reorganize around the mass of chromatin (Fig. 12/2). The process may involve the action of chromatin-bound, plus end-directed kinesin-like proteins the chromokinesins (Wang and Adler, 1995, Vernos et al., 1995). With the microtubule minus ends oriented away from the chromatin in the developing spindles, the organization of the microtubules into bipolar spindles may then be achieved by the action of multivalent, minus end-directed microtubule motor complexes that can tether parallel-oriented microtubules into bundles and stabilize converging microtubules into poles (Fig. 12/2). As Matthies et al. (1996) showed, in Drosophila oocytes the process is clearly dependent on presence of the minus end-directed motor Ncd, although there seem to be other motor proteins with redundant functions involved.

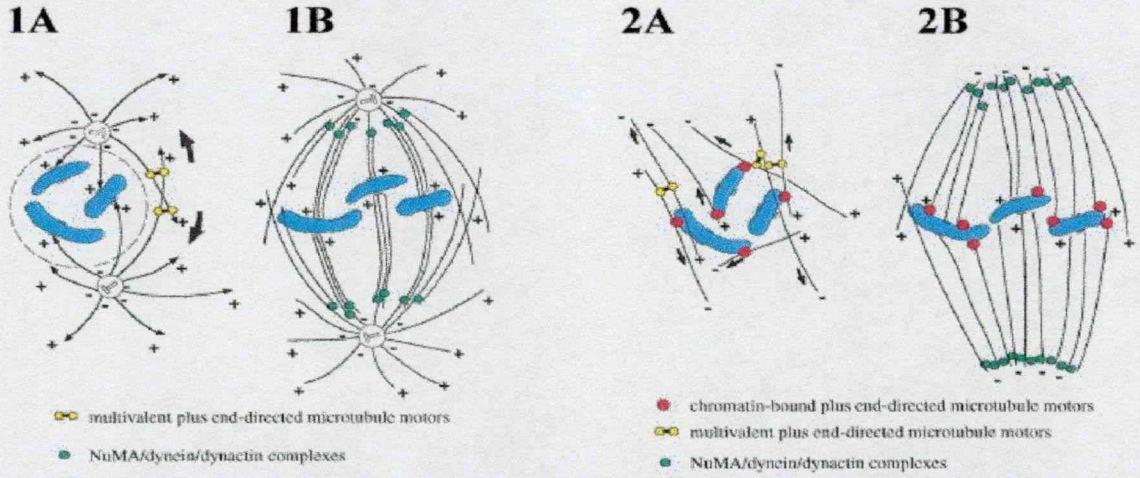


Figure 12/1. Spindle formation in centrosome-containing cells. (A) Microtubules are nucleated from the duplicated centrosomes with their growing plus ends pointing away from the centrosomes. Microtubules are captured by the kinetochores of the chromosomes. Multivalent plus end-directed motors of the BimC family may be involved in the separation of the two centrosomes and the establishment of a symmetric spindle axis *(big arrows)*. (B) In the mature spindle, microtubule minus ends disconnect from the centrosomes and are anchored to the body of the spindle by complexes of NuMA/dynein/dynactin. (The chromosomes are indicated in *blue*.)

Figure 12/2. Spindle formation in centrosome-free cells. (A) Spindle formation is driven by chromatin-associated, plus end-directed microtubule motors, orienting chromatin-attached microtubules with their minus ends outward *(arrows)*. Multivalent plus end-directed microtubule motors of the BimC family can interconnect antiparallel microtubules and establish a bipolar organization of the spindle by moving the microtubule ends apart. (B) During spindle pole formation complexes composed of NuMA, dynein, and dynactin induce convergent arrays of microtubules at the spindle poles and provide stability to the spindle by tethering the microtubule minus ends. (Adapted from Merdes and Cleveland, 1997)

Recent reports indicate that the small GTPase Ran (Ras-like nuclear protein), which plays a key role in nuclear transport, also has a role in microtubule nucleation and in spindle assembly (Kahana and Cleveland, 2000, Fig. 13). Ran in its GTP-bound form promotes the formation of microtubules. Following nuclear envelope breakdown, chromatin-bound RCC1 converts RanGDP to RanGTP which stimulate tubulin polymerization and promote spindle organization. Chromosomal influence on spindle assembly is apparent in both centrosomal and acentrosomal cells.

