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Horka^{D}, a Chromosome Instability-Causing Mutation in Drosophila, Is a Dominant-Negative Allele of *lodestar*

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ABSTRACT

Correct segregation of chromosomes is particularly challenging during the rapid nuclear divisions of early embryogenesis. This process is disrupted by *Horka*^{D}, a dominant-negative mutation in *Drosophila melanogaster* that causes female sterility due to chromosome tangling and nondisjunction during oogenesis and early embryogenesis. *Horka*^{D} also renders chromosomes unstable during spermatogenesis, which leads to the formation of diplo//haplo mosaics, including the gynandromorphs. Complete loss of gene function brings about maternal-effect lethality: embryos of the females without the *Horka*^{D}-identified gene perish due to disrupted centrosome function, defective spindle assembly, formation of chromatin bridges, and abnormal chromosome segregation during the cleavage divisions. These defects are indicators of mitotic catastrophe and suggest that the gene product acts during the meiotic and the cleavage divisions, an idea that is supported by the observation that germ-line chimeras exhibit excessive germ-line and cleavage function. The gene affected by the *Horka*^{D} mutation is *lodestar*, a member of the helicase-related genes. The *Horka*^{D} mutation results in replacement of Ala777 with Thr, which we suggest causes chromosome instability by increasing the affinity of Lodestar for chromatin.

MOST of the proteins required in early embryogenesis are maternally provided; it is generally agreed that little if any zygotic gene expression occurs during the onset of embryogenesis (Derenzo and Seydoux 2004; Tadros and Lipshitz 2005). To dissect the commencement of embryogenesis in *Drosophila melanogaster*, we isolated dominant female-sterile (*Fs*) mutants (Erdelyi and Szabad 1989; Szabad *et al.* 1989) and focused our attention on those that terminate embryogenesis at or shortly after fertilization.

Horka^{D} is one such *Fs* mutation (Erdelyi and Szabad 1989). It is a gain-of-function mutation (Erdelyi and Szabad 1989) that results in chromosome nondisjunction and renders chromosomes unstable during spermatogenesis, causing them to be lost in the resulting zygotes (Szabad *et al.* 1995). Loss of the chromosomes leads to the formation of diplo//haplo mosaics, including *X*$\underline{\boldsymbol{X}}$*//X*0, female//male mosaics, and gynandromorphs (Szabad *et al.* 1995). ($\underline{\boldsymbol{X}}$represents chromosomes derived from the *Horka*^{D} males.) In fact, *Horka*^{D} has been used as a "tool" to generate genetic mosaics (Szabad and Nothiger 1992; Zallen and Wieschaus 2004; Villanyi *et al.* 2008).

To determine the function of the gene carrying the *Horka*^{D} mutation, we first mapped *Horka*^{D} by screening for duplications that can ameliorate the Horka^{D} mutant phenotype in embryos of the *Horka*^{D}*/+/+* females. This revealed the dominant-negative (antimorphic) nature of the mutation. We generated *horka*^{rvP} *P*-element-induced alleles (hereafter called pseudorevertants) that no longer exhibit the dominant mutant phenotype and used them to map and then isolate the gene. We discovered that *Horka*^{D} is an allele of *lodestar* (*lds*), which encodes a member of the Snf2 family of the helicase-related genes (Girdham and Glover 1991; Liu *et al.* 1998; Flaus *et al.* 2006). Our results suggest that the lodestar (LDS) protein is involved in progression from metaphase to anaphase of the cell cycle. We propose that the lodestar protein altered by *Horka*^{D} disturbs chromatin organization and segregation and renders chromosomes unstable. It appears thus that the LDS protein is one of the many components engaged in maintaining genome integrity (Takada *et al.* 2003; Allard *et al.* 2004; Musacchio and Salmon 2007).

MATERIALS AND METHODS

***Horka*^{D}, *horka*^{rv}, and *Horka*^{RR} alleles:** *Horka*^{D} was induced by EMS on an isogenic third chromosome labeled with the *mwh* and the *e* recessive marker mutations (Erdelyi and Szabad 1989). For an explanation of the genetic symbols, see FlyBase at http://flybase.bio.indiana.edu. The *horka*^{rv} revertant alleles were generated through second mutagenesis of *Horka*^{D}: the *horka*^{rvE1} allele by EMS (Erdelyi and Szabad 1989) and the

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horka^{rvP} alleles through mutagenesis with the normal *P* elements. For induction of the *horka*^{rvP} alleles, dysgenic *Horka*^{D}*/ TM3, Sb Ser* males were mated with *TM3, Ser/ TM1, Me* virgin females. The *P* elements were hopping in these males and might have become inserted into the *Horka*^{D} allele. (The dysgenic *Horka*^{D}*/ TM3, Sb Ser* males were generated by crossing *M* cytotype *TM3, Sb Ser/ TM6B, Tb* females with *P* cytotype *Horka*^{D}*/ TM3, Sb Ser* males. The latter males resulted 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 resulting females, who mated with the sibling males, were screened for offspring production. Only the *horka*^{rvP}*/ TM3, Ser* and the *horka*^{rvP}*/ TM1, Me* females give rise to progeny, allowing a direct selection of the *horka*^{rvP} phenotypically revertant alleles. (To avoid the isolation of clusters of the *horka*^{rvP} alleles, groups of 10 dysgenic males were mated with *TM3, Ser/ TM1, Me* females and the descendants from the parallel crosses were screened separately.)

The *P*-element insertion sites in the *horka*^{rvP} alleles were determined by standard *in situ* hybridization on salivary gland chromosomes, using DIG-labeled *P*-element DNA probe.

To remobilize the *P* elements in the *horka*^{rvP} revertants and isolate *Horka*^{RR} alleles (***r***evertant alleles of the *horka*^{rvP} revertants), we constructed *horka*^{rvP}*/ TM3,* Δ*2-3* females and males. The *Horka*^{RR} originated most likely through precise excision of the *P* element from the *horka*^{rvP} alleles. 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 the *horka*^{rvP} alleles was analyzed in outcrosses with *y v f mal* females and measured through the frequency of *XX*// *X*0, female// male mosaics among the descending *XX* zygotes (*cf.* Szabad *et al.* 1995).

Drosophila cultures used in the study were kept at 25°.

