Horka^{D}, a chromosome instability causing mutation in Drosophila, identifies the *lodestar* gene and indicates involvement of the LDS protein in metaphase chromatin surveillance

Ph.D. Thesis

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Publications related to the Ph.D. Thesis

Scientific paper

Szalontai, Tamás, Gáspár, Imre, Belecz, István, Kerekes, Irén, Erdélyi, Miklós, Boros, Imre and Szabad, János, *Horka*^{D}, a chromosome instability causing mutation in Drosophila, is a dominant negative allele of *lodestar*.
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SUMMARY

Horka^{D}, a dominant negative mutation of *Drosophila melanogaster*, was found out to (1) bring about female sterility through the induction of chromosome tangling during both oogenesis and the commencement of embryogenesis. (2) *Horka*^{D} induces nondisjunction during spermatogenesis and - through dominant paternal effect - renders the chromosomes unstable such that they tend to be lost in the descending embryos leading to the formation of diplo//haplo mosaics, including the *X*X//*X*0 mosaics, the gynandromorphs. The mutant phenotype suggested involvement of the *Horka*^{D} identified gene of Drosophila in chromosome organization and/or segregation and initiated molecular analysis of the *Horka*^{D} identified gene.

The aim of my Ph.D. work was an understanding of the role of the *Horka*^{D} identified gene. In order to achieve the goals, we first mapped the *Horka*^{D} mutation and confirmed its dominant negative nature. We induced, through P-element mutagenesis of *Horka*^{D}, phenotypic revertant *horka*^{rP} alleles. The *horka*^{rP} alleles were used to (i) precisely locate the identified gene, (ii) to determine the mutant phenotype and (iii) molecularly clone the gene. The genetic and molecular analyses clearly showed that *Horka*^{D} and its revertant alleles identify the *lodestar* (*lds*) gene. Combined genetic, molecular and cell biology efforts revealed that the encoded Lodestar (LDS) protein, which is one of the helicase-related types of the proteins, opens the compacted chromatin during the metaphase/anaphase transition providing thus appropriate condition to remove, by a phosphatase, to the phosphate group from Histone-3-Ser10 allowing thus the decongestion of the chromatin upon the onset of anaphase. It appears that LDS action also establishes accessibility for the reparation enzymes to the DNA. It appears that the LDS protein is a component of the Checkpoint-kinase-2 pathway that ensures the elimination of the nuclei with damaged DNA, and thus LDS is a component of the system that is engaged in the maintenance of chromosome stability and genome stability.

We also determined the nature of the *Horka*^{D} allele and learnt that a single base pair exchange type mutation leads to the formation of the *Horka*^{D} encoded A777T-LDS molecules. The slight stickiness of the A777T-LDS molecules to the DNA leads to all the *Horka*^{D} related defects.

INTRODUCTION

Most of the factors required during early embryogenesis are maternally provided in the animal kingdom and there is very little if any zygotic gene expression during the onset of embryogenesis (DERENZO and SEYDOUX 2004; TADROS and LIPSHITZ 2005). To genetically dissect the commencement of embryogenesis in *Drosophila melanogaster*, there were a number of dominant female sterile (*Fs*) mutations induced and isolated in the "Szabad laboratory" (ERDELYI and SZABAD 1989; SZABAD *et al.* 1989). It was assumed that at least some of the *Fs* mutations that terminate embryogenesis at or shortly after fertilization identify genes with important functions during the commencement of embryogenesis. Understanding their molecular functions well may reveal important features of the beginning of a new life.

Horka^{D} is one of the early-defect-causing *Fs* mutations (ERDELYI and SZABAD 1989; SZABAD *et al.* 1989). Moreover, *Horka*^{D} brings about nondisjunction during spermatogenesis and renders the chromosomes (all but the Y) unstable. The unstable chromosomes tend to be lost in the descending zygotes and thus there is a dominant paternal effect associated with *Horka*^{D} (Szabad et al. 1995). Loss of the unstable chromosomes leads to the formation of diplo//haplo mosaics, including the *X*X//*X*0 female//male mosaics, the gynandromorphs (Fig. 1. SZABAD *et al.* 1995). (*X* stands for an X chromosome labeled with recessive marker mutation(s), X for the *Horka*^{D} derived X chromosome and 0 for lack of the X chromosome.) In fact, *Horka*^{D} has been used as a "tool" to generate genetic mosaics (SZABAD and NOTHIGER 1992{Zallen, 2004 #8; VILLANYI *et al.* 2008).

Horka^{D} has been reported to be a gain-of-function mutation for the following reasons. (1) Following mitotic recombination induced in the *Horka*^{D}/+ female germ line cells, perdurance of the *Horka*^{D}-encoded mutant gene product prevents the manifestation of the *Horka*^{D}-free status of the forming +/+ daughter cells (ERDELYI and SZABAD 1989). (+ stands for the normal allele.) (2) *Horka*^{D} can be reverted to loss-of-function *horka*^{r} revertant alleles (ERDELYI and SZABAD 1989).

To understand the function of the *Horka*^{D} identified gene, we mapped *Horka*^{D} by duplications and not only established its approximate location but also learnt that *Horka*^{D} does indeed belong to the dominant negative (antimorph) class of the gain-of-function mutations. The dominant negative nature of *Horka*^{D} shows that products of the *Horka*^{D} and the normal gene participate in the same process and implies that the normal gene product fulfills essential function during early Drosophila embryogenesis.

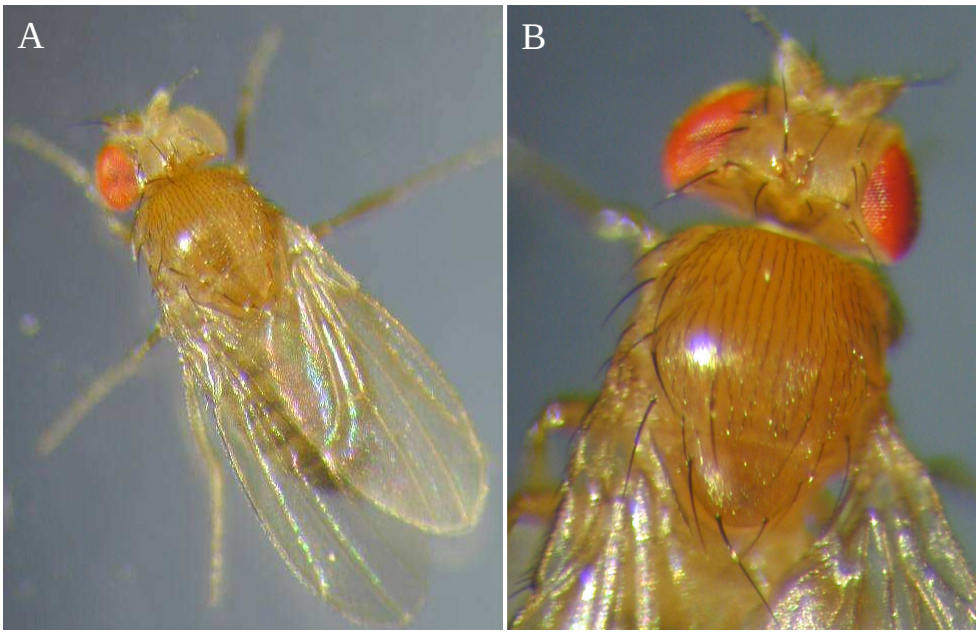


Figure 1. Picture of a *Horka*^{D}-generated gynandromorph (A) and a haplo-4 mosaic (B) Drosophila. (A) The left part of the gynandromorph is female (*X*X) and does not show the mutant phenotypes associated with the recessive marker mutations on the *X* chromosome. Upon loss of the *Horka*^{D}-derived X chromosome during early embryogenesis, phenotypes of the *X*-linked recessive maker mutations (*w*, *white* eyes and *y*, *yellow* body appear in the *X*0 male regions of the gynandromorph (see also Nature Genetics **38**, 613, 2006). Note that the line separating the *X*X female and the *X*0 male regions runs randomly in the different gynandromorphs. (B) While the bristles are normal on the left side of the head and the thorax, they are short and thin on the right side. Cells on the left side carry two of the 4th chromosomes each, and are normal. Cells on the right side carry only one 4th chromosome and show the mutant phenotype of a haplo-insufficient *Minute* mutation.

To elucidate the molecular function of the *Horka*^{D} identified gene we reverted *Horka*^{D} by P-element mutagenesis and generated *horka*^{rP} revertant alleles, one of which (*horka*^{rP2}) is a functionally null allele. Making use of the *horka*^{rP} alleles we determined the position of the locus, characterized the loss-of-function mutant phenotype and cloned the *Horka*^{D}-identified gene. It has turned out - and was confirmed through complementation analysis - that *Horka*^{D} and its *horka*^{r} revertant alleles identify the *lodestar* (*lds*) gene, a member of the Snf2 family of the helicase-related genes (FLAUS *et al.* 2006; GIRDHAM and GLOVER 1991; LIU *et al.* 1998).

Horka^{D} originated through a single base pair exchange mutation: G^{2424}→A. The consequent replacement of Ala^{777} by Tre altered the DNA-interacting protrusion such that the A777T-LDS molecules tend to disturb chromatin organization, chromosome segregation and render the chromosomes unstable.

It appears that the LDS protein is involved in cell cycle progression from meta- to anaphase for the following reasons. (1) Both the LDS and the A777T-LDS protein is present on the meta-, ana- and telophase chromosomes. (2) The mitotic catastrophe features associated with the *Horka^{D}* and the *horka^{r}* mutations are practically identical to those described for the checkpoint kinase mutants (BRODSKY *et al.* 2004; LAROCQUE *et al.* 2007; TAKADA *et al.* 2003; WICHMANN *et al.* 2006). (3) The defects are restricted to the germ line and to the cleavage divisions, as have been reported for several genes engaged in cellular damage surveillance, repair and the spindle checkpoint mutants (MUSACCHIO and SALMON 2007). It appears thus that the LDS protein is one of the several components that are engaged in the maintenance of genome integrity. Instability of the *Horka^{D}* exposed chromosomes may perhaps shed light on the relationship between chromosome instability and the origin of solid tumors as was proposed recently (THOMPSON and COMPTON 2008).

MATERIALS AND METHODS

Horka^{D}, the *horka^{r}* and the *Horka^{RR}* alleles

Horka^{D} was induced by EMS on a 3rd chromosome labeled with the *mwh* and the *e* recessive marker mutations (ERDELYI and SZABAD 1989). For explanation of the genetic symbols see the FlyBase at http://flybase.bio.indiana.edu. The *horka^{r}* revertant alleles were generated through second mutagenesis of *Horka^{D}*: the *horka^{rE1}* allele by EMS (ERDELYI and SZABAD 1989), the *horka^{rP}* alleles through P-element mutagenesis. For the induction of the *horka^{rP}* alleles, dysgenic *Horka^{D}*/*TM3, Sb Ser* males (in which the P-elements were hopping and might have become inserted into the *Horka^{D}* allele) were mated with *TM3, Ser*/*TM1, Me* virgin females. (The dysgenic *Horka^{D}*/*TM3, Sb Ser* males were generated by crossing *M* cytotype *TM3, Sb Ser*/*TM6β, Tb* females with *P* cytotype *Horka^{D}*/*TM3, Sb Ser* males. These males descended from a cross between *P* cytotype *CxD*/*TM3, Sb Ser* females and *Horka^{D}*/*TM3, Sb Ser* males.) Since the *TM3, Sb Ser*/*TM3, Ser* and the *TM3, Sb Ser*/*TM1, Me* combinations are lethal, only the *Horka^{D}*/*TM3, Ser* and the *Horka^{D}*/*TM1, Me* offspring survive. The descending females were mated with their sibling males and screened for offspring production. Only the *horka^{rP}*/*TM3, Ser* and the *horka^{rP}*/*TM1, Me* females give rise to progeny allowing thus a direct selection of the *horka^{rP}* mutant alleles. (To avoid the isolation of clusters of *horka^{rP}* alleles, groups of ten dysgenic males were mated with *TM3, Ser*/*TM1, Me* females and the descendants from the parallel crosses were screened separately.)

The *horka*^{rP} alleles are kept in balanced stocks. The P-element insertion sites in the *horka*^{rP} alleles were determined by a standard *in situ* hybridization technique on salivary gland chromosomes using DIG-labeled P-element DNA probe.

To remobilize the P-elements in the *horka*^{rP} alleles, we constructed *horka*^{rP}/TM3, *Δ2-3* females and males. (*Δ2-3* ensures constitutive production of transposase.) The so-called *Horka*^{RR} alleles (*r*evertant alleles of the *horka*^{rP} revertants) originated most likely through precise excision of the P-element from the *horka*^{rP} allele. The *Horka*^{RR} alleles, which behaved as *Horka*^{D}, were used in *in situ* hybridization studies on salivary gland chromosomes.

The chromosome destabilizing effect of *Horka*^{D} and its revertant *horka*^{r} alleles was analyzed in outcrosses with *y v f mal* females and the extent of chromosome instability was measured through the frequency of *X*X//*X*0, female//male mosaics (gynandromorphs) among the descending *X*X zygotes (cf. (SZABAD *et al.* 1995).

The Drosophila cultures were kept on 25°C.

The *Horka*^{D}/+/*Dp* combinations

To decide about the nature of *Horka*^{D}, i.e. whether it is dominant negative (antimorph) or neomorph and about its approximate location, we constructed *Horka*^{D}/+/*Dp* females and males by crossing *Dp(3;3)/TM3* females with *Horka*^{D}/*TM3, Sb Ser* males. *Dp(3;3)* stands for 18 tandem duplications, which cover - bit by bit - the right arm of the 3rd chromosome. The descending *Horka*^{D}/+/*Dp* females were mated with wild type males and fate of their descending embryos was monitored, the males were mated with *y v f mal* females and the subsequent generation was screened for *X*X//*X*0 mosaics.

Mapping the *horka*^{r} alleles and complementation analyses

To locate the *horka*^{r} alleles and to determine the loss-of-function mutant phenotype, we combined the *horka*^{r} alleles (and also *Horka*^{D}) with *Df(3R)* deficiencies and analyzed the *horka*^{r}/– (and also the *Horka*^{D}/–) flies. (The – symbol stands for any of the deficiencies that remove the *Horka*^{D} identified gene.) The *Horka*^{D}/– and the *horka*^{r}/– hemizygotes were produced by crossing *Df(3R)dsx*^{15}*/TM6β, Tb* females with *horka*^{r}/*TM6β, Tb* or with *Horka*^{D}/*TM6β, Tb* males. To decide whether the *horka*^{r} alleles identify a gene with already existing mutant alleles, we carried out complementation analyses between *horka*^{r} and mutant alleles of the nearby genes.

Immunostaining

To describe the *Horka*^{D} and the *horka*^{r} associated mutant phenotypes, ovaries, testes and eggs/embryos of *Horka*^{D}/+; *Horka*^{D}/– and *horka*^{rP2}/– females and males were dissected and fixed according to Gonzalez and Glover (GONZÁLEZ and GLOVER 1993). The stage 14 oocytes were immunostained according to Tavosanis et al. (TAVOSANIS *et al.* 1997). The eggs and the embryos were prepared as follows. The chorion was removed by Clorox. The dechorionated embryos were fixed in a mixture of 1:1 4% paraformaldehyde : heptane or in a 1:1 mixture of methanol : heptane. The vitelline membrane was removed subsequently by agitation in a mixture of heptane and methanol. To block nonspecific staining, the embryos were incubated in 1% BSA (Sigma) in PBST for 90 minutes at room temperature.

For immunological detection of the microtubules, the DM1A monoclonal anti-α-tubulin antibody was used (1:1000, overnight at 4°C; T6199; Sigma). The LDS protein was detected by polyclonal anti-LDS rabbit antibody raised against the almost complete LDS protein, a generous gift from David Glover's laboratory (GIRDHAM and GLOVER 1991). The anti-LDS antibody was present in the serum from which the non-specific components were depleted through preincubation of the serum in dechorionated and heptane-permeabilized eggs of *horka*^{rP2}/– females, which do not contain LDS protein. The anti-LDS antibody was applied at a 1:200 dilution in 1% BSA in PBST. Centrosomin, a centrosome protein, was detected by the anti-CNN antibody (HEUER *et al.* 1995), the RNA-plymerase by an anti-RNA-polymerase antibody and Histone-3-Ser10P, a marker for the compacted chromatin, by anti-Histone-3-Ser10P antibody.

