okra and *spindle-B* encode components of the *RAD52* DNA repair pathway and affect meiosis and patterning in *Drosophila* oogenesis

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okra (*okr*), *spindle-B* (*spnB*), and *spindle-D* (*spnD*) are three members of a group of female sterile loci that produce defects in oocyte and egg morphology, including variable dorsal-ventral defects in the eggshell and embryo, anterior-posterior defects in the follicle cell epithelium and in the oocyte, and abnormalities in oocyte nuclear morphology. Many of these phenotypes reflect defects in *grk-Egfr* signaling processes, and can be accounted for by a failure to accumulate wild-type levels of Gurken and Fs(1)K10. We have cloned *okr* and *spnB*, and show that *okr* encodes the *Drosophila* homolog of the yeast DNA-repair protein Rad54, and *spnB* encodes a Rad51-like protein related to the meiosis-specific *DMC1* gene. In functional tests of their role in DNA repair, we find that *okr* behaves like its yeast homolog in that it is required in both mitotic and meiotic cells. In contrast, *spnB* and *spnD* appear to be required only in meiosis. The fact that genes involved in meiotic DNA metabolism have specific effects on oocyte patterning implies that the progression of the meiotic cell cycle is coordinated with the regulation of certain developmental events during oogenesis.

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The anterior-posterior and dorsal-ventral axes of the *Drosophila* embryo are established during oogenesis by a series of intercellular signaling events that generate asymmetries in the developing egg chamber, which are subsequently transmitted to the egg. Early in oogenesis, signaling between the germ-line-derived oocyte and the overlying somatic follicle cells specifies posterior follicle cell fates. These cells, in turn, signal back to the oocyte, initiating a reorganization of the microtubule network that defines the anterior-posterior axis of the oocyte and embryo. In mid-oogenesis, signaling from oocyte to follicle cells specifies dorsal follicle cell fates, and this in turn restricts the activation of a second follicle cell to oocyte signaling process that defines the dorsal-ventral axis of the embryo (for review, see Ray and Schüpbach 1996).

Both of these oocyte to follicle cell signaling events are mediated by a single signaling system involving the *gurken* (*grk*) and *Epidermal growth factor receptor* (*Egfr*) genes. *grk* and *Egfr* are a ligand/receptor pair: *grk* en-

codes a TGF- α -like protein that is expressed in the germ-line and localized to the oocyte (Neuman-Silberberg and Schüpbach 1993, 1996); *Egfr* encodes the *Drosophila* homolog of the EGFR (Livneh et al. 1985) and is expressed in the somatic follicle cells (Kammermeyer and Wadsworth 1987; Sapir et al. 1998) in which it acts as a receptor for *grk* in these signaling events. The mutant phenotypes of *grk* and *Egfr* reflect their roles in anterior-posterior and dorsal-ventral patterning during oogenesis. Eggs produced by *grk* or *Egfr* mutant females have a duplication of anterior chorion structures at their posterior ends, reflecting a defect in the specification of posterior follicle cell fates (Schüpbach 1987; González-Reyes et al. 1995; Roth et al. 1995). The eggs also lack dorsal appendage material, reflecting a defect in the specification of dorsal follicle cell fates (Schüpbach 1987). The polarization of the anterior-posterior axis of the oocyte and embryo, and the polarization of the dorsal-ventral axis of the embryo, are also defective in the mutants. In the oocyte, RNAs that are normally localized to the anterior cortex in wild type, like the *bicoid* (*bcd*) mRNA, are found at both the anterior and posterior poles, whereas RNAs localized to the posterior pole, like the *oskar* (*osk*) mRNA, are found mislocalized to the middle (González-Reyes et al. 1995; Roth et al. 1995). In the embryo there is an expansion of ventral pattern elements at the ex-

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pense of more dorsal ones, reflecting a ventralization of the embryonic dorsal-ventral axis (Schüpbach 1987; Roth and Schüpbach 1994).

In addition to *grk* and *Egfr*, a number of other genes have been identified that are required either in the germline or the follicle cells to regulate or transmit the *grk-Egfr* signal. In the germline, several genes, in particular *fs(1)K10* (*K10*), have been shown to be required for the proper localization of the *grk* mRNA within the oocyte, and females mutant for these genes give rise to dorsalized eggshells and embryos that reflect this mislocalization of *grk*. Another germline required gene, *cornichon* (*cni*) has been shown to be involved in the secretion or activation of Grk. In the follicle cells, components of the Ras signaling pathway have been implicated in the transmission of the *grk-Egfr* signal from receptor to the nucleus (for review, see Ray and Schüpbach 1996).