The *Horka*^{D}*/Dp(3;3)* combinations: *Horka*^{D} was mapped to the right arm of the third chromosome (Erdelyi and Szabad 1989). To determine the approximate location and the nature of *Horka*^{D} (whether it is antimorphic or neomorphic), we constructed *Horka*^{D}*/Dp(3;3)* 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 third chromosome. The resulting *Horka*^{D}*/Dp(3;3)* females were mated with wild-type males and the fate of their resulting embryos was monitored. Males were mated with *y v f mal* females and the subsequent generation was screened for *XX*// *X*0 mosaics.

Localizing the *horka*^{rv} alleles and complementation analysis: To locate the *horka*^{rv} alleles and to determine the loss-of-function mutant phenotype, we combined the *horka*^{rv} alleles (as well as *Horka*^{D}) with *Df(3R)* deficiencies and analyzed the *horka*^{rv}*/*− (and the *Horka*^{D}*/*−) flies. (The − symbol stands for either of the deficiencies that remove the *Horka*^{D}-identified locus.) The studied *Horka*^{D}*/*− and the *horka*^{rv}*/*− hemizygotes were produced by crossing *Df(3R)dsx*^{15}*/ TM6B, Tb* females with *horka*^{rv}*/ TM6B, Tb* or with *Horka*^{D}*/ TM6B, Tb* males.

To determine whether the *horka*^{rv} alleles identify a gene with already existing mutant alleles, we carried out complementation analyses between *horka*^{r} and mutant alleles of the nearby genes, *lds*, *dsx*, and *CG10445* (see Figure 3). (Mutant alleles of the *CG10445* gene were generated in our laboratory; I. Belecz and J. Szabad, unpublished observations.)

Characterization of mutant phenotypes: To describe the *Horka*^{D}- and the *horka*^{rv}-associated mutant phenotypes, ovaries, testes, and eggs/embryos of *Horka*^{D}*/*+; *Horka*^{D}*/*− and *horka*^{rvP2}*/*− females and males were dissected and fixed according to González and Glover (1993). The stage 14 oocytes were immunostained according to 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 1:1 mixture of 4% paraformaldehyde:heptane or in a 1:1 mixture of methanol:heptane, and 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, St. Louis) in PBST for 90 min at room temperature.

For immunological detection of the microtubules, we used the DM1A monoclonal anti-α-tubulin antibody (1:1000, overnight at 4°; T6199, Sigma). The centrosomes were detected using an anti-centrosomin antibody (Heuer *et al.* 1995). 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 nonspecific components were depleted through preincubation of the serum in dechorionated and heptane-permeabilized eggs of *horka*^{rvP2}*/*− females, which do not contain LDS protein. The anti-LDS antibody was applied at a 1:200 dilution in 1% BSA in PBST. The embryos were incubated in secondary antibodies for 3 hr at room temperature or overnight at 4°. The secondary antibodies were either anti-mouse or anti-rabbit IgG (Sigma) and were labeled with FITC, Texas-Red, or Alexa Fluor-633. To detect DNA, the embryos were stained with DAPI following incubation with the secondary antibody. After several rinses in PBST, the embryos and the testes were mounted in Aqua PolyMount (Polysciences, Warrington, PA). 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 (1989).

Cytoplasm injections: To analyze the effect of *Horka*^{D} on the cleavage divisions, we injected ~300 pl of cytoplasm (~3% of the total egg volume) from eggs of wild-type (as the control) and *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 a maximum of 30 min old and the injected embryos were in the 9th–11th cleavage cycle of embryogenesis. Effect of the injected cytoplasm was followed in time through a series of optical sections generated in an Olympus FV1000 confocal microscope. The injections were carried out at 25°.

Germ-line chimeras: To determine whether the *Horka*^{D}-and the *horka*^{rvP2}*/*−-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. Tables 2 and 3 list the crosses from which the donor and the host embryos originated. Pole cells were collected from single blastoderm-stage donor embryos and transplanted into two to three host blastoderm embryos. 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 a normal environment for development of the received female pole cells. Pole cells of *y v f mal* embryos were transplanted into *Horka*^{D}*/*+ and *horka*^{rvP2}*/*− host embryos, and the developing adults were analyzed for the presence of the implanted *y v f mal* germ-line cells. The flies that developed following pole cell

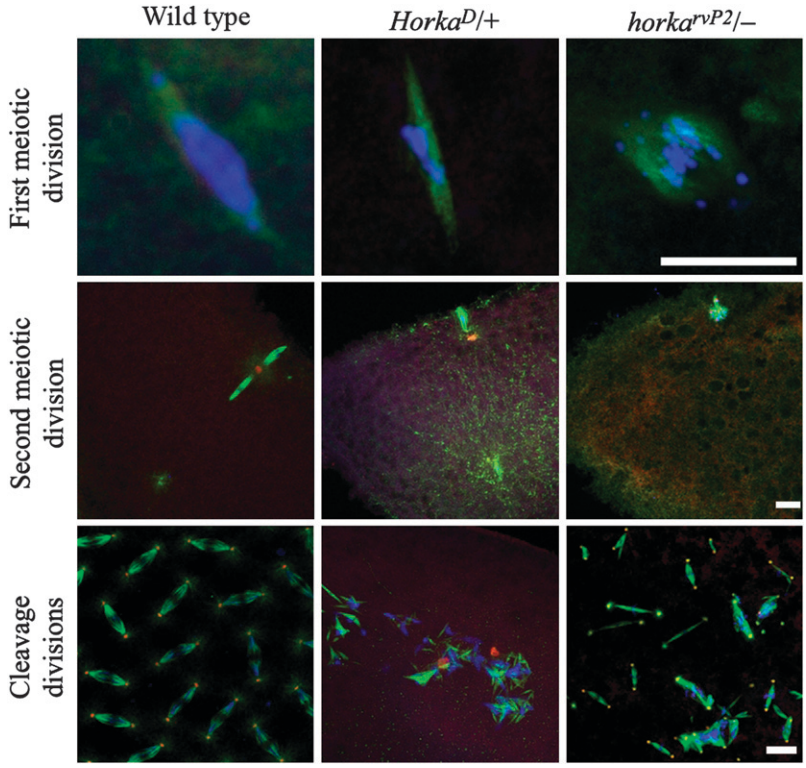

FIGURE 1.—Meiotic and cleavage divisions in the egg primordia and in eggs of wild-type, *Horka*^{D}/+, and *horka*^{rvP2}/− females. In the optical sections the microtubules appear in green, the centrosomes and the spindle pole bodies in red, and the DNA in blue. Detachment of one of the spindles (dashed circle) is a typical feature of the second meiotic division in the *Horka*^{D}/+ females. Although the meiotic divisions proceed as in wild type in ~70% of the cases, abnormal meiotic spindles develop in a number of egg primordia in the *horka*^{rvP2}/− females. Note that most centrosomes cannot nucleate astral microtubules and several of the spindles are abnormal in embryos of the *horka*^{rvP2}/− females. Bars, 10 μm.

transplantation were mated with appropriate partners, as described in Tables 2 and 3, and tested for germ-line chimerism.