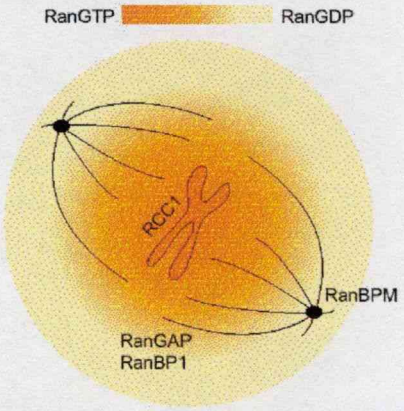


Figure 13. Model showing the proposed effects of chromosomal RCC1 on RanGTP/RanGDP levels in the surrounding cytoplasm. RCC1 converts RanGDP to RanGTP close to the chromosomes, while cytoplasmic RanGAP together with RanBP1 leads to GTP hydrolysis by Ran, generating a gradient of RanGTP that promotes microtubule nucleation and growth towards the chromosomes. (Adapted from Heald and Weis, 2000)

Dynein at the spindle poles

Dynein acts as a microtubule tethering factor at the spindle poles, irrespective of the presence or absence of centrosomes. Microtubule tethering into poles is mediated by a large complex containing NuMA, dynein, and dynactin, using the motor activity of dynein to power the complex toward the microtuble minus ends and the distinct microtubule binding sites on NuMA (Merdes et al., 2000) and the associated p150 dynactin component (Karki and Holzbaur, 1995) to provide the needed crosslinking. Microtubule tethering to spindle poles in centrosomal spindles is needed because up to 75% of the interpolar microtubules do not connect directly to the centrosome but end within a distance of>1 μm thereof (Mastronarde et al., 1993). Moreover, removal of the centrosome by micromanipulation does not grossly affect spindle integrity (Nicklas et al., 1989). A plausible model for what keeps these microtubules in place invokes the NuMA complex, which is distributed in a broad, crescent-shaped area between the centrosome and the spindle microtubule bundles, rather than focused directly at the centrosome (Merdes et al., 2000). NuMA thus is likely to be one of the connecting molecules that anchor the large number of free microtubule minus ends to the microtubules still directly nucleated by the centrosome. In a number of *Laborc*^{D} eggs the first meiotic spindle poles are divergent showing involvement of cytoplasmic dynein in spindle pole focusing (Fig. 1C), which have been

described in other model systems. The above role for cytoplasmic dynein has not been shown in Drosophila female meiosis previously.

CENTROSOME REPLICATION

Analysis of the early celeavage divisions in *Laborc*^{D} eggs suggests new and unexpected cytoplasmic dynein functions in centrosome replication, therefore I briefly discuss recent knowledge about centrosome replication in different systems.

The centrosome cycle in mammalian cells

The cell cycle is an ordered progression of events that leads to division of one cell into two, each with an identical copy of the genome. In normal divisions, both the chromosomal DNA and the centrosome must be duplicated once and only once per cycle. The structure of DNA, with its complementary strands, provides a simple mechanism for duplication. Centrosomes having no DNA must employ some other mechanism of duplication. What happens during centrosome replication (for reviews see Urbani and Stearns, 1999; Zimmermann et al., 1999)? In the G1 phase of the cell cycle, there is one centrosome per cell, consisting of a pair of centrioles and the associated pericentriolar material. The replication process begins at the G1–S transition, at approximately the same time that DNA replication is initiated (Fig. 14). The visible manifestation in the centrosome is that the centrioles move apart from each other (Fig. 14-16). Once separated, new centrioles start to grow orthogonal to the parental centrioles. By G2, there are two centrosomes lying side by side, each with a pair of centrioles within. Centrosome duplication is semi-conservative, in that each centrosome of the duplicated pair has one old and one new centriole. Typical somatic cells must have an existing centriole to create a new centriole, although there are several well-characterized situations in both animal and plant cells in which basal body/centriole formation occurs *de novo*. The lack of a fundamental requirement for an existing centriole suggests that new centrioles are not strictly templated by old centrioles, and it is not known how the very regular structure of the centriole is propagated. At the G2–M transition the duplicated centrosomes move to opposite poles of the nuclear envelope (Fig. 14). Migration of the daughter centrosomes is dependent on the action of microtubule motor proteins (see above). As the nuclear envelope breaks down, microtubules from the centrosomes interact with the chromosomes, and with overlapping microtubules from the opposite pole, creating the bipolar spindle. Segregation of chromosomes, followed by cytokinesis results in daughter cells with a single centrosome. How is centrosome duplication controlled so that it happens at the right time, and happens only once per cell cycle? Recent experiments have shown that cyclin E and its associated kinase Cdk2 are required for centrosome duplication. Activity of the cyclin E–