To identify other genes involved in this signaling process, we focused on a group of female sterile loci on the second and third chromosomes that produce ventralized eggshells similar to those produced by mutants in the *grk-Egfr* pathway. These genes include *okra* (*okr*), *deadlock* (*del*), *squash* (*squ*), *zucchini* (*zuc*), *aubergine* (*aub*), and *vasa* (*vas*) on the second chromosome (Schüpbach and Wieschaus 1991), and the spindle genes (*spnA*, *spnB*, *spnC*, *spnD*, and *spnE*) on the third (Tearle and Nüsslein-Volhard 1987). Recent studies on *aubergine* (Wilson et al. 1996) and the spindle genes (González-Reyes et al. 1997) have provided evidence that mutations in these genes affect *grk-Egfr* signaling.

We have concentrated on three genes, *okr*, *spnB*, and *spnD*. We show here that mutations in these genes produce defects in the oocyte and embryo that are consistent with a role in regulating the *grk-Egfr* signaling process. Our results indicate that many of the patterning defects produced by these mutations are the result of a failure to accumulate wild-type levels of Grk and K10 protein in the oocyte. We have cloned *okr* and *spnB*, and find that the genes encode two components of the yeast *RAD52* DNA repair pathway. In light of these homologies, we have investigated a requirement for the genes in mitotic and meiotic DNA repair, and find that *okr* is required for both mitotic DNA repair and meiotic recombination, whereas *spnB* and *spnD* are required only for recombination. These data provide evidence that the progression of meiotic events in the oocyte nucleus is providing cues to the cytoplasm that are necessary for the proper regulation or timing of developmental processes.

Results

okr, *spnB*, and *spnD* affect D/V patterning in the eggshell and the embryo

The predominant phenotype produced by females mutant for *okr*, *spnB*, and *spnD* is a ventralization of the eggshell, reflected in a loss of dorsal appendage material that is similar to the phenotype produced by mutations

in the *grk-Egfr* pathway. However, unlike *grk* and *Egfr* alleles, which produce fairly discrete ventralized phenotypes, all alleles of *okr*, *spnB*, and *spnD* produce a broad spectrum of ventralization. In addition, the mutant females also produce eggshell phenotypes that are not observed in *grk-Egfr* mutants, including dorsalized eggs with extra appendage material or multiple appendages as well as small eggs, although these phenotypes are comparatively rare. The majority of the eggs produced by *okr*, *spnB*, and *spnD* mutant females, including those that are only mildly ventralized, do not hatch and show no indication of embryonic development.

To quantify the ventralization observed in these mutants, eggshells were assigned to one of four phenotypic classes. Class 1 eggs resemble wild type with two normal dorsal appendages, class v2 eggs have two dorsal appendages that are fused at the base, class v3 eggs have a single dorsal appendage, and class v4 eggs have little or no dorsal appendage material (Fig. 1A–D, Table 1). Females mutant for alleles of *okr*, *spnB*, and *spnD* produce eggs in all four classes, and this variability is not caused by differential expressivity, as a single female produces the full range of phenotypes. Although all three genes give rise to the same range of phenotypes, differences in the distribution of eggs among the various classes reflect the severity of a particular genotype (Table 1). We have observed that the *spnB* and *spnD* eggshell phenotypes become more severe over time. Newly eclosed *spnB* and *spnD* mutant females produce 90%–95% class 1 eggs in the first day after mating, and some of these eggs hatch and give rise to viable progeny. By the fourth day, however, the mutant females produce only 10%–20% class 1 eggs, and there is a corresponding increase in the percent of class 4 eggs. We do not observe a change in the severity of the *okr* eggshell phenotype with time.

A characteristic of mutations in the *grk-Egfr* signaling pathway is that they affect patterning in both the eggshell and embryo. The embryos that develop within the ventralized eggshells produced by *grk* and *Egfr* mutant females are also ventralized, and show an expansion of the mesodermal anlage (Schüpbach 1987; Roth and Schüpbach 1994). To determine if *okr*, *spnB*, and *spnD* affect embryonic patterning as well as eggshell patterning, we examined the expression of Twist (*Tw*), a mesodermal marker (Thisse et al. 1988), in the mutant embryos. Even though only a small percentage of the mutant embryos develop to the cellular blastoderm stage, we find that those that do show a variable expansion of the mesoderm (Fig. 1E–G), ranging from cases in which the mesoderm is fairly normal to cases in which it encompasses most of the blastoderm. Notably, this expansion is always more severe at the posterior than the anterior. In addition to these phenotypes, we also see cases in which the mesoderm is normal at the anterior end of the embryo, but posteriorly splits into two independent domains that run up the lateral sides of the embryo and meet at the dorsal midline (Fig. 1G). Apart from the difference in ventralization along the anterior-posterior axis, these ventralized phenotypes are similar to those that have been observed in *grk* and *Egfr* mutant embryos,