Inverse PCR: To clone the *Horka*^{D}-identified gene, we used the inverse PCR technique and amplified DNA sequences flanking the *P* elements in three of the *horka*^{rvP} alleles. Briefly, we isolated DNA from *horka*^{rvP}-carrying males and digested the DNA with *Hin*PI or with *Msp*I. The digested genomic DNA was ligated overnight at 4°, ethanol precipitated, and resuspended in distilled water. Two PCR reactions were conducted next. In the first reaction, the outward primers were designed on the basis of the terminal sequences of the *P*-element adjacent to the cut site. (The primers are described in supplemental Table 2.) Because the first PCR did not 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. (See supplemental Table 2.) Products from the second PCR reactions were isolated, purified, and sequenced in an IBI automated sequenator on both strands. The resulting sequence information allowed us to precisely position the *P*-element insertion sites on the Drosophila genome sequence (ADAMS *et al.* 2000).

Molecular cloning and sequencing of *Horka*^{D}: DNA of *Horka*^{D}/– and *mwh e* (as the control) males served as a template in a set of PCR reactions to produce DNA fragments for sequencing. The PCR primers were designed on the basis of the *lds* gene sequence (EMBL nucleotide sequence database, accession no. X62629). Sequencing of the PCR products was carried out in an IBI sequenator on both strands.

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 Figure 3). The 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 a w^{1118} background by standard procedures. The *TG*$^{+}$ transgene became inserted into the second chromosome. The *TG*$^{+}$ transgene was combined, in appropriate genetic crosses, with the *Horka*^{D}, the *horka*^{rv}, and the *lds* mutant alleles to determine whether the *TG*$^{+}$ transgene can overcome the mutant phenotypes associated with the *Horka*^{D} and the *horka*^{rv} alleles.

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

RESULTS

***Horka*^{D} disrupts the meiotic and the early cleavage divisions:** Although the *Horka*^{D}/+ females deposit normal numbers of normal-looking eggs (fertilized as in wild type), cleavage divisions do not commence in >90% of the eggs. Moreover, when cleavage divisions are seen, only ~12 scattered chromosomes appear, along with unusual microtubule bundles (Figure 1). As expected, cuticle fragments, indicators of development beyond the blastoderm stage, never form inside the eggs of the *Horka*^{D}/+ females (Table 1). Abnormal segregation of the chromosomes is already apparent during both the first and the second meiotic divisions in egg primordia of the *Horka*^{D}/+ females (Figure 1). The mutant phenotypes suggest involvement of the *Horka*^{D}-identified normal gene product in chromosome organization, stability, and/or segregation.

Horka^{D} has been reported to be a gain-of-function mutation (ERDELYI and SZABAD 1989). We have now

TABLE 1

Features of the *Horka^{D}*- and the *horka^{rvP2}*-carrying females and males

	Analysis of the females					Analysis of the male offspring[c]		
							*XX//X*0 mosaic	
Genotype	Tested	Test period[a]	Dead embryos with cuticle (%)	Offspring	Rate of offspring production[b]	*XX*	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	6.4×10^{-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^{rvP2}/Df(3R)dsx^{15}	180	15.2	20.6	—	—	194	0	—
horka^{rvP2}/+	11	7.0	3.4	2695	35.0	3433	0	—
horka^{rvP2}/lds$^{98.1}$	85	12.3	21.0	—	—	298	0	—
TG^{+}; horka^{rvP2}/Df(3R)dsx^{15}	5	7.0	11.0	087	31.1	—	—	—
TG^{+}; horka^{rvP2}/lds$^{98.1}$	7	7.0	8	649	33.7	—	—	—

horka^{rvP2} is a functionally null allele and *lds$^{98.1}$* is a *lodestar* null allele (Girdham and Glover 1991).

[a] Average test period per female (days).

[b] Offspring/(female × day).

[c] The males were mated with *y v f mal* females (*XX*) and the *XX* offspring flies were screened for *XX//X*0 mosaics.

[d] Pooled data from 17 *Dp(3;3)* tandem duplications with the exception of *Dp(3;3)Antp^{rv8}*.

confirmed this observation by cytoplasm injection studies. When cytoplasm taken from newly deposited eggs of the *Horka^{D}/+* females was injected into *horka^{+}* embryos in which the chromosomes were highlighted by RFP-tagged histones and the microtubules by GFP-tagged tubulins, it 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. (See Videos 1 and 2 in the supplemental material.) Toxicity of the *Horka^{D}*-derived egg cytoplasm is best illustrated by the fact that not a single embryo survived the cytoplasm injections. (Injection of wild-type egg cytoplasm did not alter progression of the cleavage cycles, and larvae hatched from almost all of the injected embryos.)

***Horka^{D}* resides between 84D5–8 and 85F5–8:** *Horka^{D}* has been mapped to the right arm of the third chromosome (Erdelyi and Szabad 1989). To more accurately locate *Horka^{D}*, we generated a series of *Horka^{D}/Dp(3;3)* flies and analyzed the embryos of the females and searched for *X<u>X</u>//X*0 mosaics among the *X<u>X</u>* offspring of the males. If *Horka^{D}* is a dominant-negative mutation, (i) a less severe mutant phenotype was expected to develop inside eggs of the *Horka^{D}/Dp(3;3)$^{+}$* females (compared to the *Horka^{D}/+* control) and (ii) reduced frequency of the *X<u>X</u>//X*0 mosaics was expected to appear in the offspring of the *Horka^{D}/Dp(3;3)$^{+}$* males. We used 18 *Dp(3;3)* tandem duplications that, in aggregate, fully covered the entire right arm of the third chromosome. Of these duplications tested, only *Dp(3;3)Antp^{rv8}* ameliorated the *Horka^{D}* imposed defects, such that embryogenesis inside eggs of the *Horka^{D}/Dp(3;3)Antp^{rv8}* females proceeded well beyond the initial steps. Not only did cuticle fragments form in almost 100% of the eggs, but also three offspring were produced by the *Horka^{D}/Dp(3;3)Antp^{rv8}* females (Table 1). Thus the results of mapping located *Horka^{D}* within the 84D5–8 and 85F5–8 cytological interval (Figure 2). Moreover, the ability of *Dp(3;3)Antp^{rv8}* to ameliorate the dominant-negative nature of *Horka^{D}* argues that the mutant and normal gene products participate in the