For immunological detection of the above proteins, the embryos were incubated in secondary antibodies, after treatment with the primary antibodies, for 3 hours at room temperature or overnight at 4°C. The following secondary antibodies were either anti-mouse or anti-rabbit IgG (Sigma) and were labeled with FITC, Texas-Red or Alexa Flour-633. To detect DNA, the embryos were stained with DAPI following incubation with the secondary antibody. Following several rinses in PBST, the embryos and the testes were mounted in Aqua PolyMount (Polysciences Inc). The immunostained preparations were analyzed either in an Olympus IX71 fluorescent microscope with a cooled CCD camera or through optical sections collected in an Olympus FV1000 confocal microscope.

We also prepared and analyzed cuticles of the dead embryos as described in Wieschaus and Nusslein-Volhard (WIESCHAUS 1989).

Cytoplasm injections

To decide about the nature of *Horka*^{D}, we injected about 300 picoliters of cytoplasm (~3% of the total egg volume) from eggs of wild-type (as the control) and also from eggs of *Horka*^{D}/+

females into the posterior region of embryos in which the microtubules were highlighted by Jupiter-GFP and the nuclei by histone-RFP (KARPOVA *et al.* 2006; SCHUH *et al.* 2007). The donor embryos were at the most 30 minutes old and the injected embryos were in the 9^{th}-11^{th} cleavage cycle of embryogenesis. Effect of the injected cytoplasm was followed in time through series of optical sections generated in an Olympus FV1000 confocal microscope. The injections were carried out on 25°C.

Germ line chimeras

To test whether the *Horka*^{D} and the *horka*^{rP2}/– related defects originate from altered function of the germ line and/or the soma, we constructed different types of germ line chimeras through the transplantation of pole cells, embryonic precursor cells of the future germ line. The crosses from which the donor and the host embryos originated are listed in the headings of Table 4. Pole cells were collected from single blastoderm-stage donor embryos and transplanted into two to three host embryos, which were in the same stage. While pole cells do not develop in the embryos of the *tropomyosin-II*^{gs} (*tmII*^{gs}) homozygous females, the somatic cells function normally (ERDELYI *et al.* 1995). *Fs(1)K1237* (also known as *ovo*^{D1}) is an X-linked dominant female-sterile mutation (KOMITOPOULOU *et al.* 1983; PERRIMON 1984). Although the *Fs(1)K1237*/+ host females do not produce eggs of their own, their soma provides normal environment for development of the received female pole cells. We also transplanted pole cells of *y v f mal* embryos into *Horka*^{D}/+ and *horka*^{rP2}/– hosts and analyzed the developing adults for the presence of the implanted *y v f mal* germ line cells. The flies that developed following pole cell transplantation were mated with appropriate partners (as described in Table 4) and tested for germ line chimerism.

Inverse PCR

To clone the gene identified by *Horka*^{D} and its revertant *horka*^{rP} alleles, we made use of the inverse PCR technique and amplified DNA sequences flanking the P-elements in three of the *horka*^{rP} alleles. Briefly, we isolated DNA from *horka*^{rP} carrying males, digested the DNA with *Hin*PI or with *Msp*I. The digested genomic DNA was ligated overnight at 4°C, ethanol precipitated and resuspended in distilled water. There were two PCR reactions conducted next. In the first one, the outward primers were designed based on the terminal sequences of the P-element adjacent to the cut site. (The primers are summarized in the Table 1.) Since the first PCR did not usually yield sufficient amounts of DNA for sequencing, a second so-called nested PCR reaction was conducted using primers complementary to slightly more interior sequences in the P-element (as described in the Appendix). Products from the second PCR reactions were

isolated, purified and sequenced. In the sequencing reactions, we used the primers underlined in the Table 1. Sequencing was carried out by the dideoxy method in an IBI automated sequenator on both strands. Site of the sequences in the genome were determined based on the Drosophila genome sequence (ADAMS *et al.* 2000).

Table 1. Primers used in the inverse-PCR reactions

Enzyme	Nature of the primer	First PCR reaction	Second PCR reaction
*Msp*I	Forward outer	5'ACTGTGCGTTAGGTCCTGTTCATTGTT3'	5'GATACTGAAGAATGGTGGACAAAGAG3'
	Reverse outer	5'CACCCAAGGCTCTGCTCCCACAAT3'	5'CTCCAGTCACAGCTTTGCAGCA3'
*Hin*6I	Forward outer	5'ATACTATTCCTTTCACTCGCAC3'	5'GCATACGTTAAGTGGATGTCTC3'
	Reverse outer	5'TGTCGTATTGAGTCTGAGTGAG3'	5'TGATTAACCCTTAGCATGTCCG3'

Note: the underlined primers were used to sequence the PCR products.

Molecular cloning and the sequencing of *Horka^D*

DNA of *Horka^D*/– and *mwh e* (as the control) males served as template in a set of PCR reactions to produce DNA fragments for sequencing. The PCR primers were designed based on the *lds* gene sequence available in the EMBL nucleotide sequence database under the accession no. X62629. Sequencing of the PCR products was carried out by the dideoxy method in an IBI automated sequenator on both strands.

Constructing the *Horka$^+$* (*TG$^+$*) and the *Horka^D* (*TG^{HD}*) transgenes

To characterize the *Horka^D* identified gene, we generated a *Horka$^+$* transgene (*TG$^+$*) in which a 5.1 kb genomic segment included both the regulatory and the structural parts of the *lds* gene (see Fig. 8). The appropriate 5107 bp genomic sequence was cloned into the *CaSpeR* vector with the *mini-white* marker gene and a germ line transformant transgenic line was generated on *w^{1118}* background by standard procedures. The *TG$^+$* transgene become inserted into the 2nd chromosome and is inherited as a single Mendelian trait. The *TG$^+$* transgene was combined - in appropriate genetic crosses - with *Horka^D* (the *TG$^+$*; *Horka^D*/+ flies), with the *horka^r* (the *TG$^+$*; *horka^r*/– flies) and with the *lds* mutant alleles to determine whether the *TG$^+$* transgene can overcome the mutant phenotypes associated with the *Horka^D* and with the *horka^r* alleles.

To generate transgenes that carry *Horka*^{D} (*TG*^{HD} transgenes), we PCR amplified a 5107 and a 5499 bp genomic segment that included the promoter and also the structural parts of the *Horka*^{D} allele (see Fig. 8). The two transgene types correspond to the two *lds* mRNAs that differ by about 500 nucleotides in their 3' UTR ((GIRDHAM and GLOVER 1991). The DNA was isolated from *Horka*^{D}*/Df(3R)dsx*^{15} males. Stable germ line transformant lines were generated through standard procedures. The *TG*^{HD} lines are kept as the dominant female-sterile mutations.

Constructing transgenes that encode CFP- or RFP-tagged LDS or A777T-LDS

To make the LDS and the *Horka*^{D} encoded A777T-LDS protein visible in confocal optical sections, we generated - using standard techniques - *TG*$^{CFP\text{-}LDS}$ *TG*$^{CFP\text{-}HD}$ *TG*$^{LDS\text{-}RFP}$ *TG*$^{HD\text{-}RFP}$ transgenes, which encode the formation of CFP (cyan fluorescent protein) or RFP (red fluorescent protein) tagged LDS or A777T-LDS chimeric molecules. The CFP-tags are fused the N, the RFP to the C terminus of the LDS and A777T-LDS chimeric molecules (see Table 5).

RESULTS

***Horka*^{D} is a dominant negative mutation. Its mutant phenotype suggests involvement of the normal gene product in chromosome organization, stability and/or segregation**

Although the *Horka*^{D}/+ females deposit normal numbers of normal looking eggs (that are fertilized as in wild type) the cleavage divisions do not commence inside over 90% of their eggs, and when they do at the most a dozen or so scattered chromosomes appear along with unusual microtubule bundles (Fig. 2). Cuticle fragments never form inside the eggs of the *Horka*^{D}/+ females (Table 2). Abnormal segregation of the chromosomes is apparent already during both the first and the second meiotic divisions, as visualized in every of the egg primordia and in the freshly deposited eggs of the *Horka*^{D}/+ females (Fig. 2). In "weak" conditions, where *Horka*^{D} is present in a transgene, the *Horka*^{D} encoded and RFP-tagged mutant protein molecules highlight all the chromosomes and also the chromatin bridges that interconnect the interphase nuclei (Fig. 3). The mutant phenotype suggests an involvement of the *Horka*^{D} identified normal gene product in chromosome organization, stability and/or segregation.

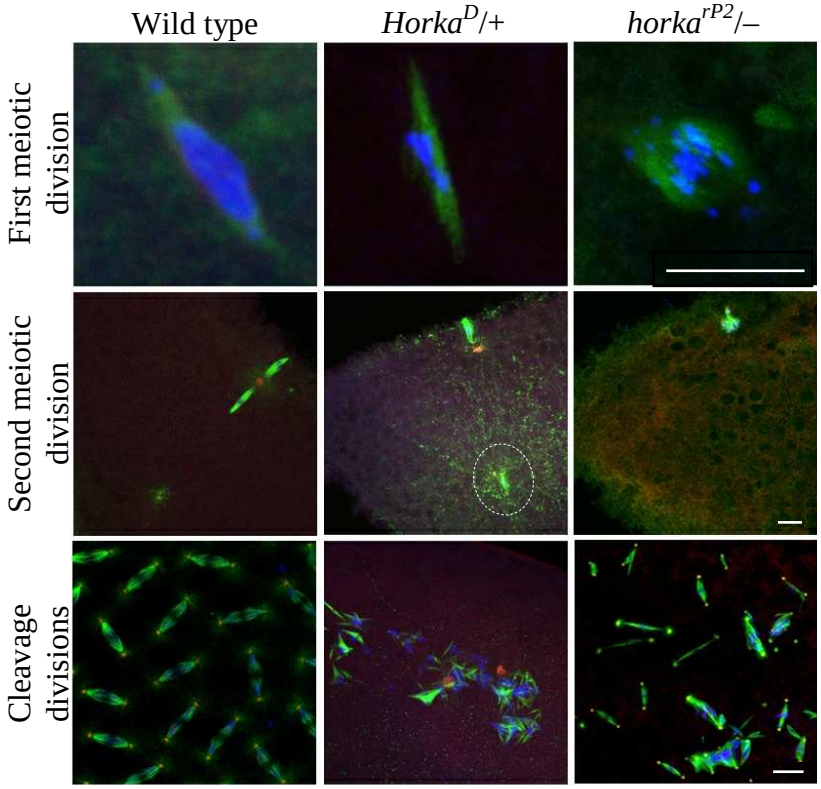


Figure 2. Meiotic and cleavage divisions in the wild type, *Horka*^{D}/+ and *horka*^{rP2}/– females. In the optical sections the microtubules appear in green, the centrosomes and the spindle pole bodies in red and the DNA in blue. Detachment of one of the spindles (encircled) is a typical feature of the second meiotic division in the *Horka*^{D}/+ females. Although the meiotic divisions proceed in most cases as in wild type, abnormal meiotic spindles develop in a few percentages of the egg primordia of the *horka*^{rP2}/– females. Note that most centrosomes can not nucleate astral microtubules and the spindles are abnormal in embryos of the *horka*^{rP2}/– females. Scale bars = 10 μm.

Horka^{D} has been reported to be a gain-of-function mutation (ERDELYI and SZABAD 1989), that was confirmed in the following experiment. When small cytoplasm samples were taken from newly deposited eggs of the *Horka*^{D}/+ females and injected into embryos in which the chromosomes were highlighted by RFP-tagged histone and the microtubules by GFP-tagged tubulins, the injected cytoplasm induced chromosome tangling during ana- and telophase, the formation of chromatin bridges, abnormally shaped and positioned nuclei (which usually drop inside the egg cytoplasm during the upcoming cleavage mitosis) and free centrosomes (Fig. 4). When injected into the vicinity of meta-, ana- or telophase cleavage nuclei, the *Horka*^{D}/+ derived egg cytoplasm exerted its effects instantaneously implying that the chromosome tangling was induced by the *Horka*^{D} encoded mutant protein. Toxicity of the *Horka*^{D} encoded mutant protein

is best shown by the fact that not a single embryo ever hatched following the injection of the *Horka*^{D}/+ derived egg cytoplasm. (Injection of wild type egg cytoplasm into the histone-GFP embryos did not alter progression of the cleavage cycles and larvae hatched from almost every of the injected embryos.) The present findings along with a formerly reported feature of *Horka*^{D}, i.e. it induces nondisjunction and renders the chromosomes unstable during spermatogenesis (SZABAD *et al.* 1995), initiated the molecular analysis of the *Horka*^{D} identified gene.

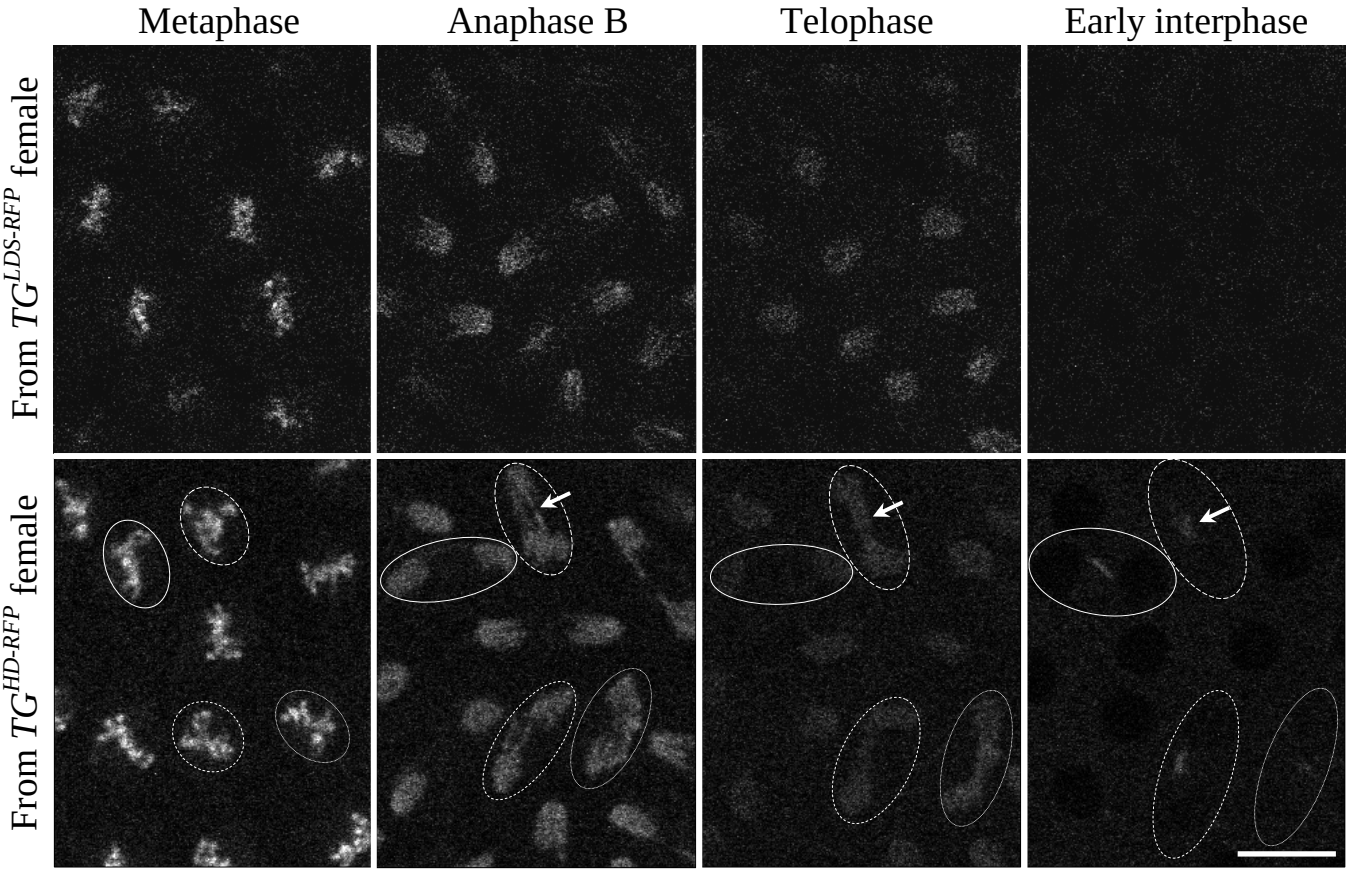


Figure 3. Abnormal metaphase plates, tangling chromosomes and chromatin bridges appear in all the cleavage embryos of the females that carry a $TG^{HD\text{-}RFP}$ transgene besides to the two normal alleles. The $TG^{HD\text{-}RFP}$ transgene contains the *Horka*^{D} mutation and encodes the formation of A777T-LDS-RFP molecules. Fate of four encircled "metaphase" nuclei are shown in four successive time lapse optical sections taken form the same live embryo. Arrows point to some of the tangling chromosomes and the chromatin bridges. Chromosome or chromatin abnormalities never appear in embryos of the females that carry a control $TG^{LDS\text{-}RFP}$ transgene, which encodes the formation of LDS-RFP molecules. Note that the nuclei, which are interconnected with the chromatin bridges are usually blurred. Scale bar = 10 μm.