Figure 1. Dorsal-ventral patterning defects in *okr*, *spnB*, and *spnD*. (A–D) Ventralized eggshells representing the four phenotypic classes described in the text. Anterior is up. (A) Class 1 (B) class v2 (C) class v3 (D) class v4. (E–G) Ventralization of the embryos in *okr*, *spnB*, and *spnD*: Cellular blastoderm embryos stained for the mesodermal marker Twist, anterior is to the left, dorsal is up, except for the embryo in G, which is shown from a ventral view. Mutant embryos show ventralization of the embryonic dorsal-ventral axis as evidenced by the expansion of the mesoderm. (E) Mutant embryo with a fairly normal mesodermal domain that is only slightly expanded at the posterior. This differential ventralization along the anterior-posterior axis is characteristic of the phenotype. (F) A more severe example than that in E with a posterior expansion of the mesoderm extending to almost 50% egg length (in some cases the entire embryo expresses Twist). (G) An example of an embryo in which the posterior expansion has resulted in a split mesodermal domain that runs up the lateral sides of the embryo and meets at the dorsal midline. Splitting of the mesodermal domain has been observed in strong combinations of *grk* and *Egfr* (Roth and Schüpbach 1994), but the process generating the duplication is not understood. (H) Anterior-posterior patterning defects in *okr*: Chorion of *okr^{AB}/Df(2L)JS17*. In up to 42% of the eggs laid by *okr^{AB}/Df(2L)JS17* females, a second micropyle is observed at the posterior pole of the egg (micropyles are indicated by arrows) indicating that the follicle cells have adopted an anterior fate.

and suggest that *okr*, *spnB*, and *spnD* affect dorsal-ventral patterning via an effect on *grk-Egfr* signaling.

okr affects anterior-posterior patterning in the egg chamber

In addition to the dorsal-ventral patterning defects described above, *okr* mutants share another phenotype with mutants in the *grk-Egfr* signaling pathway: They produce eggs that often have a second micropyle at the posterior end (Fig. 1H). This phenotype appears in ~2% of the eggs laid by females homozygous for amorphic *okr* alleles, and in 42% of the eggs laid by females mutant for the more severe antimorphic alleles. This follicle cell defect can also be visualized with molecular markers:

We find that 77% of the egg chambers from strong *okr* mutations show *dpp* expression at both the anterior and posterior poles instead of the normal restricted expression in anterior follicle cells (Twombly et al. 1996). In these mutant ovaries, we also observed a defect in *bcd* RNA localization: We find that 5% of the egg chambers show localization of *bcd* to both the anterior and posterior poles of the oocyte, indicating that the anterior-posterior polarity of the oocyte is also affected. These data are consistent with the hypothesis that *okr* affects both the early (anterior-posterior) and late (dorsal-ventral) *grk-Egfr* signaling processes. However, we have found that the appearance of second micropyles on *okr* mutant eggs does not necessarily reflect the severity of the dorsal-ventral defect: Second micropyles are sometimes observed on eggs with normal dorsal-ventral polarity (see Fig. 1H), and strongly ventralized eggs do not necessarily have a second micropyle. This uncoupling of the two phenotypes implies that *okr* can affect the early *grk* signaling process independently from the later one. In *spnB* and *spnD* mutant eggs, we do not observe a significant number of second micropyles, nor have we seen duplication of *dpp* or mislocalization of *bcd* in the mutant ovaries.