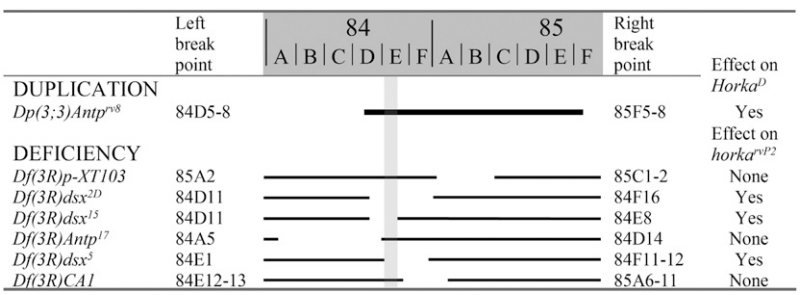

Figure 2.—Duplication and deficiency mapping of the *Horka^{D}* and the *horka^{rvP2}* mutations. The thick bar represents the *Dp(3;3)Antp^{rv8}* tandem duplication. Missing sections in the *Df(3R)* deficiencies illustrate the eliminated chromosome segments. The deficiencies located the *horka^{rv}*-identified locus between 84E1 and 84E8.

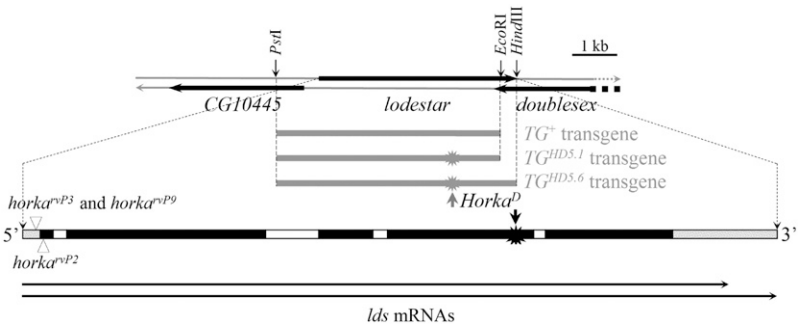

FIGURE 3.—Organization of the region around the *lodestar* gene in the 84E5 cytological region. The *lds* gene encodes the formation of two mRNAs that differ in the last ~500 nucleotides. Stippled boxes correspond to sequences that encode the 5′ and the 3′ untranslated regions of the *lds* mRNAs, and open and solid boxes represent introns and exons, respectively. The *P*-element insertion sites in $horka^{rvP3}$, $horka^{rvP9}$, and $horka^{rvP2}$ are labeled and also the position of the $Horka^{D}$ mutation (✹). The shaded lines represent different transgene types.

same process and the mutant gene product is truly antimorphic (*i.e.*, it impedes function of the normal counterpart).

We cannot discern whether the $Horka^{D}$-related dominant paternal effect is also of dominant-negative nature because the frequency of the *XX*//*X*0 mosaics varied between 2.1 and 28.8% in the *XX* offspring of the $Horka^{D}/Dp(3;3)$ males. The variation in the *XX*//*X*0 mosaic frequencies is most likely related to the different genetic backgrounds of the $Horka^{D}/Dp(3;3)$ males (SZABAD *et al.* 1995).

The $horka^{pseudorevertant}$ ($horka^{rv}$) alleles: To learn the function of the gene carrying the $Horka^{D}$ mutation we induced $horka^{rvP}$ pseudorevertant alleles through mutagenesis of $Horka^{D}$ with a normal *P* element. Of the 15,600 *P*-element-mutagenized females tested, 9 independent ones were fertile and gave rise to one $horka^{rvP}$ pseudorevertant allele each. All the $horka^{rv}$ alleles are lethal both in homozygotes and in *trans*-heterozygotes due to one or more second-site lethal mutations induced during EMS induction of $Horka^{D}$ (*cf.* ERDELYI and SZABAD 1989).

To characterize the $horka^{rvP}$ alleles, we crossed $horka^{rvP}$/*TM3, Sb Ser* males from each of the nine $horka^{rvP}$ lines with *y v f mal* females and searched the offspring for *XX*//*X*0 mosaics (see Table 1). Mosaics appeared (though with very low frequencies) among the offspring of three of the nine $horka^{rvP}$ pseudorevertants ($horka^{rvP5}$, $horka^{rvP6}$, and $horka^{rvP8}$), suggesting that these alleles retain some feature of $Horka^{D}$. Their incomplete loss of the Horka^{D} phenotype is also shown by the strongly reduced fertility of the heterozygous females. Mosaics did not appear in the offspring of the males that were heterozygous for the other six $horka^{rvP}$ alleles and larvae hatched from the vast majority of the eggs deposited by the heterozygous females, indicating the loss-of-function nature of six of the $horka^{rvP}$ mutations. One of the alleles, $horka^{rvP2}$, is a complete loss-of-function mutation (Table 1 and see below). The concurrent loss of dominant female sterility and dominant paternal effect in six of the nine $horka^{rvP}$ alleles shows that the $Horka^{D}$-related dominant mutant phenotypes stem from the same mutation.

The $horka^{r}$ mutations reside within the 84E1–84E8 cytological interval: The $horka^{rv}$ alleles (and also $Horka^{D}$) were combined with deficiencies that remove well-defined regions around the 84E cytological region (Figure 2). The $horka^{rv}$/– hemizygous combinations are viable and the flies develop with the expected frequencies. (The – symbol stands for either of the deficiencies that remove the $horka^{rv}$ identified locus.) The $horka^{rv}$/– hemizygous females either are completely sterile ($horka^{rvP2}$/–; Table 1) or possess reduced fertility: progeny develop from 4–21% of the zygotes in all the other $horka^{rv}$/– combinations. The deficiencies located the $horka^{rv}$-identified locus within the 84E1–E8 cytological region (Figure 2).