Table 2. Features of the *Horka*^{D} and the *horka*^{r} carrying females and males

Genotype	Females					Males[c]		
	Tested	Test period[a]	Dead embryos with cuticle (%)	Offspring	Rate of offspring production[b]	*X*X	*X*X//*X*0 mosaic	
							Total	%
Horka^{D}/+	851	16.3	0	0	-	4304	432	9.1
Horka^{D}/*Dp(3;3)*^{d}	1704	8.8	0	0	-	-	-	2.1 - 28.8
Horka^{D}/*Dp(3;3)Antp*^{rv8}	261	18.0	100	3	6.4 x 10^{-4}	116	20	14.7
Horka^{D}/*Df(3R)dsx*^{15}	161	19.5	0	0	-	178	14	7.3
Horka^{D}/*lds*$^{98.1}$	310	20.7	0	0	-	276	2	0.7
TG^{+}; *Horka*^{D}/+	258	19.2	93	0	-	363	14	3.7
+/+; *TG*$^{HD5.1}$	147	16.7	28	0	-	167	13	7.2
+/+; *TG*$^{HD5.5}$	188	15.5	25	0	-	246	8	3.2
horka^{r}/*Df(3R)dsx*^{15}	Semi-sterile combinations					1233	0	-
horka^{rP2}/*Df(3R)dsx*^{15}	180	15.2	20.6	-	-	194	0	-
TG^{+}; *horka*^{rP2}/*Df(3R)dsx*^{15}	5	7.0	11	1087	31.1	-	-	-
horka^{r}/*lds*$^{98.1}$	Semi-sterile combinations					2313	0	-
horka^{rP2}/*lds*$^{98.1}$	85	12.3	21.0	-	-	298	0	-
TG^{+}; *horka*^{rP2}/*lds*$^{98.1}$	7	7.0	8	1649	33.7	-	-	-

[a] Average test period per female (days).
[b] Offspring/(female x day).
[c] The XY; *Horka*^{D} carrying males were mated with *y v f mal* females (*XX*) and the *X*X offspring flies were screened for *X*X//*X*0 mosaics
[d] Pooled data from seventeen *Dp(3;3)* tandem duplications except *Dp(3;3)Antp*^{rv8}

Notes
- *lds*$^{98.1}$ is a *lodestar* null allele (GIRDHAM and GLOVER 1991)
- *horka*^{r} represents pooled data for the following revertant alleles *horka*^{rE1}, *horka*^{rP1}, *horka*^{rP3}, *horka*^{rP4}, *horka*^{rP7} and *horka*^{rP9}.

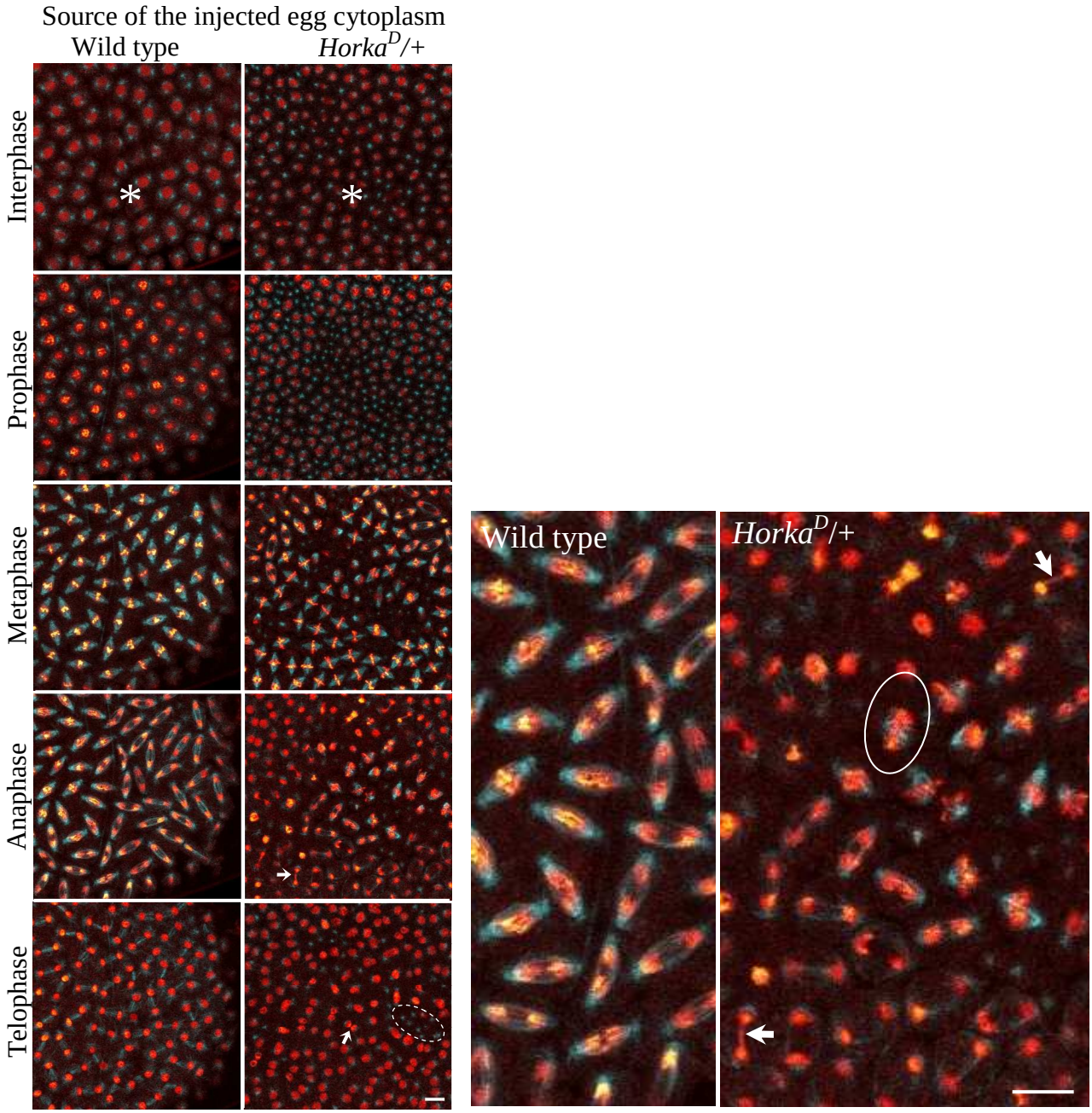


Figure 4. Effect of the injected, *Horka*^{D}/+ derived egg cytoplasm on the cleavage divisions. While injection of wild type egg cytoplasm did not alter progression of the cleavage divisions, the injection of *Horka*^{D}/+ derived egg cytoplasm induced chromosome tangling in ana- and in telophase, abnormal arrangement of the nuclei (most of which drop back inside the egg during the upcoming division, as shown an encircled nucleus on the right side blown up picture), chromatin bridges (arrows) and free centrosomes (encircled with dashed line). The injections were administered at the * sites. The chromosomes were highlighted by histone-RFP and the microtubules by tubulin-GFP. The pictures are time lapse optical sections on live embryos. The two pictures on the right side show higher magnifications of the cleavage nuclei. Scale bar = 10μm.

Horka^{D} resides between 84D5-8 and 85F5-8

Horka^{D} has been mapped to the right arm of the 3rd chromosome (ERDELYI and SZABAD 1989). To more accurately locate *Horka*^{D}, we generated a series of *Horka*^{D}/+/*Dp(3;3)* flies and (1) analyzed the embryos of the females and (2) searched for *X*X//*X*0 mosaics among the offspring of the males. Some of the duplications (labeled *Dp*$^{+}$) might have included a normal (+) copy of the *Horka*^{D} identified gene. Should *Horka*^{D} be a dominant negative mutation (i) less severe mutant phenotype was expected to develop inside eggs of the *Horka*^{D}/+/*Dp*$^{+}$ females (as compared to the *Horka*^{D}/+ control) and (ii) reduced frequency of the *X*X//*X*0 mosaics was expected to appear in the offspring of the *Horka*^{D}/+/*Dp*$^{+}$ males. There were eighteen *Dp(3;3)* tandem duplications used that covered - bit by bit - the entire right arm of the 3rd chromosome. Of the *Dp(3;3)* duplications *Dp(3;3)Antp*^{rv8} eased the *Horka*^{D} imposed defect. Embryogenesis proceeded well beyond the initial steps of embryogenesis inside eggs of the *Horka*^{D}/+/*Dp(3;3)Antp*^{rv8} females and not only cuticle fragments formed in 100% of the eggs but a few offspring descend (Table 2). Results of the analysis located *Horka*^{D} within the 84D5-8 and 85F5-8 cytological interval (Fig. 5) and confirmed the dominant negative nature of *Horka*^{D} implying that the *Horka*^{D}-encoded and the normal gene products participate in the same process such that the mutant gene product impedes function of the normal counterpart.

	Left break point	84 A B C D E F	85 A B C D E F	Right break point	Effect on *Horka*^{D}
DUPLICATION					
Dp(3;3)Antp^{rv8}	84D5-8			85F5-8	Yes
					Effect on *horka*^{rP2}
DEFICIENCY					
Df(3R)p-XT103	85A2			85C1-2	None
Df(3R)dsx^{2D}	84D11			84F16	Yes
Df(3R)dsx^{15}	84D11			84E8	Yes
Df(3R)Antp^{17}	84A5			84D14	None
Df(3R)dsx^{5}	84E1			84F11-12	Yes
Df(3R)CA1	84E12-13			85A6-11	None

Figure 5. Duplication and deficiency mapping of the *Horka*^{D} and the *horka*^{r} mutations. Thick bar represents the *Dp(3;3)Antp*^{rv8} tandem duplication. Missing sections in the *Df(3R)* deficiencies illustrate the eliminated chromosome segments. The deficiencies located the *horka*^{r} identified locus between 84E1 and 84E8.

Whether the *Horka*^{D} related dominant paternal effect is also of dominant negative nature could not be determined since the frequency of the *X*X//*X*0 mosaics varied between 2.1 and 28.8% in the *X*X offspring of the *Horka*^{D}/*Dp(3;3)* males (Table 2). The variation in the *X*X//*X*0 mosaic frequencies is most likely related to the different genetic backgrounds of the males (SZABAD *et al.* 1995).

The *horka*^{revertant} (*horka*^{r}) alleles

Attempts to isolate X-ray-induced revertants of *Horka*^{D} failed. When exposed to 4000 Rad of X-rays, a dose that effectively reverts the dominant female-sterile mutations (ERDELYI and SZABAD 1989) the *Horka*^{D}/*TM3, Sb Ser* males are sterile. This observation reveals the X-ray or perhaps mutagen-sensitivity of *Horka*^{D}, a feature waits for detailed analysis.

To understand the molecular function of the *Horka*^{D} identified gene, we induced *horka*^{rP} phenotypic revertant alleles through P-element mutagenesis of *Horka*^{D}. Any allele that allowed some fertility was classified as revertant. Among the 15,600 females tested nine were fertile and gave rise to a *horka*^{rP} revertant allele each. The nine females appeared in different sub-lines of the screen and thus the *horka*^{rP} alleles originated independently. The previously EMS-induced *horka*^{rE1} (ERDELYI and SZABAD 1989) and the P-element induced *horka*^{rP} alleles are lethal both in homo- and in trans-heterozygous combinations most likely due to second site lethal mutation(s). There were about three such mutations induced on an EMS-mutagenized chromosome along EMS-induction of the dominant female-sterile mutations (ERDELYI and SZABAD 1989).

To characterize the *horka*^{rP} alleles, *horka*^{rP}/*TM3, Sb Ser* males were crossed from each of the *horka*^{rP} lines with *y v f mal* females and the offspring were analyzed for *X*X//*X*0 mosaics. Mosaics appeared (though with very low frequencies) among the offspring of three of the *horka*^{rP} revertants (*horka*^{rP5}, *horka*^{rP6} and *horka*^{rP8}) suggesting that they are partial revertant alleles that kept some feature of *Horka*^{D}. Their partial revertant nature is also shown by the strongly reduced fertility of e.g. the *horka*^{rP5}/+ females. Mosaics did not appear in the offspring of the males that were heterozygous for the either of the six other *horka*^{rP} alleles and larvae hatched from the vast majority of the eggs deposited by e.g. the *horka*^{rP2}/+ females indicating the loss-of-function nature of six of the *horka*^{rP} mutations. The concurrent loss of dominant female sterility and dominant paternal effect in six of the nine *horka*^{rP} alleles shows that the *Horka*^{D} related dominant mutant phenotypes are consequences of the same mutation.

The *horka*^{r} mutations reside within the 84E1-84E8 cytological interval

The *horka*^{r} alleles (and also *Horka*^{D}) were combined with deficiencies that remove well defined parts of the 84D-F cytological region (Fig. 5). The *horka*^{r}/– hemizygous combinations are viable and the *horka*^{r}/– flies develop with the expected frequencies. (The – symbol stands for either of the deficiencies, remove the *horka*^{r}-identified locus; Fig. 5.) The *horka*^{r}/– females are either completely sterile (*horka*^{rP2}/–) or possess reduced fertility and progeny develop only from 4-21% of the zygotes (Table 2). The deficiencies located the *horka*^{r}-identified locus within the 84E1-E8 cytological region.

Fertility of the *horka*^{rP2}/– males is very strongly reduced (Table 3): crosses in which several hundred *horka*^{rP2}/– males were mated with several hundred *y v f mal* females yielded only few offspring, none of which was *X*X/*X*0 mosaic (Table 2).

The *Horka*^{D}/– flies are also viable and emerge with the expected frequencies. The females deposit normal numbers of normal looking eggs in which, although normally fertilized, embryogenesis never commence. Fertility of the *Horka*^{D}/– males is also strongly reduced (Table 3). However, a few offspring derived from a cross between several hundred *Horka*^{D}/– males and *y v f mal* females and 6.7% (14/192) of the *X*X offspring flies were *X*X//*X*0 mosaics (Table 2).

It appears that reduced fertility of the *Horka*^{D} carrying males is also of dominant negative nature since when sired by *Horka*^{D}/–, *Horka*^{D}/+ or by *Horka*^{D}/+/ *Dp(3;3)Antp*^{rv8} males 92, 71 and 59% of the embryos perished during embryogenesis (Table 3). Since there was no sperm inside in practically 100% of the eggs in which embryogenesis did not commence, the reduced fertility of the *Horka*^{D} carrying males is most likely the consequence of abnormal spermatogenesis. Remarkably, the egg production rate of the partner *y v f mal* females was not significantly different from the control (Table 3) and thus *Horka*^{D} does not seem to affect other fertility related features than sperm production suggesting that *Horka*^{D} has little if any effect on the somatic cells (LIU and KUBLI 2003).