okr, *spnB*, and *spnD* affect *grk* RNA localization and protein accumulation

To more precisely establish the role of *okr*, *spnB*, and *spnD* in *grk-Egfr* signaling, we have looked more directly at their effects on the signaling process. Specifically, because the three genes are required in the germline (our observations, for *spnB* and *spnD* see also González-Reyes et al. 1997), we have examined the effect of the mutants on the expression and localization of *grk* RNA and protein. In wild-type ovaries, *grk* RNA is localized to the oocyte during the early stages of oogenesis, and then, in mid-oogenesis, it is localized within the oocyte, first transiently in an anterior-cortical ring (stage 8), and then to a dorsal-anterior patch overlying the oocyte nucleus (stages 9 and 10). In *okr* mutant ovaries, *grk* RNA is correctly localized to the oocyte in early stages. In mid-oogenesis, however, we find instances of persistent localization of the RNA in an anterior-cortical ring. The *spnB* and *spnD* mutant phenotypes are more severe. In stage 9, 85% of the mutant egg chambers show persistent localization of *grk* RNA in the anterior-cortical ring (see also, González-Reyes et al. 1997). In stage 10, the *spnB* and *spnD* mutant egg chambers show a range of phenotypes including cases in which the RNA is normally localized, others in which it is only partially localized, others in which it is still present in an anterior cortical ring, and others in which the level of RNA is reduced or undetectable (data not shown).

In addition to their effects on *grk* mRNA localization in the oocyte, *okr*, *spnB*, and *spnD* also affect the accumulation of *grk* protein. In wild-type egg chambers, Grk is restricted to the oocyte, and in mid-oogenesis, it is localized to a dorsal-anterior patch (Neuman-Silberberg and Schüpbach 1996; Fig. 2A,B). In *okr* mutant ovaries,

Table 1. Phenotypes produced by *okr*, *spnB*, and *spnD* mutant females

A. Eggshell phenotypes of representative alleles as homozygotes and hemizygotes					
Genotype	N ^a	Eggshell phenotype (%)			
		1	v2	v3	v4
<i>okr^{RU}/okr^{RU}</i>	960	43	30	22	5
<i>okr^{RU}/Df(2L)JS17</i>	866	65	27	6	2
<i>okr^{AB}okr^{AB}</i>	479	10	45	43	2
<i>okr^{AB}/Df(2L)JS17</i>	497	79	14	4	3
<i>spnB¹⁵³/spnB¹⁵³</i>	543	29	32	24	15
<i>spnB¹⁵³/Df(3R)trxE12</i>	888	38	32	29	1
<i>spnB^{BU}/spnB^{BU}</i>	828	19	11	10	60
<i>spnB^{BU}/Df(3R)trxE12</i>	929	48	19	23	10
<i>spnD¹⁵⁰/spnD¹⁵⁰</i>	383	14	7	15	64
<i>spnD¹⁵⁰/Df(3R)trxE12</i>	903	8	4	9	79

B. Eggshell phenotypes in double mutant combinations with fs(1)K10							
Genotype	N	Eggshell phenotype (%)					
		d3 ^b	d2 ^b	1	v2	v3	v4
<i>K10</i>	374	99	1				
<i>K10; grk^{HK}/+</i>	511	61	28	9	1	1	
<i>K10; spnB¹⁵³/+</i>	150	92	8				
<i>K10; spnB¹⁵³/spnB⁰⁵⁶</i>	218	39	31	22	6	2	
<i>K10/+; spnB¹⁵³/spnB⁰⁵⁶</i>	325	—	—	9	8	41	42
<i>K10; okr^{AA}/Df(2L)JS17</i>	581	75	13	1	3	6	2

^a(N) Total number of eggs scored that fall into the four classes shown in the table. Dorsalized and small eggs, which account for <5% of the total, were not scored in this experiment. For *spnB* and *spnD*, collections were made after at least 5 days at 25°C.

^b(d2, d3) Dorsalized eggshells were classified as follows: (d2) intermediate dorsalization resulting in eggs with two dorsal appendages spaced far apart, i.e., shifted laterally; and (d3) strongly dorsalized eggs with dorsal appendage material extending around the lateral and ventral side of the egg (Wieschaus et al. 1978).

levels of Grk are variably reduced throughout oogenesis. Within a single ovariole, egg chambers expressing Grk can alternate with egg chambers that do not (Fig. 2C,D). In *spnB* and *spnD* mutant egg chambers, the early accumulation of Grk in the oocyte is normal (Fig. 2E). By mid-oogenesis, however, the level of Grk in the oocytes is reduced and is often undetectable (Fig. 2F).

Relationship between *okr*, *spnB*, and *spnD* and the dorsalizing mutant *K10*

The effects of *okr*, *spnB*, and *spnD* on Grk accumulation in the oocyte place these genes upstream of *grk* in the genetic hierarchy controlling dorsal-ventral patterning in the egg chamber. To assess the relationship between *okr*, *spnB*, and *spnD* and a different class of genes in the patterning hierarchy that are required for the localization of *grk* RNA and produce dorsalizing phenotypes, we

have analyzed the phenotypes produced by double mutants with *K10*.