The fertility of $horka^{rvP2}$/– males is also very strongly reduced (see supplemental Table 2). The cross in which several hundred $horka^{rvP2}$/– males were mated with several hundred *y v f mal* females yielded only few offspring, none of which was *XX*//*X*0 mosaic (Table 1).

The $Horka^{D}$/– flies are also viable and emerge with the expected frequency. The females deposit normal numbers of normal-looking eggs in which, although normally fertilized, embryogenesis never commences. Fertility of the $Horka^{D}$/– males is also strongly reduced (supplemental Table 2). 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 *XX* offspring flies were *XX*//*X*0 mosaics (Table 1).

It appears that reduced fertility of the $Horka^{D}$ also exhibits a dominant-negative effect on male fertility, as evidenced by the observation that, when sired by $Horka^{D}$/–, $Horka^{D}$/+, or $Horka^{D}/Dp(3;3)Antp^{rv8}$ males, 92, 71, and 59% of the embryos perished during embryogenesis (supplemental Table 2). Because there was no sperm in 98.5% (446/453) 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 (supplemental Table 2) and thus $Horka^{D}$ and $horka^{rvP2}$ do not seem to affect other fertility-related features than sperm production, suggesting that $Horka^{D}$ has little if any effect on the somatic cells (*cf.* LIU and KUBLI 2003).

***In situ* hybridizations confirm that the $Horka^{D}$-identified gene resides in 84E:** To locate the gene carring the $Horka^{D}$ mutation we probed salivary gland chromosomes

in the nine *horka*^{rvP} revertants with labeled *P*-element DNA. There were three to six *P*-element insertions in the right arm of the third chromosome in the different *horka*^{rvP} alleles. The only common *P*-element insertion site appeared in 84E, suggesting that the gene resides in 84E.

The *P* elements of the *horka*^{rvP7} and the *horka*^{rvP9} alleles were successfully remobilized. As a result, two *Horka*^{RR} alleles (***r***evertant alleles of the *horka*^{rvP} mutations) emerged from the 135 chromosomes tested. The *Horka*^{RR} alleles behaved as *Horka*^{D}: *Horka*^{RR}/+ females are sterile and embryos perish inside their eggs, essentially as described for the *Horka*^{D}/+ females. Among the progeny of the *Horka*^{RR}/+ males and *y v f mal* females, 13.9% (14/85) and 13.0% (25/164) of the *X*$\underline{\boldsymbol{X}}$ zygotes developed as *X*$\underline{\boldsymbol{X}}$//*X*0 mosaics in the two *Horka*^{RR} alleles. More important, the *P*-element insertions in 84E were absent in the *Horka*^{RR} alleles, suggesting that the gene carrying the *Horka*^{D} mutation indeed resides at 84E. 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:** We exploited the *P* elements in *horka*^{rvP2}, *horka*^{rvP3}, and *horka*^{rvP9} (each with as few as three *P* elements inserted into 3R) to identify the gene carrying *Horka*^{D}. We amplified sequences adjacent to the *P* elements in an inverse PCR. The DNA sequence of the PCR products was determined, and we analyzed only those originating from 84E. The *P* element resides in the leader sequence of the *lds* gene in *horka*^{rvP3} and in *horka*^{rvP9} (Figure 3 and supplemental Figure 3). In *horka*^{rvP2}, the *P* element is inserted between nucleotides 3,746,261 (G) and 3,746,262 (A) in the first exon of the open reading frame of the *lds* gene. On the basis of this mutant lesion and the observation that the LDS protein is also missing from ovaries of the *horka*^{rvP2}/− females (data not shown), we conclude that *horka*^{rvP2} is a null allele of the *lds* gene.

The positions of the *P* elements in the *horka*^{rvP} alleles identify the *lds* gene (Girdham and Glover 1991). Indeed, the *horka*^{rv} and the *lds* alleles do not complement, so we conclude that *Horka*^{D} is a dominant-negative *lds* allele (Table 1). The *horka*^{rvP2}/*lds*$^{98.1}$ and the *horka*^{rvP2}/*lds*$^{298.8}$ combinations are female sterile and are as strong as the *horka*^{rvP2}/− condition. The *lds*$^{98.1}$ and the *lds*$^{298.8}$ alleles are complete loss-of-function alleles as there is no LDS protein in ovaries of the *lds*$^{98.1}$/− and the *lds*$^{298.8}$/− hemizygous females (Girdham and Glover 1991). Although embryogenesis proceeds beyond the blastoderm stage in ~60% of the eggs of the *horka*^{rvP2}/−, the *horka*^{rvP2}/*lds*$^{98.1}$, and the *horka*^{rvP2}/*lds*$^{298.8}$ females and fragments of cuticles appear in ~21% of their eggs, larvae never hatch. The females are semisterile in all the further *horka*^{rv}/*lds* combinations.

We crossed several hundred *horka*^{rvP2}/*lds*$^{98.1}$ males, which are almost completely sterile, with several hundred *y v f mal* females. None of the recovered 298 *X*$\underline{\boldsymbol{X}}$ offspring were *X*$\underline{\boldsymbol{X}}$//*X*0 mosaic (Table 1). However, *X*$\underline{\boldsymbol{X}}$//*X*0 mosaics appeared among offspring of the *Horka*^{D}/*lds*$^{98.1}$ males (Table 1).