The different *horka*^{r}/– and the *Horka*^{D}/– combinations not only located the identified gene within the 84E1-E8 cytological region but confirmed the dominant negative nature of *Horka*^{D} whereby the most severe defects appear in the *Horka*^{D}/– hemizygous condition and decrease along with the increase of the number of the wild type (+) copies. It may be suggested furthermore, since the *horka*^{rP2}/– combination is viable but seriously affects female and male fertility, that function of the identified gene is needed during oo-, spermato- and embryogenesis.

Table 3. Hatching rate of the embryos from *y v f mal* females that were mated with different types of males

Type of experiment	Genotype	Male fertility		
		Eggs/day[a]	Addle eggs (%)	n
Control	*mwh e/mwh e*	34 ± 11	13 ± 4	10
	mwh e/Dp(3;3)Antp^{rv8}	32 ± 13	19 ± 5	13
	mwh e/Df(3R)dsx^{15}	30 ± 4	23 ± 9	9
	TG^{+}; mwh e/+	34 ± 11	9 ± 4	15
	TG^{+}; mwh e/Df(3R)dsx^{15}	31 ± 10	13 ± 6	14
Horka^{D}	*Horka^{D}/mwh e*	28 ± 10	60 ± 16	10
	Horka^{D}/Dp(3;3)Antp^{rv8}	31 ± 9	71 ± 12	11
	Horka^{D}/Df(3R)dsx^{15}	29 ± 8	92 ± 13	10
	TG^{+}; Horka^{D}/+	31 ± 10	67 ± 12	14
	TG^{+}; Horka^{D}/Df(3R)dsx^{15}	30 ± 7	75 ± 20	15
TG^{HD}	*+/+; TG$^{HD5.1}$*	30 ± 9	49 ± 13	18
	+/+; TG$^{HD5.5}$	34 ± 10	48 ± 14	13
horka^{rP2}	*horka^{rP}/mwh e*	35 ± 12	15 ± 7	12
	horka^{rP2}/Dp(3;3)Antp^{rv8}	39 ± 11	12 ± 6	15
	horka^{rP2}/Df(3R)dsx^{15}	30 ± 9	96 ± 10	17
	TG^{+}; horka^{rP2}/Df(3R)dsx^{15}	34 ± 10	16 ± 7	15

[a] Single *y v f mal* females were mated with single males. Egg production and the hatching rates were determined throughout one week. (Average ± standard deviation.)
Notes
- *Horka^{D}* was induced (by EMS) on a chromosome labeled with the *mwh* and the *e* recessive marker mutations.
- *TG^{+}* stands for a transgene (inserted into a 2nd chromosome) with a normal *lds* gene inside.

The loss-of-function mutant phenotype

Of the *horka^{r}*/– combinations *horka^{rP2}*/– is the strongest. Cytological analysis revealed abnormal chromosome segregation already during the first meiotic division in some of the *horka^{rP2}*/– egg primordia (Fig. 2). However, most meiotic divisions appear normal, at least on the cytological level. Similarly, although several of the second meiotic divisions appear normal, abnormal chromosome segregation and unusual spindles are also frequent (Fig. 2).

All the eggs of the *horka^{rP2}*/– females appear normal and are fertilized as in wild type, and although cleavage divisions commence inside about 60% of the eggs larvae never hatch. Once

started, the cleavage divisions proceed more or less normally and cells form sometimes over relatively large areas and differentiate as indicated by the cuticle fragments which form inside about 21% of the eggs (Table 2). Although the cuticle fragments are usually poorly differentiated, however every larval cuticle landmark emerges though in different embryos.

Detailed analysis of the cleavage divisions revealed that although the daughter centrosomes separate appropriately, several of them lose the ability to nucleate microtubules. The centrosome defects lead to the formation abnormal spindles, which then bring about distorted arrangement of the chromosomes, a defect resembling "mitotic catastrophe" (Figures 2 and 6; SIBON *et al.* 2000; TAKADA *et al.* 2003; WICHMANN *et al.* 2006). While the nuclei close to the abnormal centrosomes drop from the egg cortex inside the egg cytoplasm, the centrosomes remain at their original place. Several of the free centrosomes nucleate microtubules during the oncoming cleavage cycles and bring about further disturbances through interfering with the formation of the nearby cleavage spindles. The impaired centrosome function may be related to one or more of the following problems: DNA damage, incomplete replication of the DNA, abnormal chromatin condensation and/or chromosome segregation and thus the loss-of-function mutant phenotype suggests involvement of the *horka*^{rP2} identified gene in the maintenance of genomic integrity.

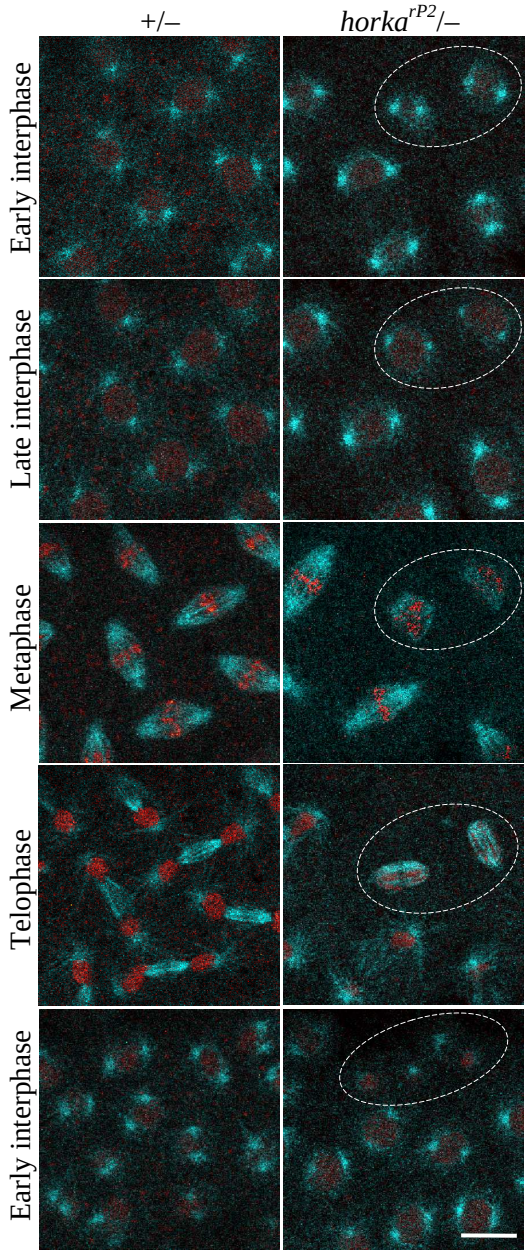


Figure 6. Impaired centrosome function develops in late cleavage embryos of the *horka*^{rP2}/– females. Time lapse optical sections were collected from embryos that derived from +/– (control) and from *horka*^{rP2}/– females. The chromosomes appear in red (were labeled by histone-RFP), the microtubules and the centrosomes in cyan (highlighted by Jupiter-GFP). Nuclei associated with abnormal centrosomes are encircled. Note that while the nuclei drop into the interior of the embryo the free centrosomes remain in the egg cortex. Scale bar = 10 μm.

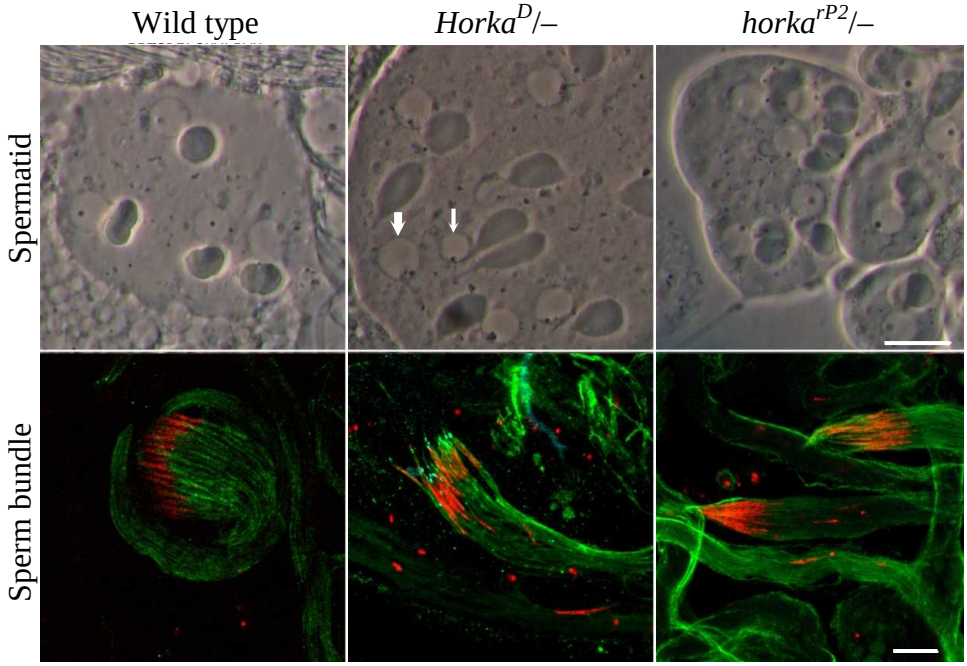


Figure 7. Onion stage spermatid and sperm bundles in wild type, *Horka*^{D}/– and *horka*^{rP2}/– males. White arrows point to nuclei with higher (thick) and fewer than normal (narrow) chromosomes, indicators of nondisjunction (cf. (GONZALEZ *et al.* 1989). In the sperm bundles the nuclei appear red, the sperm tail tubulin green and the LDS protein blue in the acrosomes. Scale bar = 10 µm.

Three characteristic types of defects appear during spermatogenesis in the *Horka*^{D}/– males. (1) Because of nondisjunction, larger-, and smaller-than-normal onion stage spermatid nuclei appear side by side (Fig. 7; SZABAD *et al.* 1995). (2) Several of the sperm nuclei are displaced from their sperm tip position and in fact, a good number of sperm tail bears no nucleus (Fig. 7; SZABAD *et al.* 1995). (3) The anti-lodestar serum identified the *Horka*^{D} encoded mutant protein in the acrosomes of every of the sperm of the *Horka*^{D}/– males, even in those sperm that bear no nucleus or the sperm nucleus has been displaced along the sperm tail (Fig. 7). This signal must be *Horka*^{D} specific as it is missing from sperm of the wild type and from the *horka*^{rP2}/– males.

Although the onion stage spermatid nuclei appear in the *horka*^{rP2}/– males as in wild type, the sperm bundles are far from normal: individualization of the sperm is incomplete, a few of the sperm heads are dislocated and the sperm head is missing from several sperm tails (Fig. 7). Yet some of the sperm are functional since the *horka*^{rP2}/– males are not completely sterile (Table 3).

Germ line chimeras revealed altered germ line function in *Horka*^{D}/+ and in *horka*^{rP2}/–

Viability and sterility of the *Horka*^{D}/+ and the *horka*^{rP2}/– females and reduced fertility of the males suggest requirement of the *Horka*^{D} identified gene only in the gonads. To find out whether function of the gene is required in the germ line or in the somatic components of the gonads, we constructed germ line chimeras through the transplantation of pole cells. First, pole cells of *Horka*^{D}/+ embryos were transplanted into host embryos that did not have pole cells on their own and yet provided normal environment for development and function of the received pole cells

(Table 4A). Three of the female germ line chimeras produced eggs and fate of the embryos inside these eggs was essentially identical to those described for the embryos of the *Horka*^{D}/+ females. There were three sibling male germ line chimeras recovered. They were mated with *y v f mal* females. On the average, 3.1% of their *X*X zygotes developed as *X*X//*X*0 mosaics (Table 4A). Features of the former chimeras clearly show that the *Horka*^{D} related defects originate from altered function of the germ line cells. We also used *Horka*^{D}/+ females and males as host for normal germ line cells. Apparently fully functional germ cells developed form the transplanted pole cells in the *Horka*^{D}/+ environment and offspring derived from the chimeras that carried normal germ line cells (besides their own) and *Horka*^{D}/+ soma (Table 4A). Features of the latter types of germ line chimeras not only revealed the germ line autonomous nature effect of *Horka*^{D} but also the normal function of the *Horka*^{D}/+ gonadal soma.

Table 4A. Features of the *Horka*^{D}/+ germ line chimeras

Cross to produce the donor embryos *mwh e*/*mwh e* ♀♀ x *Horka*^{D}/*TM3* ♂♂ ↓ Cross to produce host embryos *tmII*^{gs}/*tmII*^{gs} ♀♀ x *tmII*^{gs}/*TM6* ♂♂			Stock to produce the donor embryos *y v f mal* ↓ Cross to produce host embryos *mwh e*/*mwh e* ♀♀ x *Horka*^{D}/*TM3* ♂♂		
Genotype of the transplanted pole cells	Germ-line chimera		Genotype of the host embryos	Germ-line chimera	
	Female[a]	Male[b]		Female[c]	Male[c]
mwh e/*Horka*^{D}	3	3	*mwh e*/*Horka*^{D}	4	2[d]
mwh e/*TM3*	8	4	*mwh e*/*TM3*	5	2

[a] The females were mated with *mwh e*/*mwh e* males.
[b] The males were mated with *y v f mal* females.
[c] Mated with *y v f mal* partner
[d] Many more offspring originated from the *y v f mal* than from their own *Horka*^{D}/+ germ line cells.

In the second set of experiments, pole cells of *horka*^{rP2}/− embryos were transplanted into *Fs(1)K1237*/+ host embryos. Of the developing chimeras three carried *horka*^{rP2}/− germ line cells (Table 4B). They deposited normal looking eggs from which larvae never hatched. Cuticle fragments were present in 21% of the eggs, as inside eggs of the *horka*^{rP2}/− females. (See below.) We also transplanted normal pole cells into *horka*^{rP2}/− host embryos and analyzed the developing female and male germ line chimeras. The *horka*^{rP2}/− flies produced offspring from the implanted germ line cells (and only from that source) showing that the *horka*^{rP2}/− soma provides full support for the normal germ line cells (Table 4B). It appears thus that function of the *horka*^{rP2} identified gene is primarily required in the germ line. However, the germ line

chimera results do not exclude function of the gene in the soma under special circumstances. It can furthermore be concluded that the maternally provided normal gene products present in the cytoplasm of the *horka*^{rP2}/+ females make life of the *horka*^{rP2}/– zygotes possible.

Table 4B. Features of the *horka*^{rP2}/− germ line chimeras

Cross to produce donor embryos *horka*^{rP2}*/TM6β* ♀♀ x *Df(3R)dsx*^{15}*/TM3* ♂♂ ↓ Cross to produce host embryos *w/w* ♀♀ x *Fs(1)K1237*/Y ♂♂		Stock to produce donor embryos *y v f mal* ↓ Cross to produce host embryos *horka*^{rP2}*/TM6β* ♀♀ x *Df(3R)dsx*^{15}*/TM3* ♂♂		
Genotype of the transplanted pole cells	Germ line chimera	Genotype of the host embryos	Germ line chimera	
			Female[a]	Male[a]
horka^{rP2}*/TM3*	8	*horka*^{rP2}*/TM3*	2	2
Df(3R)dsx^{15}*/TM6β*	5	*Df(3R)dsx*^{15}*/TM6β*	3	4
TM3/TM6β	1	*TM3/TM6β*	1	2
horka^{rP2}*/Df(3R)dsx*^{15}	3	*horka*^{rP2}*/Df(3R)dsx*^{15}	1	1

[a] The chimeras produced *y v f mal* offspring following test crosses with *y v f mal* partners.

Notes
- Arrows symbolize the direction of pole cell transplantations.
- The chromosome labeled with the *mwh* and the *e* recessive marker mutations is isogenic. *Horka*^{D} was induced on an *mwh* and *e* labeled chromosome by EMS (ERDELYI and SZABAD 1989).

In situ hybridizations confirm that the *Horka*^{D} identified gene resides in 84E

To precisely locate the *Horka*^{D} identified gene, we carried out *in situ* hybridizations in which labeled P-element DNA probe highlighted the P-elements in the salivary gland giant chromosomes in each of the nine *horka*^{rP} revertants. There were three to six P-element insertions in the right arm of the 3rd chromosome in the different *horka*^{rP} alleles. The only common P-element insertion site appeared in 84E suggesting that the *Horka*^{D} identified gene resides in 84E.