For all three double-mutant combinations, we find that rather than producing all ventralized or all dorsalized eggs, the mutant females produce a broad spectrum of phenotypes ranging from completely dorsalized to completely ventralized (Table 1). Given that these experiments do not reveal a simple epistatic relationship between *okr*, *spnB*, *spnD*, and *K10*, the three genes must affect Grk activity by a pathway that is at least partially independent of *K10*. Significantly, as this same spectrum of phenotypes is produced by *K10* mutant females that have only one wild-type copy of *grk* (Table 1; see also Forlani et al. 1993), these results are consistent with a role for these genes in directly affecting the accumulation of Grk in the oocyte.

Given that the mislocalization of *grk* mRNA that is observed in *okr*, *spnB*, and *spnD* mutant egg chambers is similar to that observed in *K10* mutant egg chambers (Neuman-Silberberg and Schüpbach 1993; Roth and Schüpbach 1994), we have also looked for a defect in the accumulation of *K10* protein in *okr*, *spnB*, and *spnD* ovaries. We find that in all three mutant genotypes, there is a reduction in the level of *K10* in the oocyte nucleus (Fig. 2G,H). Thus, *okr*, *spnB*, and *spnD* are necessary for normal accumulation of both *K10* and Grk in the oocyte. The failure to accumulate wild-type levels of *K10* leads to the mislocalization of *grk* mRNA in mid-oogenesis, whereas the failure to accumulate wild-type levels of Grk leads to ventralization of the eggshell and embryo. The former defect may also account for the production of rare dorsalized eggs by *okr*, *spnB*, and *spnD* mutant females. Moreover, the fact that we observe both ventralized and dorsalized eggs suggests that the two effects are to some degree independent: Ventralized eggs arise from cases in which Grk levels are reduced and *K10* levels are normal or reduced, whereas dorsalized eggs arise from cases in which Grk levels are fairly normal and *K10* levels are reduced.

okr, *spnB*, and *spnD* ovaries show defects in the morphology of the oocyte nucleus

Oocytes from *okr*, *spnB*, and *spnD* mutants also have defects in nuclear morphology. Studies on chromosome behavior in wild-type ovaries have shown that during stage 3, the DNA in the oocyte nucleus condenses into a compact spherical structure, the karyosome, which is maintained through the later stages in oogenesis until the onset of metaphase I (Spradling 1993). In ovaries stained with a DNA dye, this structure appears as a bright spot within which there is a spot of greater intensity (Fig. 2I). In *okr*, *spnB*, and *spnD* mutant egg chambers, this condensation is aberrant and a variety of defective structures are observed. In some cases, the DNA assumes an ellipsoidal shape that is larger than the normal spot (Fig. 2J), and in others, the DNA is present in clumps that line the inside of the nuclear membrane (Fig. 2K). As this defect is not seen in *grk* or *K10* mutant egg chambers (data not shown), it does not arise from a

(red) and DNA (green). In wild type (H), the DNA in the oocyte nucleus is condensed into a tight sphere. In ovaries mutant for *okr*, *spnB*, *spnD*, or a number of other genes (including *aub*, *del*, *squ*, *vas*, *zuc*, *spnA*, *spnC*, and *spnE*), the DNA is more diffuse (J) or threadlike and fragmented (K).

defect in *grk-Egfr* signaling. Our findings corroborate those of a previous study on the spindle genes (González-Reyes et al. 1997), and more recent studies on *vas* have shown that mutations in this gene have a similar nuclear defect (Styhler et al. 1998; Tomancak et al. 1998). We have examined ovaries from females mutant for the remaining loci in this group, and find that *del*, *squ*, *zuc*, and *aub* produce the phenotype as well. Thus, the nuclear defect appears to be a phenotype common to all the genes in this class.