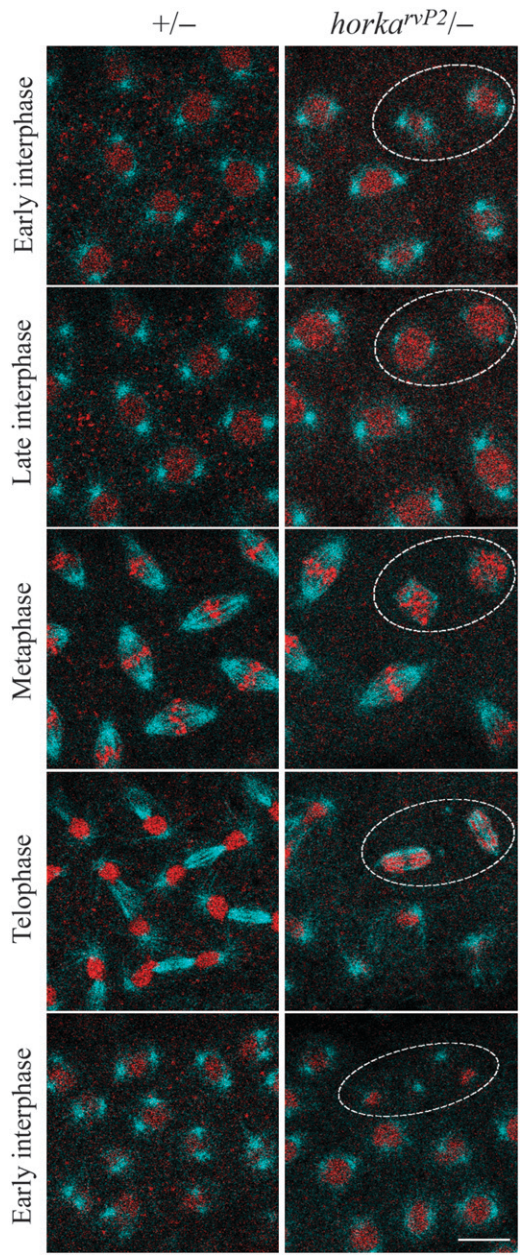

Figure 4.—Impaired centrosome function develops in late cleavage embryos of the *horka*^{rvP2}/− females. Time-lapse optical sections were collected from embryos that derived from +/− (control) and from *horka*^{rvP2}/− females. The chromosomes were labeled by histone-RFP and appear in red, and the microtubules and the centrosomes were highlighted by Jupiter-GFP and are shown in cyan. Nuclei associated with abnormal centrosomes are within dashed circles. Note that while the nuclei drop into the interior of the embryo, the free centrosomes remain in the egg cortex. Bar, 10 μm.

An *lds*$^{+}$-bearing transgene (denoted *TG*$^{+}$) rescues loss-of-function *horka* mutants: To confirm that *Horka*^{D} and *horka*^{rv} alleles indeed identify the *lds* gene we

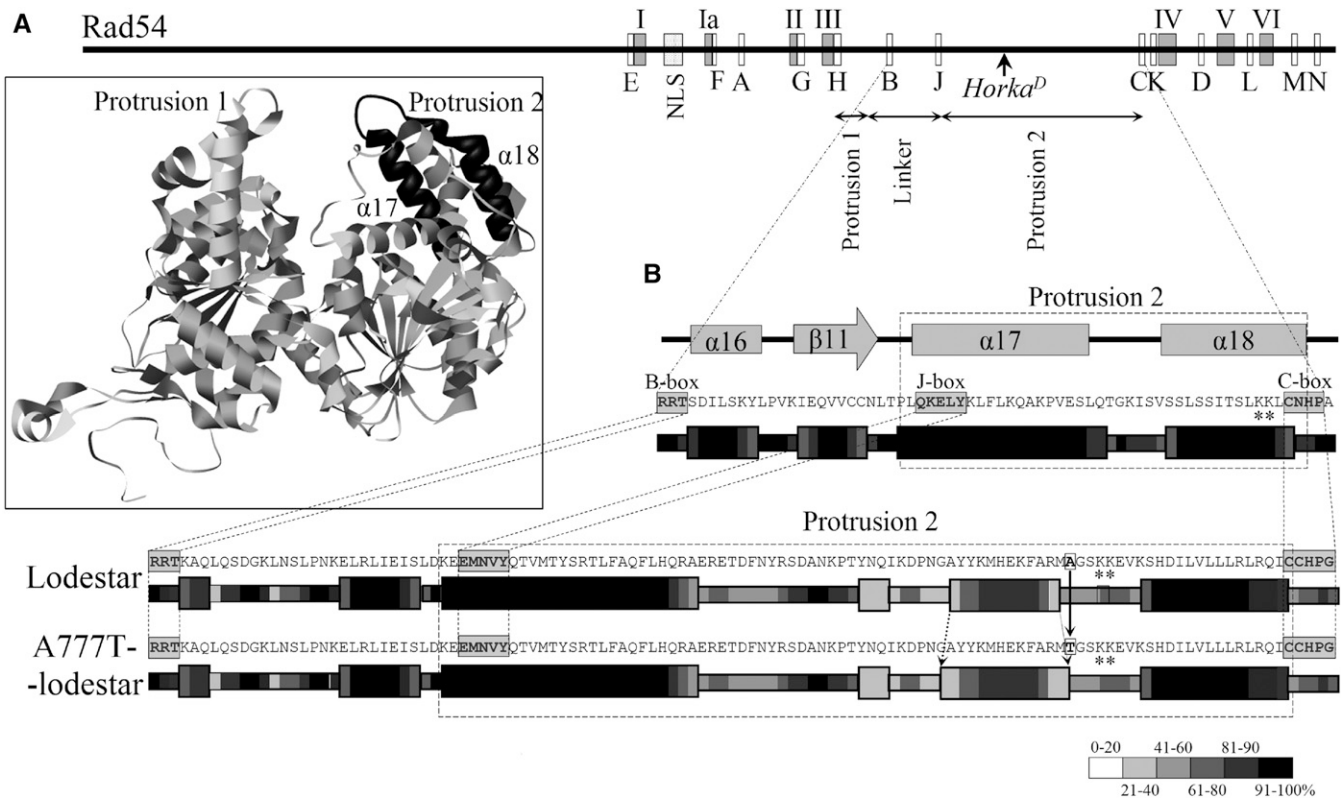

FIGURE 5.—Domain organization of the Rad54, part of the LDS, and the A777T-LDS proteins. (A) The nucleotide triphosphate-binding so-called helicase motifs (I–VI) appear in shaded boxes and the E–N conserved domains are in open boxes in zebrafish Rad54A, a typical member of the Snf2 family of the helicase-related proteins (FLAUS *et al.* 2006). (NLS, putative nuclear localization signal.) (B) The region including the J and the C boxes forms protrusion 2 that is composed of the α17 and α18 helices and the connecting short stretch of amino acids. Protrusion 2 was proposed to interact with the DNA (THOMA *et al.* 2005; FLAUS *et al.* 2006). (See the inset and see www.sanger.ac.uk/cgi-bin/Pfam/swisspfamget.pl?name=P34739.) The presence of the B, the J, and the C boxes and the α17 and α18 helices is apparent in the LDS protein. The KK amino acids near the C box (labeled ** and also present in LDS) 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 α-helix is predicted inside this interconnecting region in the LDS protein. This α-helix became longer by two amino acids in the *Horka*^{D} encoded A777T-LDS protein as compared to LDS. The shaded scale at the bottom right illustrates the likelihood (as determined by the PSIPRED software: http://bioinf.cs.ucl.ac.uk/psipred/) that any amino acid is part of an α-helix.