Cdk2 complex reaches a peak in activity at the G1–S transition, and is also responsible for initiating DNA replication, consistent with the similar timing of DNA and centriole replication. *In vitro* experiments have shown that separation of the centrioles is the first step in centrosome duplication requiring cyclin E–Cdk2 activity, although the relevant substrates of the kinase in centriole separation are not yet known. An interesting difference between DNA replication and centrosome duplication is that DNA replication has a stringent once-and-only-once control that involves a mechanism termed 'licensing', which depends on selective access of replication factors to the DNA. Centrosome duplication seems to be less tightly controlled, as in both embryonic and somatic systems it is possible to have multiple rounds of centrosome duplication within one cycle if the time spent in S phase is extended artificially (Urbani and Stearns, 1999).

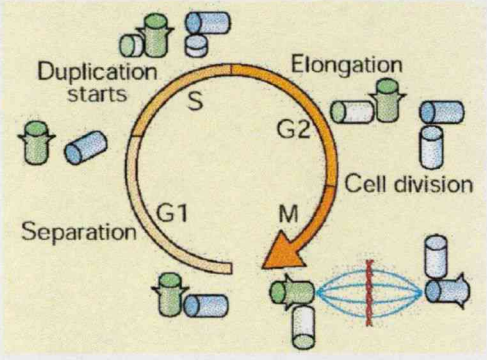


Figure 14. The centrosome duplication cycle. After cell division, each animal cell has one centrosome that contains a pair of centrioles (green and blue). The centrioles separate during the G1 phase. During S phase, a 'procentriole' (light green and light blue) forms at each centriole. The originally small procentrioles grow longer during the G2 phase and migrate to form the poles of the mitotic spindle (bright blue) during mitosis. The cell divides to make two cells that each contain one centrosome. (Adapted from Urbani and Stearns, 1999)

Centrosome cycles during the embryonic divisions in Drosophila

Cell divisions

Distinct steps in the regulation of the postblastoderm centrosome cycles have been described lately (Vidwans et al., 2000; Fig. 15) Activation of Cdc20 *fizzy*-dependent degradation triggers the metaphase-anaphase transition and allows exit from mitosis. As cells progress through mitosis, mother and daughter centrioles disengage and separate and centrosomes split into two. Unlike mammalian cells, each interphase cell contains two centrosomes. The postblastoderm mitoses (of cycles 14 and 15) are followed immediately by the S phase, during which procentrioles form. G2 cells enter mitosis upon developmentally controlled expression of Cdc25 *string* (Widvans et al. 2000). Completion of daughter centriole assembly occurs as cells progress to metaphase. Cdc25 *string* is required for completion of daughter centriole assembly, Cdc20 *fizzy* is required for timely centriole disengagement and mitotic cyclin/Cdk1 needs to be downregulated for procentriole formation (Widvans et al. 2000).