Molecular analysis of *okr* and *spnB*

The *okr* locus was cloned from a genomic walk spanning the 23C interval to which the gene was localized (see Materials and Methods; Fig. 3). Within this region, a 4.7-kb genomic fragment was found to rescue the *okr* mutant phenotype. Northern analysis of ovarian poly(A)⁺ RNA identified three transcripts of 4.5, 2.7, and 1.6 kb, of which only the 2.7-kb transcription unit is completely contained within the rescuing fragment. A cDNA corresponding to this RNA was isolated, sequenced, and found to be identical to the previously characterized *Drosophila* gene *DmRad54* (Kooistra et al. 1997). Thus, *okr* is the *Drosophila* homolog of the yeast *RAD54* gene, a DNA helicase of the *RAD52* epistasis group that is required for double-strand break (DSB) repair. In situ hybridization to wild-type ovaries by use of the *okr* cDNA as a probe indicates that the RNA is widely expressed at

Figure 2. (A–F) Expression of Grk in *okr*, *spnB*, and *spnD* mutant ovaries. Ovarioles (A,C,E) and stage 10 egg chambers (B,D,F) with Grk in green and cortical actin detected with Phalloidin in red. In wild-type ovaries, Grk is detected in the oocyte throughout oogenesis (A), and becomes localized to the presumptive dorsal–anterior corner in stages 9 and 10 (B). In *okr* mutant ovaries, Grk expression is reduced or undetectable in many egg chambers, and these are interspersed among egg chambers that have apparently normal Grk expression (C,D). In *spnB* and *spnD* mutant ovaries, Grk expression is apparently normal in the early stages, but less and less protein is detectable in the oocyte as oogenesis proceeds (E). The majority of stage 10 *spnB* and *spnD* mutant egg chambers have no detectable Grk (F). (G,H) Expression of *fs(1)K10* in *okr*, *spnB*, and *spnD* mutant ovaries. Triple stainings of egg chambers with K10 shown in red and cortical actin and DNA shown in green. In wild type (G), K10 protein is observed in the oocyte nucleus, and is particularly concentrated around the karyosome. In the mutant egg chambers (H) K10 protein is reduced or absent. (I–K) Defects in oocyte nuclear morphology in *okr*, *spnB*, and *spnD*. Stage 8 egg chambers stained for cortical actin

all stages of oogenesis (data not shown). The sequence of the seven *okr* alleles was determined, and all showed single nucleotide changes in the coding region of the 2.7-kb transcript that resulted either in missense or nonsense mutations (Table 2). Two of the alleles, *okr*^{AA} and *okr*^{AG}, appear to be molecular nulls. In *okr*^{AA}, the ninth codon is mutated to a stop codon, thus truncating the protein after the eighth amino acid, and in *okr*^{AG}, the initial methionine is mutated to an isoleucine. The other five alleles were found to contain lesions in regions that are conserved among all members of the Snf2 DNA helicase family of proteins (Fig. 3C).

The *spnB* locus was cloned from an existing genomic walk in the 88B region (see Materials and Methods, Fig. 4, and legend). The *spnB* mutant phenotype was rescued by a 5.9-kb genomic fragment that was found to hybridize to at least four transcripts (Fig. 4B). Smaller rescue constructs specific for the 550-nucleotide and 1.35-kb RNAs were tested, and it was found that only constructs containing the complete 1.35-kb transcription unit were able to rescue *spnB* mutants. Multiple cDNAs were isolated corresponding to the 550-nucleotide and the 1.35-kb transcripts, and cDNAs for each gene, as well as the entire genomic region were sequenced (see Materials and Methods). In situ hybridization to wild-type ovaries with both cDNAs revealed that, like *okr*, the genes are uniformly expressed throughout oogenesis (data not shown). The *spnB* cDNA was found to encode a protein of 341 amino acids with an apparent molecular weight of 38

Figure 3. Molecular characterization of *okr*. (A) Physical map of the 23C region of 2L. (Shaded boxes) Regions deleted in *Df(2L)C144*, *Df(2L)JS17*, and *Df(2L)JS7*. *Df(2L)JS17* extends off the map both proximally and distally. (Stippled boxes) Regions of uncertainty. Simplified restriction map showing only *Xba*I (Xb) sites, was derived from the maps of genomic phage that are indicated as bars below the map. A more detailed restriction map of λ 7-4 is shown with all *Hind*III (H), *Eco*RI (E), *Sal*I (S), and *Xba*I sites indicated. Three previously characterized genes, *Rbp9*, *Rrp1*, and γ *Tub23C* are shown as open boxes with arrows indicating the direction of transcription. (Hatched boxes) Unmapped ovarian transcripts defined by Northern analysis of poly(A)⁺ RNA using corresponding genomic fragments. Approximate sizes are shown below each box. The rescue construct, pRRa54E4.7w+, which rescues the *okr* mutant phenotype, is shown beneath the 2.7-kb transcription unit which it includes. (B) Restriction map of the 4.7-kb *Eco*RI fragment included in the rescue construct shown in A. The structure of the *okr* gene was derived from the existing sequences for *DmRad54* (Kooistra et al. 1997), and our genomic and cDNA sequences (see Materials and Methods). The 4.3-kb cDNA was identified by Northern analysis of ovarian poly(A)⁺ RNA with a fragment extending from the left *Eco*RI site to just before the transcriptional start of *okr*. The 1.6-kb transcript that overlaps with *okr* was identified by Northern analysis by use of either the entire rescue fragment or the *okr* cDNA, and the overlap was verified by sequencing the 3' ends of the EST corresponding to this gene. (C) Protein structure of Okr. (Light shading) Six domains of homology shared with other members of the Snf2 family of DNA helicases (Bork and Koonin 1993). (Black bars) Positions of mutation in the alleles indicated (Table 2).