That the *Horka*^{D} identified gene does indeed reside in 84E is supported by results of the so-called *Horka*^{RR} experiment. Some of the P-elements were successfully remobilized in the *horka*^{rP7} and the *horka*^{rP9} alleles. As the outcome of P-element remobilization, two so-called *Horka*^{RR} alleles (revertant alleles of the *horka*^{rP} mutations) emerged among the altogether 135 tested chromosomes. The *Horka*^{RR} alleles behaved as *Horka*^{D}: the *Horka*^{RR}/+ females were sterile and embryos perished inside their eggs essentially as described for the *Horka*^{D}/+ females. Among progeny of the *Horka*^{RR}/+ males and *y v f mal* females 13.9% (14/85) and 13.0% (25/164) of the *X*X zygotes developed as *X*X//*X*0 mosaics in the two *Horka*^{RR} alleles. More

importantly, the P-element insertions in 84E were not present in the *Horka*^{RR} alleles making it very likely that the *Horka*^{D} identified gene does indeed reside in 84E. Existence of the *Horka*^{RR} alleles underline the common origin of the *Horka*^{D} related dominant defects.

Horka^{D} and its revertant alleles identify the *lodestar* gene

To molecularly clone the gene identified by *Horka*^{D} and its *horka*^{r} alleles, we made use of the P-elements in *horka*^{rP2}, *horka*^{rP3} and *horka*^{rP9} (that each carry as few as three P-elements inserted into 3R) and the inverse-PCR technique. Starting from the P-element sequences we PCR amplified sequences adjacent to the P-elements in three of the *horka*^{rP} alleles. The PCR products were sequenced and only those were further analyzed that originated from 84E. Apparently, a P-element is present in the same position in the leader sequence coding region of the *lodestar* (*lds*) gene in *horka*^{rP3} and in *horka*^{rP9} (Figures 7 and 8). In *horka*^{rP2}, the P-element is inserted into the open-reading-frame-coding region of the *lds* gene (Figures 8 and 9). The P-element in *horka*^{rP2} brought about a frame-shift mutation that leads to the formation of a four amino acid long nonfunctional product and thus *horka*^{rP2} is a null allele. Positions of the P-elements in the *horka*^{rP} alleles suggest that *Horka*^{D} and its *horka*^{r} revertant alleles identify the *lodestar* (*lds*) gene (GIRDHAM and GLOVER 1991). Indeed, the *horka*^{r} and the *lds* alleles do not complement (Table 2) and thus *Horka*^{D} is a dominant negative *lodestar* allele. The *horka*^{rP2}/*lds*$^{98.1}$ and the *horka*^{rP2}/*lds*$^{298.8}$ combinations are female-sterile and as strong as *horka*^{rP2}/−. The *lds*$^{98.1}$ and the *lds*$^{298.8}$ alleles have been reported to be complete loss-of-function alleles; there is no lodestar protein (LDS) in ovaries of the *lds*$^{98.1}$/− and of the *lds*$^{298.8}$/− hemizygous females (GIRDHAM and GLOVER 1991). The LDS protein is also missing from ovaries of the like *horka*^{rP2}/− females. (Data not shown.) Although embryogenesis proceeds beyond the blastoderm stage inside about 60% of the eggs of the *horka*^{rP2}/−, the *horka*^{rP2}/*lds*$^{98.1}$ and the *horka*^{rP2}/*lds*$^{298.8}$ females and fragments of cuticles appear in about 21% of their eggs larvae never hatch. The females are semi-sterile in all the further *horka*^{r}/*lds* combinations (Table 2).

We crossed several hundred *horka*^{rP2}/*lds*$^{98.1}$ males (that are almost completely sterile) with several hundred *y v f mal* females and none of the recovered 298 *X*X offspring was *X*X//*X*0 mosaic (Table 2). However, *X*X//*X*0 mosaics appeared among offspring of the *Horka*^{D}/*lds*$^{98.1}$ males (Table 2).

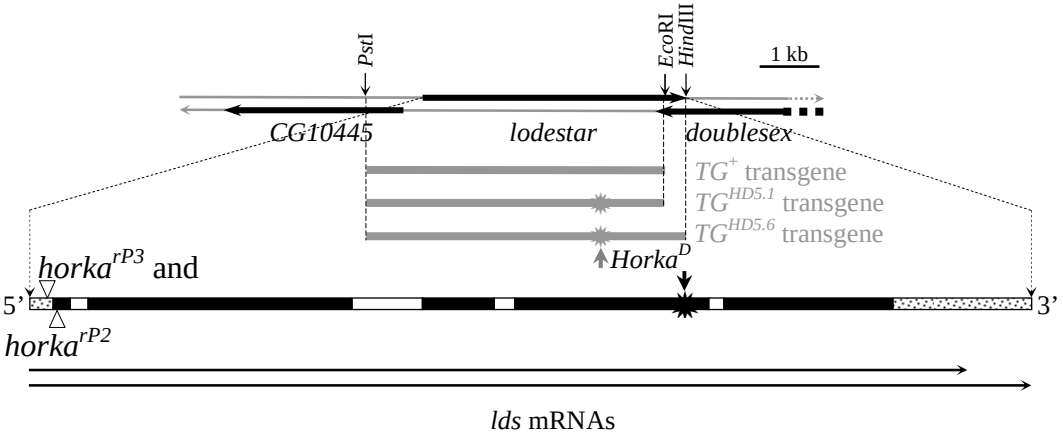
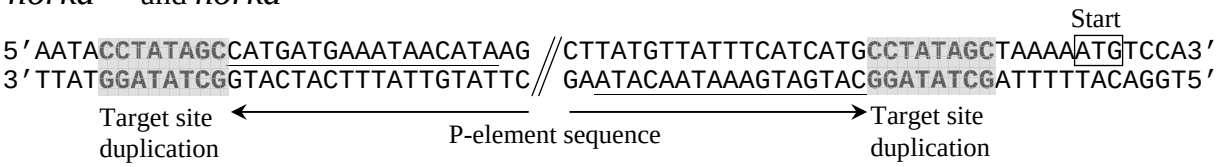


Figure 8. Organization of the region around the *lodestar* gene in the 84E5 cytological region. The *lds* gene encodes the formation of two mRNAs that differ in the last about 500 nucleotides. Dotted boxes correspond to sequences that encode the 5' and the 3' untranslated regions of the *lds* mRNAs, open and dark boxes represent introns and exons, respectively. The P-element insertion sites in *horka*^{rP3} and in *horka*^{rP2} are labeled and the position of the *Horka*^{D} mutation (✹). The grey lines represent the different types of the transgenes.

horka^{rP3} and *horka*^{rP9}



horka^{rP2}

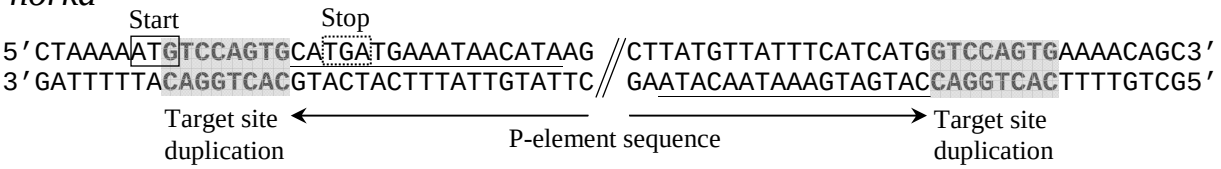


Figure 9. The molecular nature of three *horka*^{rP} alleles. The inserted P-element is bordered by the target site duplications (O'HARE and RUBIN 1983). The ATG start code is enboxed. Parts of the inverted repeat sequences of the P-element are underlined (BEALL and RIO 1997). In the independently induced *horka*^{rP3} and *horka*^{rP9} alleles, the P-element is inserted in the promoter region. In *horka*^{rP2}, the ATG start site is shortly followed by a TGA stop code.

The *TG*$^{+}$ transgene rescues the *lds* mutant phenotype

To show that *Horka*^{D} and the *horka*^{r} alleles do indeed identify the *lds* gene, we generated a stable transgenic line (*TG*$^{+}$, inserted into a 2nd chromosome) that covers a 5.1 kb genomic sequence and includes the normal *lds* gene (except the last 500 bps; Fig. 8). The *TG*$^{+}$; *Horka*^{D}/+ females are sterile and although fragments of cuticle develop inside 93% of their eggs larvae never hatch (Table 2). The *TG*$^{+}$ transgene overcomes sterility of the *horka*^{rP2}/−, the *horka*^{rP2}/*lds* and the *lds*/*lds* females: in the presence of *TG*$^{+}$ the females are fertile, larva hatch from most of their eggs and develop to adulthood (Table 2). In the presence of *TG*$^{+}$, fertility of the *horka*^{rP2}/− and the

horka^{rP2}/*lds*$^{98.1}$ males is essentially as in wild type (Table 3). Evidently, *Horka*^{D} and its *horka*^{r} alleles identify the *lodestar* gene.

Horka^{D} originated through a transition

To decide about the molecular nature of *Horka*^{D}, we sequenced the mutant allele and also the wild type *lds* gene in the *mwh e* labeled founder chromosome. Comparison of the sequences revealed a single nucleotide exchange in position 2424 where the G was replaced by an A. The G^{2424}→A transition results in the replacement of Ala^{777} by Tre in the *Horka*^{D} encoded mutant A777T-LDS molecules (Fig. 8).

To prove that *Horka*^{D} did indeed originate through the G^{2424}→A transition we generated two types of *TG*^{HD} transgenes: *TG*$^{HD5.1}$ and *TG*$^{HD5.5}$ (Fig. 8). The *TG*^{HD} transgenes render the *TG*^{HD} carrying females sterile. Although cuticle fragments appear inside 25-28% of their eggs, larvae never hatch (Table 2). Crosses between *y v f mal* females and +/+; *TG*^{HD} males yielded *X*X//*X*0 mosaics among the *X*X offspring (Table 2). Features of the *TG*^{HD} transgenes prove that *Horka*^{D} is a dominant *lodestar* mutant allele and that the *Horka*^{D} related mutant phenotypes originated from the same mutation.

The *TG*$^{CFP\text{-}LDS}$ *TG*$^{CFP\text{-}HD}$ *TG*$^{LDS\text{-}RFP}$ *TG*$^{HD\text{-}RFP}$ transgenes

To visualize the LDS and the *Horka*^{D} encoded A777T-LDS molecules, in confocal optical sections prepared from live embryos, we generated the following types of transgenes. The *TG*$^{CFP\text{-}LDS}$ and the *TG*$^{LDS\text{-}RFP}$ transgenes, which encode the formation of CFP- (on the N terminus) or RFP- (on the C terminus) tagged LDS molecules (Table 5). The *TG*$^{CFP\text{-}HD}$ and the *TG*$^{HD\text{-}RFP}$ transgenes code for the formation of CFP- (on the N terminus) or RFP-tagged (on the C terminus) A777T-LDS molecules (Table 5). The CFP- and the RFP-tagged molecules are functional since (1) the CFP-LDS and the LDS-RFP molecules (i) overcome sterility of the *horka*^{rP2}/– females and (ii) increase immensely the fertility of the *horka*^{rP2}/– males (Table 5). (2) The CFP-A777T-LDS and the A777T-LDS-RFP molecules act as weak *Horka*^{D} mutations as they (i) bring about strong reduction in female fertility and (ii) once in the males, they induce the formation of gynandromorphs (Table 5).

The *TG*$^{CFP\text{-}LDS}$ *TG*$^{CFP\text{-}HD}$ *TG*$^{LDS\text{-}RFP}$ *TG*$^{HD\text{-}RFP}$ transgenes contain the yeast UAS (upstream activation sequence) and allow thus "driving" the expression of the transgenes in desired organs with the Gal4/UAS system (BRAND and PERRIMON 1993); (DUFFY 2002). Once expression of the *TG*$^{CFP\text{-}HD}$ was ensured by the *α1Tub-Gal4*, an all-over type of the Gal4 drivers, the females become practically sterile showing that the CFP-A777T-LDS molecules are functional (Table 5).

Table 5. Features of the $TG^{CFP\text{-}LDS}$ $TG^{CFP\text{-}HD}$ $TG^{LDS\text{-}RFP}$ $TG^{HD\text{-}RFP}$ transgenes that encode the formation of CFP- or RFP-tagged LDS or A777T-LDS molecules

Transgene	TG in chromosome	Female genotype and percentage of addle eggs among the eggs deposited by the transgene carrying females Average ± SD (n)		Fertility of the transgene carrying males[a]				Percentage of the *X*X//*X*0 mosaics in the offspring of the $TG^{CFP\text{-}HD}$ or the $TG^{HD\text{-}RFP}$ transgene carrying males following mating with *y v f mal* females					
				+/+; *TG*		$horka^{rP2}$/–; *TG*		+/+; *TG*			$horka^{rP2}$/–; *TG*		
		+/+; *TG*	$horka^{rP2}$/–; *TG*	Eggs/day	Addle eggs (%)	Eggs/day	Addle eggs (%)	*X*X	*X*X//*X*0	%	*X*X	*X*X//*X*0	%
None	-	8± 3 (16)	100 (180)	34±11	13± 4 (10)	30± 9	96±10 (17)	N.d.			N.d.		
TG^{+}	2nd	15± 8 (18)	26±12 (16)	34±11	9± 4 (15)	34±10	16± 7 (15)	N.d.			194	0	-
$TG^{CFP\text{-}LDS}$	1st	22±10 (9)	47±17 (10)	28± 8	20± 9 (12)	30±10	41±11 (12)	N.d.			N.d.		
	1st	20±11 (9)	47±15 (13)	N.d.									
	3rd	17± 9 (11)	N.d.	N.d.									
$TG^{CFP\text{-}HD}$	2nd	52±14 (14) *90± 5 (10)*	82± 8 (15)	30±10	41±11 (10)	29 ±11	47±14 (7)	357 *604*	2 *0*	0.6 -	398	54	13.6
	2nd	76± 8 (14) *99± 1 (16)*	84±11 (11)	N.d.				244 *732*	11 *1*	4.3 *0.1*	87	15	14.6
	3rd	36±22 (16) *87± 6 (10)*	N.d.	N.d.				212 *335*	6 *0*	2.7 -	N.d.		
$TG^{LDS\text{-}RFP}$	1st	18± 8 (13)	50±20 (4)	33±11	22±10 (7)	27±13	36±14 (8)	N.d.			N.d.		
	1st	20± 8 (12)	38±12 (5)	N.d.									
	2nd	17± 5 (10)	47±14 (13)	N.d.									
$TG^{HD\text{-}RFP}$	3rd	48±11 (19)	39±15 (7)	30±10	27±10 (15)	29±11	39±15 (10)	3650	1	0.06	513	1	0.2

[a] Single males were mated with single *y v f mal* females and the egg production rate of the *y v f mal* females was followed for one week; average ± standard deviation (n)
N.d. = not determined

Notes:
- The – symbol stands for $Df(3R)dsx^{15}$, a deficiency that removes the *lds* and some of the adjacent loci.
- Data in *Italic* refer to experiments in which the *α1Tub-Gal4* driver ensured all-over type of expression of the transgene

LDS localizes to mainly to the meta- and the anaphase chromosomes

To reveal the position of the LDS protein throughout the cleavage cycles, we analyzed embryos of the females with carried two transgenes: one encoded the formation of CFP-LDS, the other ensured the production of GFP-tagged α1-tubulin (GRIEDER *et al.* 2000). As Figure 10 shows, the CFP-LDS molecules enter the nucleus during prometaphase and highlight the entire chromosomes throughout meta- and anaphase. The telophase chromosomes are still highlighted by CFP-LDS, though at a reduced level. There is no CFP-LDS in the nuclei by the end of mitosis.

To more precisely describe the localization of the CFP-LDS molecules throughout mitosis, we fixed CFP-LDS containing embryos, stained their DNA and made the CFP visible by making use of an anti-GFP antibody (Sigma-Aldrich) that also recognizes CFP, a mutant version of GFP (Fig. 11). As stated above, the LDS protein is cytoplasmic throughout the interphase, localizes over the entire length of the chromosomes during meta- and anaphase and almost completely leaves the chromosomes by telophase (Fig. 11). Remarkably, there is hardly any LDS protein along the spindle apparatus. This observation is in contrast to the results of Girdham and Glover (1991), who - by making use of a polyclonal serum raised against the nearly full length LDS protein - detected a strong LDS signal over the spindle apparatus.