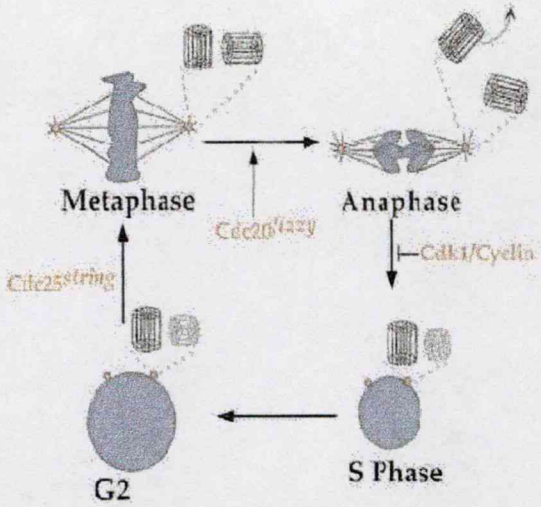


Figure 15. The centrosome cycle of the postblastoderm Drosophila embryo. Chromatin: blue, centrosomes: red, and centrioles as segmented cylinders. (Adapted from Widvans et al. 2000)

Cleavage divisions

The centrosome cycle of the early Drosophila embryo is characterized by precocious separation of the centrioles before their replication (Fig. 16). This peculiarity is perhaps a characteristic feature of the syncytial divisions, which consist essentially of rapid alternate M and S phases (Foe at al., 1993). In a typical somatic cell division cycle, nucleation of the daugter centriole usually precedes the final separation of the parent centrioles (that is, the duplication of the centrosome as observable at the optical level). The most reliable and detailed description of the centrosome cycle in the syncytial Drosophila embryo has been done by Debec et al. (1999) who used energy filtering transmission electron microscopy. During the first part of mitosis, and until metaphase, the two centrioles are attached to each other by a fibrillar link. During anaphase, the link disappears and the two centrioles start to separate. During late telophase, the two separated centrioles bind to the newly formed nuclear envelope. Duplication of the centrosomes occurs concomittantly. The separation and the migration of the two centrosomes, then constituted of only one centriole, takes place during most of interphase. During interphase the centrosomes are attached to the nuclei by a fibrogranular connection. The duplication of the centrioles occurs only at the end of interphase. The two centrosomes that lie at opposite poles of the nuclei are then composed of two centrioles, and the connections with the nuclei disappear. Soon after, the mitotic spindle is established with a centriolar duplex at each pole. The speed of the syncytial divisions allow a very short time, a few minutes, to assemble a daughter centriole, which is remarkable for such a complex molecular structure. With an interphase consisting only S phase, the early Drosophila embryo has to generate and separate two centriolar duplexes in a minimum of time. If the signal triggering the duplication of the centrioles is generated during S phase, a precocious separation of the parent centrioles may reduce the generation time of the new centrosomes. The singularity of the centrosome cycle in the early Drosophila embryo can then be

related to the extreme shortening of the interphase. As described below, *Laborc*^{D} probably affects centrosome replication during the early cleavage divisions.

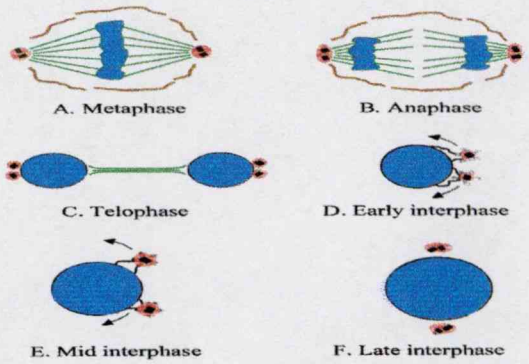


Figure 16. The centrosome cycle of the early Drosophila embryo. Chromatin: blue, centrosomes: red, centrioles: black rectangles, spindle envelope: brown, and MTs: green. (Redrawn after Debec et al., 1999)

Laborc^{D} *results in the formation of gonomeric spindles with multiple centrosomes*

In fertilized *Laborc*^{D} eggs the first cleavage mitosis (that is gonomeric in Drosophila) forms as in wild type with one centrosome at each spindle pole (Fig. 3A; Foe et al., 1993). In wild type the centrosomes do not replicate prior to completion of mitosis (Foe et al., 1993). In fertilized *Laborc*^{D} eggs, however, multiple centrosomes developed at the spindle poles suggesting that the centrosomes begin to replicate prior to accomplishment of mitosis (Belecz et al., 2001). Although the centrosomes are sometimes smaller and variable in size than normal, they are probably functional since they nucleate MTs. Analysis of the first cleavage spindles suggests previously unknown involvement of the cytoplasmic dynein in the prevention of centrosome replication and/or in the coupling of chromosome and centrosome cycle. Which of the following possibilities lead to the formation of multiple centrosomes at the poles of the first cleavage spindle is not clear at present.