generated a stable transgenic line (*TG*$^{+}$, inserted into a second chromosome) that covers a 5.1-kb genomic sequence and includes the normal *lds* gene (except the last 500 bp; Figure 3). Although the *TG*$^{+}$; *Horka*^{D}/+ females are sterile, the effects of *Horka*^{D} are ameliorated and cuticle fragments develop inside 93% of their eggs (Table 1). The *TG*$^{+}$ transgene overcomes the sterility of the *horka*^{rvP2}/−, *horka*^{rvP2}/*lds*, and *lds*$^{98.1}$/*lds*$^{298.8}$ females (Table 1). In the presence of *TG*$^{+}$, fertility of the *horka*^{rvP2}/− and the *horka*^{rvP2}/*lds*$^{98.1}$ males is essentially as in wild type (see supplemental Table 2). Evidently, *Horka*^{D} and *horka*^{rv} are alleles of the *lds* gene.

***Horka*^{D} originated through a transition:** The *Horka*^{D} mutation is a single-nucleotide change G^{2424} → A, resulting in the replacement of Ala^{777} by Thr in the lodestar protein (Figures 3 and 5).

Lodestar transgenes that carry the G^{2424} → A mutation (Figure 3) render females sterile. Although cuticle fragments appear inside 25–28% of their eggs, larvae never hatch (Table 1). Crosses between *y v f mal* females and +/+; *TG*^{HD} males yielded *X*$\underline{\mathbf{X}}$//*X*0 mosaics among the *X*$\underline{\mathbf{X}}$ offspring (Table 1). Thus, *Horka*^{D} is a dominant *lds* mutant allele and the *Horka*^{D} phenotypes originated from the same mutation.

Phenotypic analysis of the *horka*^{rvP2} null allele: Cytological analysis revealed abnormal chromosome segregation in ~30% (14/47) of the *horka*^{rvP2}/− egg primordia during the first meiotic division (Figure 1). Similarly, ~38% (5/13) of the second meiotic divisions are unusual as shown by the abnormalities in both chromosome segregation and the formation of unusual spindles (Figure 1).

All eggs of the *horka*^{rvP2}/− females appear normal and are fertilized as in wild type, and although cleavage divisions commence inside ~60% of the eggs, larvae never hatch. Once started, the cleavage divisions proceed more or less normally and cells may form over relatively large areas in the egg cortex and differentiate as indicated by the cuticle fragments that form inside 20.6% of the eggs (Table 1). Although the cuticle

TABLE 2

Features of the *Horka^{D}*/+ germ-line chimeras

Cross to produce the donor embryos: *mwh e/mwh e* ♀♀ × *Horka^{D}/TM3* ♂♂ → Cross to produce the host embryos: *tmII^{gs}/tmII^{gs}* ♀♀ × *tmII^{gs}/TM6* ♂♂			Stock to produce the donor embryos: *y v f mal* → Cross to produce the host embryos: *mwh e/mwh e* ♀♀ × *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.

fragments are usually poorly differentiated, every larval cuticle landmark develops, albeit in different embryos.

Although daughter centrosomes separate appropriately, several of them lose the ability to nucleate microtubules. The centrosome defects lead to the formation of abnormal spindles, which then bring about a distorted arrangement of the chromosomes, a defect known as mitotic catastrophe (Figures 1 and 4; 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 in place. Most of the free centrosomes nucleate microtubules and bring about further abnormalities by disturbing the nearby cleavage spindles. The impaired centrosome function may be related to one or more problems: DNA damage, incomplete replication of the DNA, abnormal chromatin condensation, and/or chromosome segregation. Thus, the loss-of-function mutant phenotype suggests involvement of *lodestar* in the maintenance of genomic integrity.

Characteristic types of defects appear during spermatogenesis in the *Horka^{D}*/− males. As a consequence of nondisjunction, larger- and smaller-than-normal onion stage spermatid nuclei appear side by side (see supplemental Figure 2; *cf.* Szabad *et al.* 1995). Several of the sperm nuclei are displaced from their sperm tip position, and a good number of sperm tails bear no nucleus (see supplemental Figure 2).

Although the onion stage spermatid nuclei appear in the *horka^{rvP2}*/− males as in wild type, the sperm bundles are abnormal: individualization of the sperm is incomplete, a few of the sperm heads are dislocated, and the sperm head is missing from several sperm tails (see supplemental Figure 2). Yet some of the sperm must be functional as the *horka^{rvP2}*/− males are not completely sterile (supplemental Table 2).

The analysis of germ-line chimeras in *Horka^{D}*/+ and *horka^{rvP2}*/− flies: The viability and sterility of the *Horka^{D}*/+ and the *horka^{rvP2}*/− females and reduced fertility of the males suggest that the function of *lodestar* is required

TABLE 3

Features of the *horka^{rvP2}*/− germ-line chimeras

Cross to produce the donor embryos: *horka^{rvP2}/TM6B* ♀♀ × *Df(3R)dsx^{15}/TM3* ♂♂ → Cross to produce the host embryos: *w/w* ♀♀ × *Fs(1)K1237*/Y ♂♂		Stock to produce the donor embryos: *y v f mal* → Cross to produce the host embryos: *horka^{rvP2}/TM6B* ♀♀ × *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^{rvP2}/TM3	8	*horka^{rvP2}/TM3*	2	2
Df(3R)dsx^{15}/TM6B	5	*Df(3R)dsx^{15}/TM6B*	3	4
TM3/TM6B	1	*TM3/TM6B*	1	2
horka^{rvP2}/Df(3R)dsx^{15}	3	*horka^{rvP2}/Df(3R)dsx^{15}*	1	1

Arrows symbolize the direction of pole cell transplantations. *Horka^{D}* was induced by EMS on an *mwh*- and *e*-labeled isogenic chromosome (Erdelyi and Szabad 1989).
[a] The chimeras produced *y v f mal* offspring following test crosses with *y v f mal* partners.

only in the gonads. To determine 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 yet provided a normal environment for development and function of the donor pole cells (Table 2). Three of the female germ-line chimeras produced eggs, and the fate of embryos inside these eggs was essentially identical to that described for embryos of the *Horka*^{D}/+ females. Three sibling male germ-line chimeras were generated and then mated with *y v f mal* females. On average, 3.1% of their *XX* zygotes developed as *XX*//*X*0 mosaics (Table 2). Features of the chimeras clearly show that the *Horka*^{D}-induced 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 from 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 2). Features of the latter types of germ-line chimeras not only revealed the germ-line autonomous nature effect of *Horka*^{D} but also showed that the *Horka*^{D}/+ gonadal soma functions normally.