kD. Motif searches revealed that the protein contained a region homologous to the consensus for the P loop, a short glycine-rich sequence that is a portion of the nucleotide binding pocket of a diverse group of GTP or ATP hydrolyzing proteins, including the Ras oncogene and its relatives, Dynein and other motors, and the bacterial RecA protein and its eukaryotic homologs (Walker et al. 1982; Story et al. 1993). Consistent with its inclusion in the latter class of genes, homology searches through DNA and protein databases revealed significant homology between *spnB* and *Saccharomyces cerevisiae* *DMC1*, *RAD51*, *RAD57*, and *RAD55* genes, all of which, like *RAD54*, have been shown to be involved in DSB

repair. *SpnB* is most homologous to the meiosis-specific *Dmc1* protein (28% identity/51% similarity) and the regions of greatest homology correspond to the domains that comprise the nucleotide binding pocket, including the P-loop region. However, *spnB* is most likely not the *Drosophila* homolog of *DMC1* insofar as the percent identity between yeast *Dmc1* and its human homolog (54%) is significantly higher than that between either of these proteins and *SpnB* (28% and 27%, respectively).

The coding regions of the five *spnB* mutants were sequenced, and each was found to contain a unique single-nucleotide change resulting in either a missense or nonsense codon (Table 2). Four of the five mutations fall into the amino-terminal portion of the P-loop sequence, and two of the four mutations, *spnB*¹⁵³ and *spnB*⁰⁵⁶, are changes in glycine residues that are conserved in all family members. The remaining two alleles, *spnB*^{BU} and *spnB*^{CN}, are associated with the same nucleotide change, and these affect another glycine residue that is common to *SpnB* and yeast *Rad57*. The clustering of mutants around the P-loop motif suggests that nucleotide binding is an essential property of the *spnB* protein. The final mutant, *spnB*^{BC}, is a stop codon that truncates the protein after the 233rd residue (Fig. 4C).

Effects on mitotic and meiotic DSB repair

In yeast, components of the *RAD52* epistasis group are required for the recombinational repair of DSBs in both mitotic and meiotic cells. In mitotic cells, mutations in these genes interfere with the cell's ability to repair

Table 2. Molecular lesions associated with alleles of *okr* and *spnB*

Allele	Residue	Alteration
<i>okr</i> ^{AA}	9	Q → ochre
<i>okr</i> ^{AB}	325	S → F
<i>okr</i> ^{AG}	1	M → I
<i>okr</i> ^{AK}	619	C → opal
<i>okr</i> ^{AO}	601	A → V
<i>okr</i> ^{RU}	391	Q → amber
<i>okr</i> ^{WS}	579	G → D
<i>spnB</i> ⁰⁵⁶	113	G → R
<i>spnB</i> ¹⁵³	102	G → R
<i>spnB</i> ^{BC}	234	R → opal
<i>spnB</i> ^{BU}	107	G → E
<i>spnB</i> ^{CN}	107	G → E

responding genomic sequences. (Dark shading) Coding region of each transcript. c5.5, c1.0/c1.2 are ovarian transcripts defined by Northern analysis using small subclones of the complete 5.9-kb fragment. The unidentified ORF was deduced from the genomic sequence. (C) Protein structure of SpnB as compared with the related protein Dmc1. (Light shading) Region of homology between the two proteins, (dark shading) A- and B-type nucleotide binding consensus sequences (Walker et al. 1982). Black bars in the SpnB diagram indicate the positions of the lesions associated with *spnB* alleles (cf. Table 2). An alignment of the A-type nucleotide binding domains of SpnB, Dmc1, yeast Rad57, *Drosophila* Rad51, yeast Rad51, and human Rad51, is shown, indicating the location of the *spnB*¹⁵³, *spnB*^{BU}, *spnB*^{CN}, and *spnB*⁰⁵⁶ mutations. The P-loop motif corresponds to the GXXXXGKT/S at the right end of the alignment.