(i) *Precocious replication of the centrosomes.*

We propose that the multiple centrosome phenotype shed light on an unknown mechanism that prevents centrosome replication prior to completion of mitosis. Cytoplasmic dyneins with *Laborc*^{D}-encoded mutant heavy chain do not respond to a negative control on centrosome replication, allow and perhaps even contribute to centrosome replication before completion of the cleavage cycle. It is generally believed, that proper centrosome replication requires the replication of centrioles. Therefore detection of centrioles at the *Laborc*^{D} gonomeric spindle poles would be crucial to prove this possibility. Unfortunately there are no Drosophila anti-centriole antibodies available yet. Electron microscopic detection of the centrioles at the gonomeric spindle poles would be feasible theoretically to elucidate the former possibility. Altough the size of the multiple centrosomes is uneven, at later stages all the centrosomes

become identical, normally shaped and probably functional suggesting the presence of centrioles in all of the *Laborc*^{D} centrosomes.

(ii) *Assembly of extra centrosomes without centrioles.* It is also possible however, that uncontrolled mutant dyneins assemble pericentriolar components without centrioles inside. This hypothesis is supported by the assembly of the oversized central spindle pole body of the second meiotic spindle in *Laborc*^{D} eggs.

(iii) *Centrosome fragmentation.* The amount of pericentriolar components at the spindle poles is more, than required for a normal sized centrosome. Altough assembly of a large centrosome followed by it's fragmentation may cause the fragmented centrosome phenotype, we do not favor this hypothesis, because such oversized centrosomes were never observed, and probably acentriolar centrosome pieces would not be able to nucleate wild type looking MT asters later on.

(iv) *„Perdurance" of oocyte centrosomes.* Because no centrosomes are present in *Laborc*^{D} oocytes (see above) the perdurance possibility can be excluded.

(v) *Uncoupling of the chromosome and the centrosome cycles* along with delayed chromosome cycles or with faster than normal centrosome cycles can in principle also account for the multiple centrosomes at poles of the first cleavage spindle in the fertilised *Laborc*^{D} eggs. The possibility of speeded up centrosome cycle is rather unlikely since centrosomes appear in the egg cortex at about the same time following fertilization as in wild type. Although the exact mechanism of multiple centrosome formation is not clear, the *Laborc*^{D}-associated mutant phenotypes clearly show involvement of cytoplasmic dynein in establishment of harmony between the chromosome and the centrosome cycles. The above detailed new cytoplasmic dynein functions could not have been revealed with loss-of-function dynein mutations, because loss-of-function dynein heavy chain mutations are zygotic lethal (Gepner et al., 1996). With gain-of-function female sterile mutations new functions of essential zygotic genes were elucidated.

6. PERSPECTIVES

The „*Laborc* story" is far from being complete. Determining the nucleotide sequence of the *Laborc*^{D} identified mutant cytoplasmic dynein heavy chain gene could shed light on the nature of *Laborc*^{D}. As discussed above, *Laborc*^{D} is most likely a point mutation. Identification of the point mutation may help to reveal the new – cleavage division restricted – regulator of cytoplasmic dynein (altough identification of a point mutation in the enormous16,6 kb *dhc* gene [cDNA=14,3 kb] will not be easy). Development of Drosophila anti-centriolar antibodies, immunostaining of the *Laborc*^{D} first cleavage spindle should elucidate whether cytoplasmic dynein does indeed have a role in centrosome replication. Alternatively with energy filtering transmission electron microscopy one may determine the exact number of centrioles in the *Laborc*^{D} first cleavage spindle, also a more detailed description of the rudimentary centrioles in unfertilized *Laborc*^{D} eggs may be given. Hopefully technical development of the confocal microscopes will allow real time imaging of the early embrionic divisions, which is not possible at present.

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