The localization of the LDS protein on the meta-and the anaphase chromosomes suggest a chromosome-associated function of the LDS protein during meta and anaphase and raises the possibility of LDS participating in progression of the cell cycle from G2 through mitosis, possibly from metaphase to anaphase.

The possible function of LDS protein

The LDS protein was proposed to be TTF2, transcription termination factor 2, which removes RNA-polymerase form the chromatin upon the onset of mitosis (MARSHALL and PRICE 1995), (XIE and PRICE 1996), (XIE and PRICE 1997), (XIE and PRICE 1998), (LIU *et al.* 1998), (JIANG and PRICE 2004). Should this assumption be correct, the RNA-polymerase molecules would remain on the chromosomes in absence of the LDS protein, i.e. in embryos of the *horka*^{rP2}/– females. (Note that expression of the zygotic genes, including those which encode the formation of the RNA-polymerase subunits, commences only at, or shortly before the blastoderm stage (TADROS and LIPSHITZ 2005). To determine whether this proposed function of LDS is correct, we analyzed the DNA and the RNA-polymerase pattern in embryos that derived from wild type or from *horka*^{rP2}/– females (Fig. 12). Since there is no difference in the DNA and the RNA-polymerase related signals in the two types of embryos, it is rather unlikely that LDS functions as TTF2.

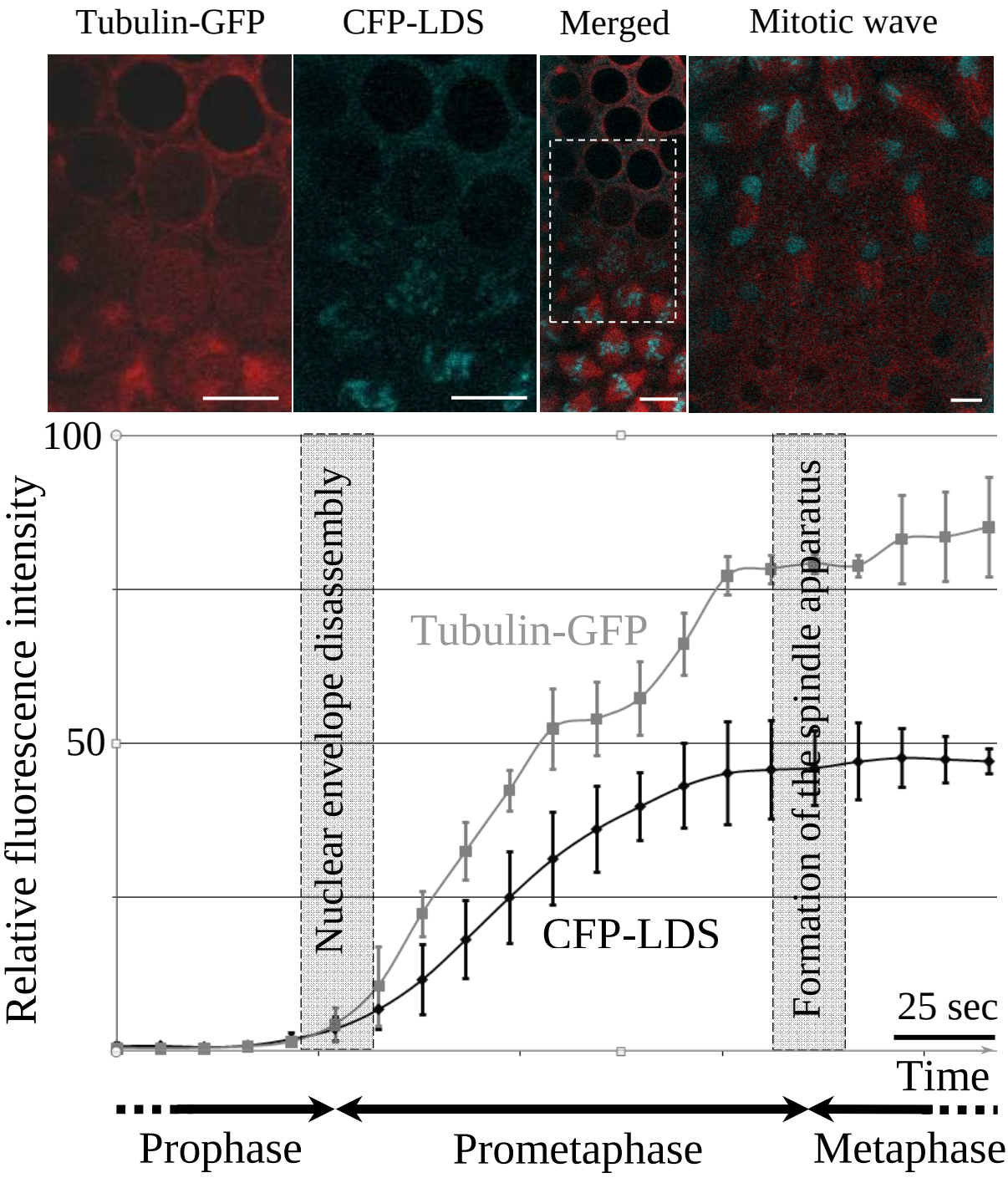


Figure 10. The appearance and the localization of the CFP-LDS protein in the nuclei of cleavage Drosophila embryo. The α1-tubulin molecules were highlighted by GFP and the emitted signal appears red (after computer-aided coloration for convenient illustration). Prometaphase begins with nuclear envelope breakdown (that is partial in Drosophila) and formation of the spindle envelope (**Foe** *et al.* 1993). Prometaphase is marked by the uniform distribution of the α1-tubulin-GFP molecules over the studied region. The CFP-LDS molecules enter the nucleus upon breakdown of the nuclear envelope and highlight the chromosomes over their entire length. Formation of the spindle apparatus is a hallmark of metaphase. The mitotic wave offers a convenient analysis of the nuclei in the different stages of the cleavage mitoses. Scale bar = 10 μm.

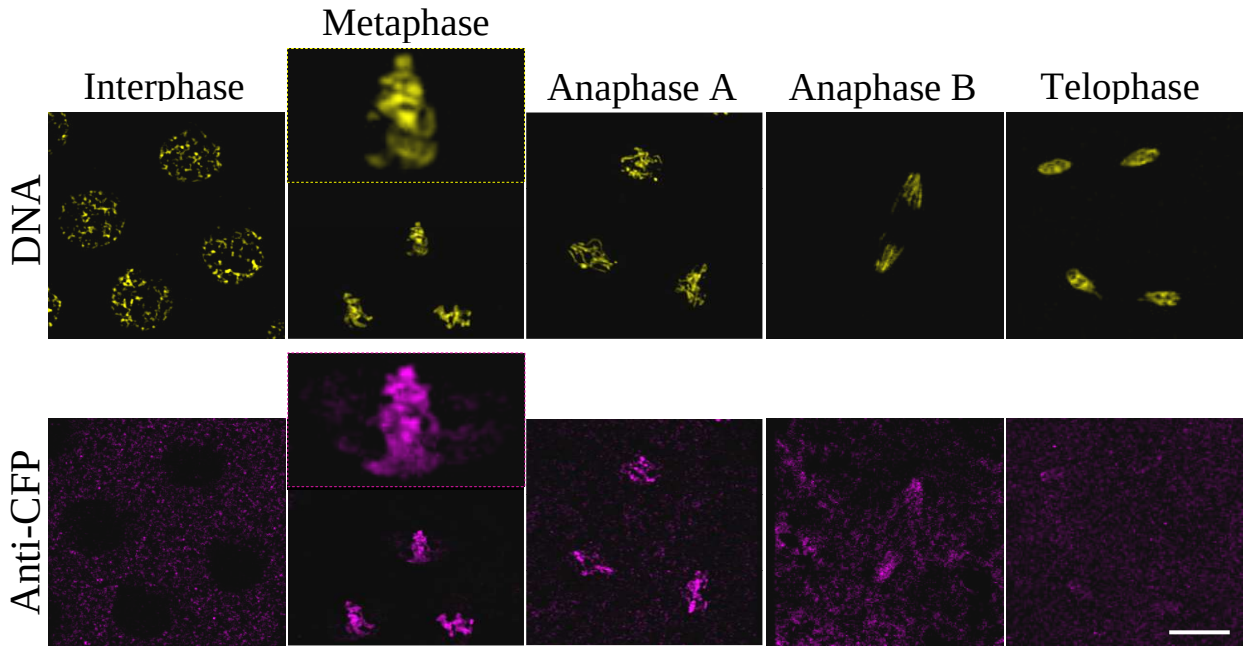


Figure 11. Localization of the LDS protein throughout a cleavage cycle. The DNA was highlighted by Hoechst 33342, and the emitted blue florescence appears yellow in the upper panel. LDS, as part of the CFP-LDS chimeric protein, was visualized through an anti-GFP primary and a fluorescent secondary antibody and appears purple in the lower panel. Scale bar = 10 µm. The metaphase inlets represent a 3.3-fold magnification of the metaphase figures.

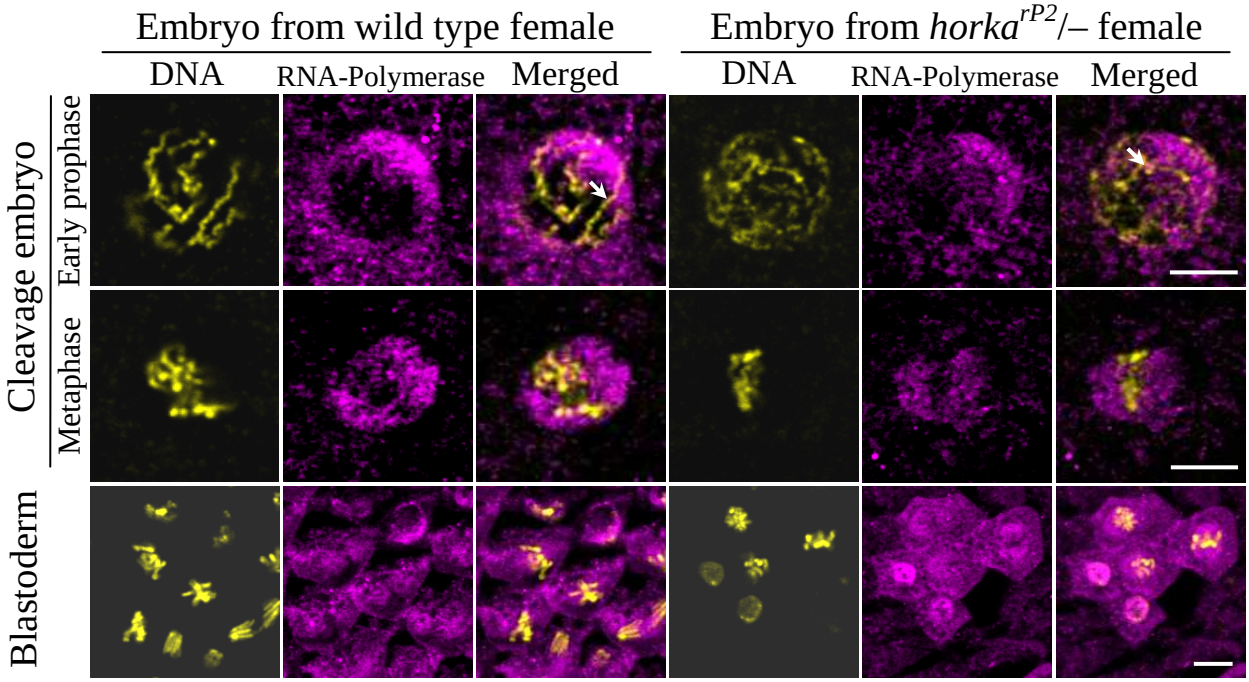


Figure 12. Localization of the DNA and the RNA-polymerase in cleavage and blastoderm stage embryos of wild type and *horka*^{rP2}/– females. The DNA was highlighted by Hoechst 33342 and the emitted blue florescence appears yellow in the stacked optical sections. RNA-polymerase was identified by an anti-RNA-polymerase primary antibody. The fluorescence emitted by the secondary antibody appears purple. Co-localization of the DNA and the RNA-polymerase related signals appear pink (shown by arrows). Note that the overall signal patterns do not appear different in the two types of embryos making it very unlikely that LDS functions as TTF2. Scale bar = 10 µm.

The LDS protein well may be engaged in cell cycle progression control

The (i) localization of the LDS protein over the meta- and the anaphase chromosomes and (ii) the mitotic catastrophe phenotype in embryos of the *horka*^{rP2}/− females suggested involvement of the LDS protein in cell cycle progression control. Largely identical defects were described for the checkpoint kinase 1 (*grapes*, *grp*), the checkpoint kinase 2 (*lok* or *maternal nuclear kinase*, *mnk*) and the Ataxia telangiectasia related *mei-41* mutant alleles (BRODSKY *et al.* 2004; LAROCQUE *et al.* 2007; MASROUHA *et al.* 2003; ROYOU *et al.* 2005; TAKADA *et al.* 2003; TAKADA *et al.* 2007; WICHMANN *et al.* 2006). For example, in embryos of the *mnk*/*mnk* females, i.e. in the absence of checkpoint kinase 2, and especially under genotoxic stress, abnormal chromosomes escape the checkpoint kinase 2 imposed control (LAROCQUE *et al.* 2007; SIBON *et al.* 2000), (TAKADA *et al.* 2007). To confirm the present hypothesis, we irradiated *horka*^{rP2}/− (and as control +/− larvae) with 2000 Rad of X-rays (150 kV; 0.5 mm Al filter and 500 Rad/min), fixed the imaginal discs 100 minutes following irradiation and stained with the anti-H3S10P antibody, which specifically reacts with those histone-3 molecules in which Ser^{10} is phosphorylated. H3S10P has been regarded as a marker of the mitotic chromatin (PRIGENT and DIMITROV 2003). While there was no indication of mitotic chromosomes in the wild type imaginal discs, the presence of mitotic chromosomes was apparent in the imaginal discs of the *horka*^{rP2}/− larvae (Fig. 13). It appears thus that the checkpoint control mechanism, which is expected to prevents the progression through mitosis of the damaged DNA before complete reparation, is absent in the *horka*^{rP2}/− larvae, suggesting the involvement of the LDS protein in checkpoint control.

This assumption is further supported by the following observation.

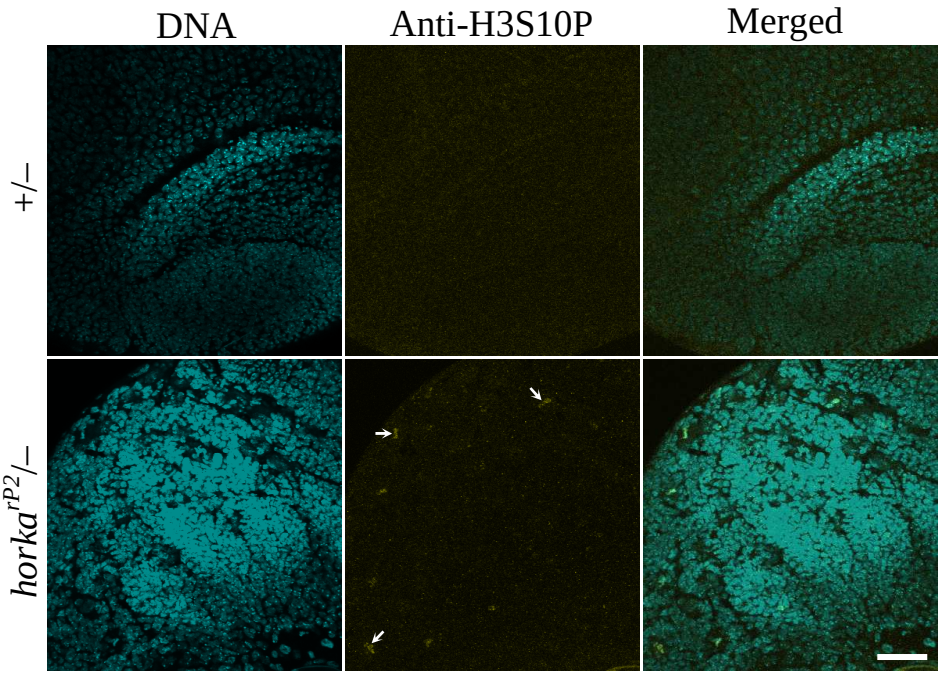


Figure 13. Mitotic chromosomes escape cell cycle progression control indicating involvement of the LDS protein in the process. Control (+/−) and *horka*^{rP2}/− larvae were irradiated by 2000 Rad of X-rays, dissected after 100 minutes, fixed and stained with both Hoechst 33342 and an anti-H3S10P primary and a fluorescent secondary antibody to detect mitotic chromatin. Nuclei that escaped control are shown by arrows. Scale bar = 100 µm.