In the second set of experiments, pole cells of *horka*^{rvP2}/− embryos were transplanted into *Fs(1)K1237*/+ host embryos. Of the developing chimeras three carried *horka*^{rvP2}/− germ-line cells (Table 3). 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*^{rvP2}/− females (Table 1). We also transplanted normal pole cells into *horka*^{rvP2}/− host embryos and analyzed the developing female and male germ-line chimeras. The *horka*^{rvP2}/− flies produced offspring from the implanted germ-line cells (exclusively), showing that the *horka*^{rvP2}/− soma provides full support for the normal germ-line cells (Table 3). It appears that function of *lodestar* is primarily required in the germ line.

DISCUSSION

Nature of the *Horka*^{D}-encoded A777T-LDS protein: *Horka*^{D} is an allele of *lodestar*, which encodes a member of the Snf2 family of the helicase-related proteins that are involved in transcription regulation, DNA repair, recombination, and chromatin unwinding (Flaus *et al.* 2006). The helicase motifs and the other conserved domains contribute to distinctive features in the Snf2 protein family (Figure 5). In Rad54, the only member of the family of known structure, two of the α-helices (α17 and α18) and a short interconnecting region compose protrusion 2, the part of the protein that interacts with DNA (Thoma *et al.* 2005; Flaus *et al.* 2006; Figure 5). The α17 and the α18 helices are present in the LDS protein but the interconnecting region is longer than in Rad54 and contains an α-helix (Figure 5). *Horka*^{D} is a $G^{2424} \rightarrow A$ transition that results in replacement of Ala^{777} by Thr in the interconnecting region. It appears that this amino acid replacement expands the α-helix by two amino acids (see supplemental Figure 1).

Possible function of the LDS protein: The LDS protein is cytoplasmic during interphases of the cleavage mitoses, enters the nucleus during prometaphase, and becomes associated with the chromosomes throughout mitosis, suggesting an involvement of the LDS protein in chromatin/chromosome surveillance during mitosis (Girdham and Glover 1991; supplemental Figure 1). This idea is supported by the loss-of-function mutant phenotype in embryos of the *horka*^{rvP2}/− females: abnormal assembly of the chromosomes during meiosis and mitosis, formation of anastral centrosomes and abnormal spindle apparatus, failures in the cleavage mitoses, fallout of the abnormal cleavage nuclei, and eventual death of the embryos. Similar, if not identical, defects have been reported in embryos of those females defective in (i) spindle assembly checkpoint functions or (ii) the mitotic catastrophe avoidance mechanism (Castedo *et al.* 2004; Musacchio and Salmon 2007; Vakifahmetoglu *et al.* 2008; Yuen and Desai 2008). The latter mechanism operates through the activation of checkpoint kinase 2 (Chk2), whereby the damaged or the incompletely replicated DNA leads to Chk2 activation and resulting inactivation of the centrosomes and the spindles. These events in turn result in blocked chromosome segregation during anaphase and the eventual elimination of those nuclei from the embryonic precursor pool. The Chk2-based mechanism is especially important for maintaining genomic stability during genotoxic stress (Masrouha *et al.* 2003; Takada *et al.* 2003; Brodsky *et al.* 2004; Wichmann *et al.* 2006; Larocque *et al.* 2007). Defects in the Chk2-based mechanism cause mitotic catastrophe (Vakifahmetoglu *et al.* 2008).

LDS does not likely function in the spindle assembly checkpoint because—in contrast to the LDS and the Chk2 proteins—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*^{rvP2}/− females exhibit all the distinctive features of mitotic catastrophe. Largely identical defects have been described for checkpoint kinase 1 (*grapes*, *grp*), checkpoint kinase 2 (*lok* or *maternal nuclear kinase*, *mnk*), and Ataxia telangiectasia-related *mei-41* mutant alleles (Masrouha *et al.* 2003; Brodsky *et al.* 2004; Royou *et al.* 2005; Takada *et al.* 2003, 2007; Wichmann *et al.* 2006; Larocque *et al.* 2007). Functions of the corresponding genes have been implicated in the G2/M checkpoint by "assaying" the status of the DNA and/or the chromatin and in the

elimination of inappropriate nuclei from the pool that serves as a source of the blastoderm cells following the cleavage cycles (Takada *et al.* 2003; Larocque *et al.* 2007). It is possible that the LDS protein might be involved in the same pathway as Chk2, because a few of the embryos of the *mnk/mnk*; *horka*^{rvP2}/– females (lacking both the Chk2 and the LDS proteins) develop to adulthood (our unpublished results). Such an event never happens to embryos of the *horka*^{rvP2}/– females. The role of the LDS protein in chromatin surveillance and cell-cycle progression regulation, however, remains to be clarified.

The requirement of *lodestar* in the germ line and in the soma: Remarkably, *lds* gene function is indispensable in the germ line but not in the soma: although flies develop normally without LDS protein, the meiotic divisions are abnormal in *horka*^{rvP2}/– females, as are the cleavage mitoses in their embryos (Girdham and Glover 1991 and this article). These defects are germline autonomous. In fact, complete or almost complete maternal-effect lethality is a characteristic feature of females that are homozygous for mutant alleles of the genes engaged in the G2/M transition control (Henderson 1999). For example, only ~20% of embryos hatch from eggs of females that are both homozygous for the strong *mnk* mutant alleles and lack Chk2 function (Xu *et al.* 2001; Masrouha *et al.* 2003; Takada *et al.* 2003; Xu and Du 2003; Brodsky *et al.* 2004). Similarly, the *grp* homozygous females, which lack checkpoint kinase 1, are sterile; their embryos suffer from abnormal cortical nuclear divisions and do not cellularize (Yu *et al.* 2000; Jaklevic *et al.* 2006; Takada *et al.* 2007). Females homozygous for the Ataxia telangiectasia-related *mei-41* strong mutant alleles are, in effect, sterile (Laurencon *et al.* 2003; Larocque *et al.* 2007).

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