To further elaborate on the "mitosis escaper" phenomenon, we X-irradiated in the *horka*^{rP2}/– (and, as control, +/–) late third instar larvae by 2000 Rad, dissected their ventral ganglia 100 min following irradiation and stained for DNA (with Hoechst 33342) and an anti-H3S10P antibody to detect mitotic chromatin. As expected, anaphase figures were not present in the neuroblast cells, due to the cell cycle progression control mechanism that ensures the reparation of the damaged DNA before allowing progression in the cell cycle (Fig. 14). There were not only anaphase figures in the *horka*^{rP2}/− neuroblasts but the escaper chromosomes were highlighted by the anti-H3S10P antibody (Fig. 14). This observation clearly shows that the phosphate group is not removed in absence of the LDS protein, and hence the anaphase chromatin remains compacted, implying that the LDS protein is not only engaged in cell cycle progression control but also in removal of the phosphate group from H3-S10 and thus bringing the chromatin into a less compacted state as during metaphase.

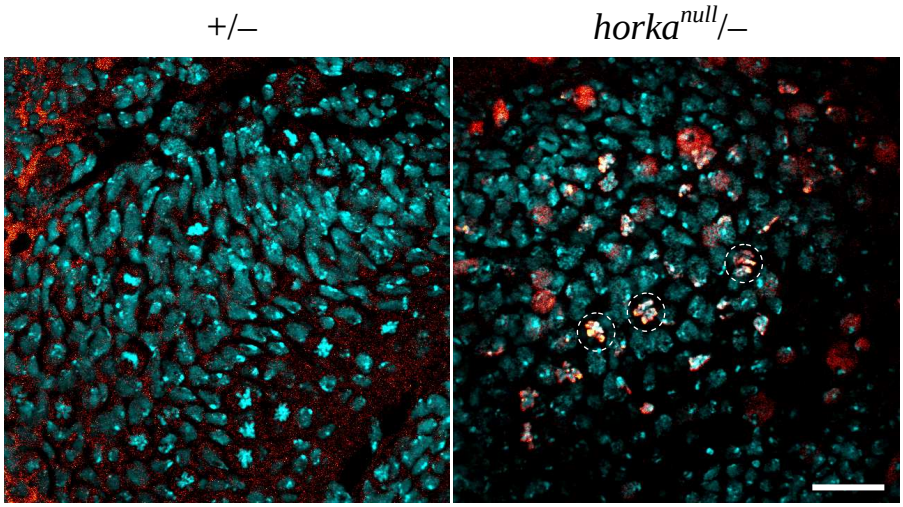


Figure 14. Neuroblasts in control (+/–) larva with one copy of the *lds* gene and in *horka*^{rP2}/− larva without *lds* gene. The larvae were X- irradiated by 2000 Rad, their ventral ganglia were dissected 100 minutes after irradiations, fixed and stained with Hoechst 33342 to label DNA and with an anti-H3S10P antibody to detect mitotic chromatin. The DNA and the anti-H3S10P related signals appear in blue and in red in the optical sections. Note the absence of the anaphase plates in the control neuroblasts and the (i) presence of anaphase figures that (ii) contain highly compacted, H-3S10P containing chromatin (encircled). Scale bar = 25 µm.

DISCUSSION

Along genetic dissection of the commencement of embryogenesis in Drosophila, the Szabad laboratory isolated several *Fs* mutations, which although allow the formation and fertilization of seemingly normal eggs, yet embryogenesis either does not commence inside the eggs or comes to an end during the initial steps. When the *Fs* mutation is dominant negative, the corresponding normal gene well may have important function during the commencement and/or the progression of embryogenesis. *Horka*^{D} is one of the *Fs* mutations (ERDELYI and SZABAD 1989). Abnormal chromosome organization and/or segregation during the meiotic divisions and in the embryos of the *Horka*^{D}/+ females suggest an involvement of the normal gene product in establishing proper chromosome structure and/or segregation, essential features in the maintenance of genome integrity (ALLARD *et al.* 2004; MUSACCHIO and SALMON 2007; TAKADA *et al.* 2003). This assumption is supported by the finding that during spermatogenesis *Horka*^{D} induces nondisjunction and renders the chromosomes unstable such that they tend to be lost in the descending embryos leading to the formation of diplo//haplo mosaics, including the gynandromorphs (SZABAD *et al.* 1995; SZABAD and NOTHIGER 1992; VILLANYI *et al.* 2008; ZALLEN and WIESCHAUS 2004).

Nature of the *Horka*^{D} encoded A777T-LDS protein

As described in (SZALONTAI *et al.* 2008), *Horka*^{D} is a dominant negative mutation and thus the *Horka*^{D} identified gene, based on the mutant phenotypes, is expected to be engaged in chromatin surveillance and/or chromosome segregation. It is also described in (SZALONTAI *et al.* 2008) that *Horka*^{D} and its *horka*^{r} revertant alleles identify the *lodestar* gene, which has been known to encode the formation of a 155 kD LDS protein composed from 1061 amino acids (GIRDHAM and GLOVER 1991). LDS is a member of the Snf2 family of the helicase-related proteins, which have been known to be involved in transcription regulation, DNA repair, recombination and chromatin unwinding. (FLAUS *et al.* 2006) grouped 1306 proteins of the Snf2 family into 24 subfamilies, one of which is lodestar (Fig. 15). Helicase motifs and several further conserved domains, all with characteristic functions, contribute to distinctive features of the Snf2 family proteins (Fig. 15). In Rad54, the only member with known structure in the Snf2 family, two of the alpha helices (α17 and α18) plus the short interconnecting region form protrusion 2, the DNA interacting part of the protein (FLAUS *et al.* 2006; THOMA *et al.* 2005); Fig. 16).

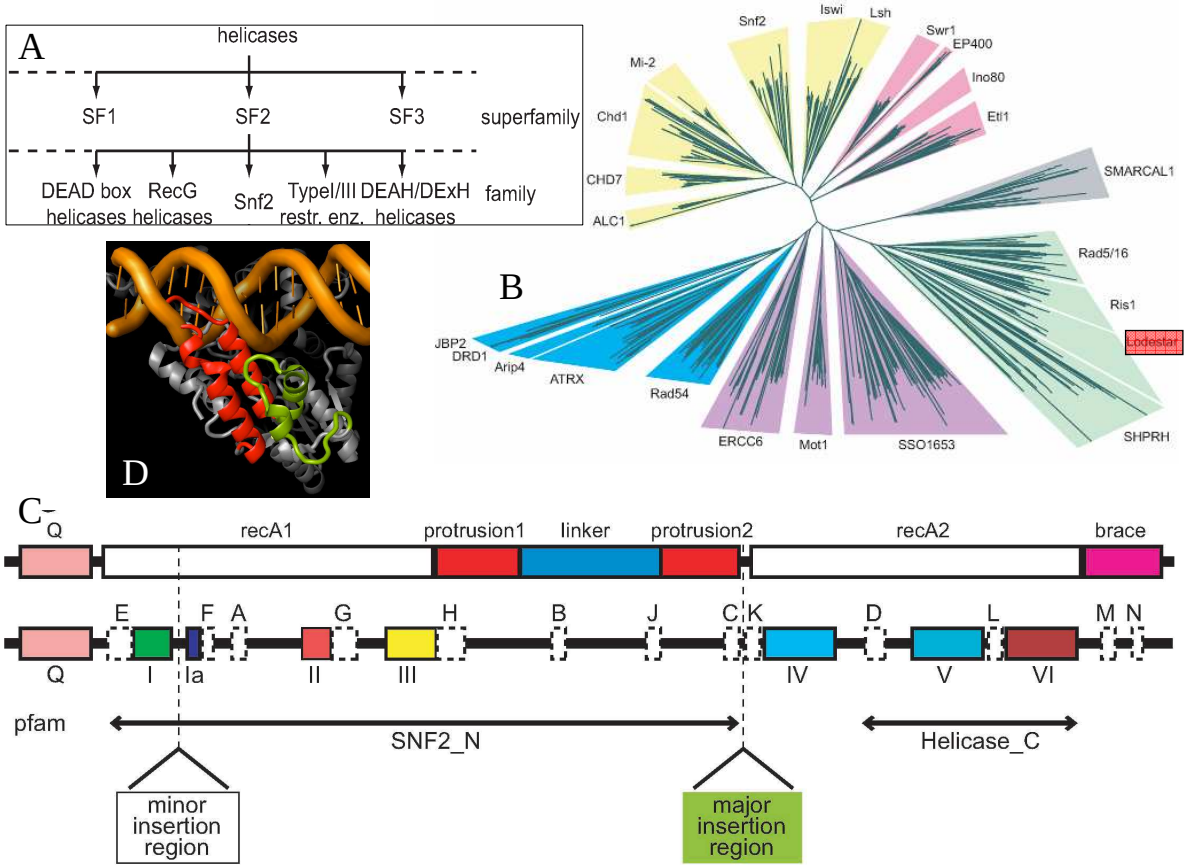


Figure 15. Features of the Snf2 family of the helicase-related proteins. (A) Schematic diagram illustrating hierarchical classification of the helicase superfamilies and members of the SF2 families. (B) Unrooted radial neighbor-joining tree from a multiple alignment of the helicase-like region sequences of the Snf2 family members of the helicase-related proteins. (C) Schematic diagram showing location of structural elements and helicase motifs in the Snf2 family members. The nucleotide triphosphate binding so-called helicase motifs appear as I through VI. The conserved domains of the Snf2 family proteins are shown from A through N. (D) Protrusion 2 interacts with the DNA. After (FLAUS *et al.* 2006; THOMA *et al.* 2005) and (FLAUS *et al.* 2006).

Presence of the α17 and the α18 alpha helices is apparent in the LDS protein. However, the interconnecting region is longer than in Rad54 and contains an alpha helix (Fig. 15). *Horka*^{D} originated through the transition of G^{2424} to A, which brought about the replacement of Ala^{777} by Tre in the aforementioned interconnecting region. It appears that the Ala^{777}→Tre replacement expanded the alpha helix by two amino acids and rendered the *Horka*^{D} encoded A777T-LDS molecules slightly sticky. Stickiness of the A777T-LDS protein well may account for abnormal chromatin organization, chromosome tangling and instability, the formation of chromatin bridges and diplo//haplo mosaics and the eventual death of the embryos derived from the *Horka*^{D} carrying females. *Horka*^{D} is thus an example of the dominant negative mutations, which originated through a single base pair exchange mutation and have a dramatic impact on genome

integrity. The present finding has special importance in the lights of the relationship between chromosome instability and the origin of most of the solid tumors (MUSACCHIO and SALMON 2007; YUEN and DESAI 2008).

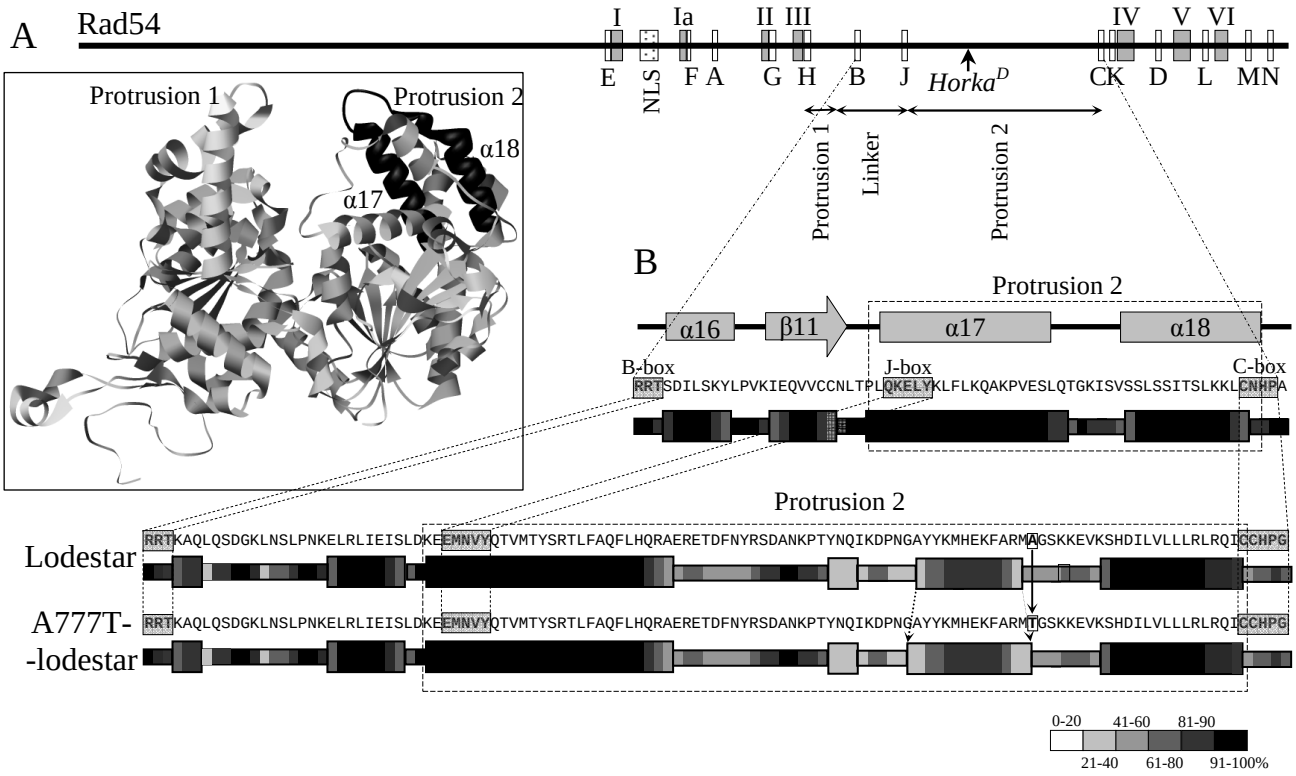


Figure 16. Domain organization of the Rad54, parts of the LDS and the A777T-LDS proteins. (A) The nucleotide triphosphate binding so-called helicase motifs (I-VI) appear in shaded, the E-N conserved domains, with well-specified functions, in open boxes in the zebrafish Rad54A, a typical member of the Snf2 family of the helicase-related proteins (FLAUS *et al.* 2006). (NLS stands for a putative nuclear localization signal.) (B) The region, including the J and the C boxes forms protrusion 2 that is composed from the α17 and the α18 helices and an interconnecting short stretch of amino acids. Protrusion 2 was proposed to interact with the DNA (FLAUS *et al.* 2006; THOMA *et al.* 2005); see the framed inlet and the web site at www.sanger.ac.uk/cgi-bin/Pfam/swisspfamget.pl?name=P34739). Presence of the B, the J and the C boxes and the α17 and the α18 helices are apparent in the LDS protein. The KK amino acids, near the C box, have been implemented in protein-DNA interaction (THOMA *et al.* 2005). In the LDS protein, more amino acids compose the sequence that connects the α17 and the α18 helices as in Rad54. Presence of an alpha helix is predicted inside this interconnecting region and this alpha helix became longer by two amino acids in the *Horka*^{D} encoded A777T-LDS protein as compared to LDS. The grey scale at the bottom right of the figure illustrates the likelihood (as determined by the PSIPRED - http://bioinf.cs.ucl.ac.uk/psipred/ software) that any amino acid is part of an α-helix.

Possible function of the LDS protein

The LDS protein is cytoplasmic during interphases of the cleavage mitoses, enters the nucleus during prometaphase and is associated with the chromosomes throughout mitosis, suggesting an involvement of the LDS protein in chromatin/chromosome surveillance during mitosis (GIRDHAM and GLOVER 1991). This assumption is supported by the complete-loss-of function mutant phenotype in embryos of the *horka*^{rP2}/− females: abnormal assembly of the chromosomes during meiosis and mitosis, formation of anastral centrosomes and abnormal spindle apparatus, failures of the cleavage mitoses, fall out of the abnormal cleavage nuclei and the eventual death of the embryos. Practically identical types of defects have been reported for embryos of the females that are defective in (i) spindle assembly checkpoint functions or (ii) in the so-called mitotic catastrophe mechanism (CASTEDO *et al.* 2004; MUSACCHIO and SALMON 2007; VAKIFAHMETOGLU *et al.* 2008; YUEN and DESAI 2008). The latter mechanism operates trough the activation of checkpoint kinase 2 (Chk2): damaged or incompletely replicated DNA lead to activation of Chk2 and the consequential inactivation of the centrosomes and the spindles, which then result in blocked chromosome segregation during anaphase and the eventual elimination of those nuclei from the embryonic precursor pool that are aneuploid or carry damaged DNA (Fig. 17). In absence of Chk2, the damaged chromosomes, nuclei escape the Chk2imposed block and proceed along mitosis leading to the formation of a condition known as genetic imbalance. The Chk2-based mechanism is especially important in the maintenance of genomic stability during genotoxic stress (BRODSKY *et al.* 2004; LAROCQUE *et al.* 2007; MASROUHA *et al.* 2003; TAKADA *et al.* 2003; WICHMANN *et al.* 2006). Defects in the Chk2 based mechanism are known as the mitotic catastrophe traits (VAKIFAHMETOGLU *et al.* 2008).

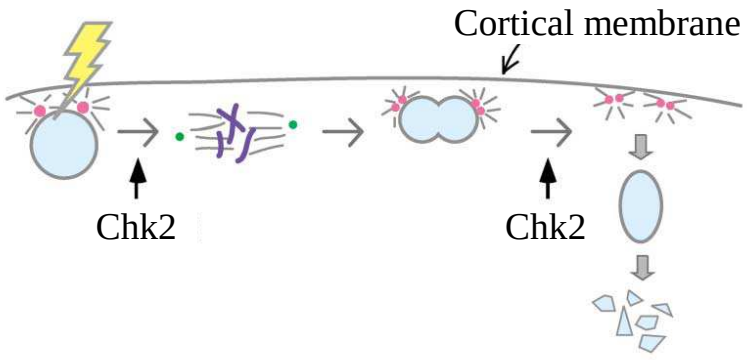


Figure 17. Two-step model for the Chk2-mediated response to DNA damage during mitosis. When DNA lesions are induced during interphase (↯) and persist into mitosis, Chk2 is activated, localizes to the centrosomes, and disrupts centrosome function. This leads to anastral spindle assembly and anaphase chromosome segregation failures. After failed mitotic division, Chk2 mediates a second DNA damage response that disrupts the link between centrosomes and nuclei, or prevents reestablishment of this link. As a result, the defective products of division failure drop into the interior of the embryo and are not incorporated into cells when the blastoderm forms. This two-step response to DNA damage thus blocks propagation of defective nuclei and prevents their transmission to the embryonic precursor pool. After (TAKADA *et al.* 2003).

In principle, the LDS protein could also be engaged in the spindle assembly checkpoint (SAC) machinery (see Fig. 18), since similar mutant phenotypes emerge upon the loss of SAC (e.g. instability and loss of the chromosomes; (MUSACCHIO and SALMON 2007) as was described for the *lds* homozygous mutant condition (GIRDHAM and GLOVER 1991); (SZALONTAI *et al.* 2008).

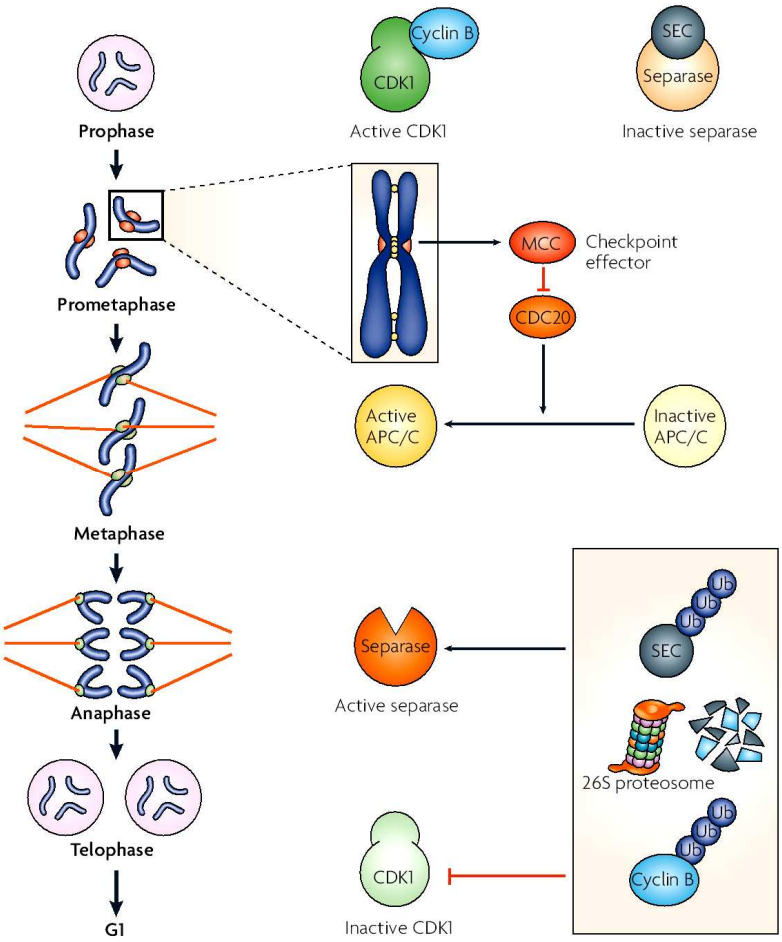


Figure 18. The relationship of spindle assembly checkpoint (SAC) with the cell-cycle machinery. Mitosis is subdivided into five consecutive phases: prophase, prometaphase, metaphase, anaphase and telophase. To enter mitosis, the cell requires the activity of the master mitotic kinase, cyclin-dependent kinase-1 (CDK1), which depends strictly on the binding of cyclin B to CDK1. Separase is a protease. Its activity is required to remove sister-chromatid cohesion at the metaphase-to-anaphase transition (cohesin is indicated in yellow on the expanded view of the chromosome). Prior to anaphase, separase is kept inactive by the binding of a protein known as securin (SEC). Unattached kinetochores (red hemi-circles) contribute to the creation of the mitotic checkpoint complex (MCC), which inhibits the ability of CDC20 to activate the anaphase-promoting complex/cyclosome (APC/C). The attachment of all sister-kinetochore pairs to kinetochore microtubules, and their bi-orientation – which produces congression to the spindle equator - negatively regulates the SAC signal. This releases CDC20, which can now activate the APC/C. This results in the polyubiquitylation of anaphase substrates such as cyclin B and securin, and their subsequent proteolytic destruction by the proteasome. The degradation of SEC results in the activation of separase, which targets the cohesin ring that is holding the sister chromatids together, thus causing the loss of sister-chromatid cohesion and the separation of sister chromatids. The degradation of cyclin B at this stage also inactivates the master mitotic kinase CDK1–cyclin B, initiating cytokinesis and the mitotic-exit programme. Attached kinetochores are shown in green. From: Musacchio and Salmon (2007).

However, it is very unlikely that LDS functions in the spindle assembly checkpoint since, and unlike the LDS and the Chk2, the spindle checkpoint proteins have been shown to bind to the kinetochores (GILLETT *et al.* 2004; MUSACCHIO and SALMON 2007). The abnormalities that

emerge in embryos of the *horka*^{rP2}/− females posses all the distinctive features of mitotic catastrophe. Largely identical defects were described for the checkpoint kinase 1 (*grapes*, *grp*), the checkpoint kinase 2 (*lok* or *maternal nuclear kinase*, *mnk*) and the Ataxia telangiectasia related *mei-41* mutant alleles (BRODSKY *et al.* 2004; LAROCQUE *et al.* 2007; MASROUHA *et al.* 2003; ROYOU *et al.* 2005; TAKADA *et al.* 2003; TAKADA *et al.* 2007; WICHMANN *et al.* 2006). Functions of the corresponding genes have been implicated in G2/M checkpoint by "assaying" status of the DNA and/or the chromatin and the elimination of the inappropriate nuclei from the pool that will serve as source of the blastoderm cells following the cleavage cycles (LAROCQUE *et al.* 2007; TAKADA *et al.* 2003). The LDS protein appears to be involved in the same pathway as Chk2, because a few of the embryos that derive from *mnk*/*mnk*; *horka*^{rP2}/– females, which lack both the Chk2 and the LDS proteins, develop to adulthood (our unpublished result), an event that never happens to embryos of the *horka*^{rP2}/– females. However, the role of the LDS protein in chromatin surveillance and cell cycle progression regulation has yet to be elaborated.

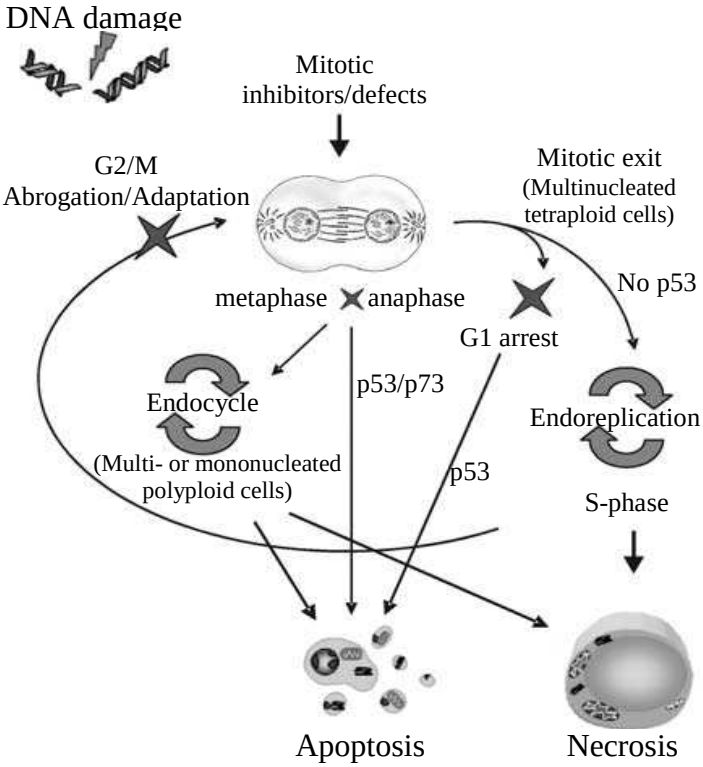


Figure 19. Schematic illustration of the pathways leading from mitotic catastrophe to cell death. Premature entry into mitosis as a consequence of abrogated G2/M arrest or adaptation in the presence of DNA damage or direct mitotic damage leads to arrest at the metaphase–anaphase transition due to spindle checkpoint and to catastrophic mitosis. During mitotic arrest, cells can die through caspase-dependent or caspase-independent apoptosis. mitotic catastrophe cells can undergo endocycle and become polyploid. These cells can die by either necrosis or apoptosis. Cells being arrested at the metaphase-anaphase transition can escape mitosis through mitotic slippage and become tetraploid. Cells that cannot be arrested at the metaphase-anaphase transition due to defects in the spindle checkpoint also become tetraploid. These tetraploid cells either can arrest at G1 and die through p53-dependent apoptosis or do not arrest at G1 and enter S-phase (endoreplication) and die through necrosis. From (VAKIFAHMETOGLU *et al.* 2008).

The requirement of *lodestar* in the germ line and in the soma

A remarkable, though not unusual, feature of the *lodestar* gene is that its function is indispensable in the germ line but not in the soma: although the flies develop normally in absence of the LDS protein, the meiotic divisions are abnormal in the *lds*/*lds* females and also the cleavage mitoses in their embryos (GIRDHAM and GLOVER 1991). The defects, as analysis of germ line chimeras revealed, are germ line autonomous, and in fact, the *lds* mutant soma is good enough to support development of the normal germ line cells. Similar feature, i.e. complete or almost complete maternal-effect lethality is a characteristic feature of the females that are homozygous for mutant alleles of the genes engaged in the G2/M transition control. For example, only about 20% of the embryos hatch from eggs of the females that - being homozygous for strong *mnk* (*lok*) mutant alleles - lack Chk2 (BRODSKY *et al.* 2004; MASROUHA *et al.* 2003; TAKADA *et al.* 2003; XU and DU 2003; XU *et al.* 2001). The *grp* homozygous females, which lack checkpoint kinase 1, are sterile; their embryos suffer from abnormal cortical nuclear divisions and do not cellularize (JAKLEVIC *et al.* 2006; TAKADA *et al.* 2007; YU *et al.* 2000). Females homozygous for the Ataxia telangiectasia related *mei-41* strong mutant alleles are basically sterile (LAROCQUE *et al.* 2007; LAURENCON *et al.* 2003). Females homozygous for mutant alleles of the *Bub1-related kinase* gene - and hence are defective in spindle assembly checkpoint control - are also sterile (PEREZ-MONGIOVI *et al.* 2005).

Sensitivity to genetoxic stress is a common feature of the above-mentioned mutants. In fact, several of the mutagen-sensitive mutations bring about maternal-effect lethality (HENDERSON 1999). Remarkably, *lodestar* also possesses X-ray sensitivity, a feature awaits for further exploration.

The most feasible explanations in the above-mentioned mutations for the disturbance of the germ line and the cleavage divisions and leaving the soma unaffected are as follows. (1) The enormous "genetic requirement" in the female germ line and - through maternal effect - during the cleavage divisions as compared to the somatic cells: while function of 67-73% of the genes is required in the germ line, this proportion is only 8-12% in the soma (SZABAD *et al.* 1989). (2) The different types of proliferation control mechanisms during the cleavage cycles and in the imaginal cells well may involve - at least in part - different sets of genes (DERENZO and SEYDOUX 2004; TADROS and LIPSHITZ 2005). (3) Function of several, if not all of the above mentioned genes is also required in the soma. However, the requirement of the gene becomes apparent only when the somatic cells are exposed to genotoxic stress (LAROCQUE *et al.* 2007; VAKIFAHMETOGLU *et al.* 2008; WICHMANN *et al.* 2006). Unlike the cleavage nuclei that rely largely on mitotic catastrophe and spindle assembly checkpoint control mechanisms to eliminate the nuclei with genetic imbalance, the cells are equipped with means that ensure apoptosis or

necrosis to achieve the maintenance of genetic integrity (VAKIFAHMETOGLU *et al.* 2008). During Drosophila development and under normal conditions, only few cells become aneuploid and/or carry inappropriately replicated or damaged DNA. These cells are removed through apoptosis or necrosis from the populations of the diploid cells (VAKIFAHMETOGLU *et al.* 2008). The eliminated cells are replaced through intercalary regeneration and thus the developing flies appear normal. In mutant zygotes, which lack e.g. Chk1, Chk2 or ATR gene function, some of the normally eliminated cells may survive, however have little if any impact on development of the soma. However, when exposed to genotoxic stress and being mutagen sensitive, a significant portion of the diploid cells with genetic imbalance are removed such that the mutant larvae fail to develop to adulthood (JAKLEVIC *et al.* 2006; LAROCQUE *et al.* 2007; XU *et al.* 2001). Since loss of the diploid cells in the mutant larvae is restricted to the diploid cells and the non-dividing larval cells are not affected, the genotoxic stress-caused death leads to death toward the end of the larval and/or at the beginning of the pupal life and is characteristic feature of the mutagen-sensitive mutant larvae (HENDERSON 1999). That the *lodestar* gene does indeed belong to the *grp*, *mnk* and *mei-41* genes is further supported by the finding that without *lodestar* gene function and upon genotoxic stress, intensive apoptosis emerges among the diploid cells and the larvae perish toward the end of the larval life (unpublished result from the Szabad laboratory). However, the latter feature of the *lds* mutant needs further studies.

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