DNA damage, whereas in meiotic cells, they block genetic recombination resulting from the failure to repair DSBs associated with crossing over (Resnick 1987; Petes et al. 1991). In light of the homology of *okr* and *spnB* to genes in this epistasis group, we have determined whether mutations in *okr*, *spnB*, and *spnD* affect mitotic and meiotic DSB repair. To look for a requirement in mitotic DSB repair, we have tested various mutant genotypes for sensitivity to DNA damage. To look for a requirement in meiotic DSB repair, we have tested mutant genotypes for a reduction in meiotic exchange.

To test for sensitivity to DNA damage, crosses producing *okr*, *spnB*, and *spnD* mutant larvae were fed a solution of 0.08% methylmethanesulfonate (MMS), a chemical mutagen that induces DSBs. The survival of MMS-treated larvae was compared with that of mutant larvae from an untreated control cross (Fig. 5). We find that *okr* mutants are sensitive to MMS, showing a significant reduction in survival in MMS-treated crosses relative to control crosses. *spnB* and *spnD*, on the other hand, are not sensitive, showing equal percentages of expected progeny in both crosses. The MMS sensitivity of *Dmrad54* mutations has been shown previously (Kooistra et al. 1997), and our data on *okr* alleles corroborate this finding. The fact that *spnB* and *spnD* mutants do not

show MMS sensitivity suggests that they may not be required for mitotic DSB repair.

To test the effect of *okr*, *spnB*, and *spnD* mutations on meiotic DSB repair, we have measured the effects of

Figure 5. MMS sensitivity of *okr*, *spnB*, and *spnD* mutants. Graph of fraction of percent expected (+MMS) to percent expected (control) for *okr*, *spnB*, and *spnD* mutants.

these mutants on meiotic exchange as reflected in the frequency of recombination and X-chromosome nondisjunction. For these experiments, we took advantage of the fact that females mutant for even the strongest *spnB* and *spnD* alleles produce escaper progeny in the first days after mating. In the case of *okr*, it was not possible to use the strongest alleles because they are almost completely sterile. Instead, we used a weak allele, *okr^{AO}*, which is fertile as a homozygote and hemizygote (25% and 50% hatching, respectively). In *spnB* or *spnD* mutant females heterozygous for X chromosomal markers, the frequency of recombination is 10%–25% of normal levels, whereas for the weak *okr* allele the frequency of recombination is at 50% of normal levels (Table 3). In crosses that allowed us to score the exceptional progeny classes produced by X chromosome nondisjunction, we observed an ~100-fold increase in X chromosome nondisjunction in both *spnB* and *spnD* mutant females, and a 17- to 20-fold increase in the crosses involving *okr* (Table 4). Although the results for *okr* are not as dramatic as those for *spnB* and *spnD*, it is likely that stronger *okr* alleles would show a more severe effect. In summary, the data are consistent with a requirement for *spnB*, *spnD*, and *okr* in meiotic DSB repair.

Discussion

Mutations in *okr*, *spnB*, and *spnD* cause specific patterning defects during oogenesis, resulting in eggs and embryos that show variable alterations along the dorsal-ventral and anterior-posterior axes. These defects can be explained by the failure to accumulate wild-type levels

of several developmentally important proteins. In addition, mutations in these genes cause a reduction in the level of meiotic recombination and an increase in the frequency of nondisjunction. The effect of these mutations on recombination can be accounted for by a requirement for these genes in DSB repair.

A role for *okr*, *spnB*, and *spnD* in meiotic recombination

Analysis of recombination and nondisjunction frequencies in the progeny of *okr*, *spnB*, and *spnD* females indicates that the ability of these females to generate recombinant chromosomes is compromised. *okr* encodes the *Drosophila* homolog of Rad54, and *spnB* encodes a Rad51-like protein, two types of proteins required for DSB repair. It is generally thought that meiotic exchange is initiated by the formation of DSBs, and that repair of DSBs is required for the formation of heteroduplex DNA and chiasmata. The former is a necessary intermediate in the production of recombinant chromosomes, and the latter is necessary for proper disjunction of homologous chromosomes at anaphase of meiosis I (for review, see Cummings and Zolan 1998; Moore and Orr-Weaver 1998). As *okr* and *spnB* encode proteins presumed to act in the repair of DSBs, it is likely that the reduction in the frequency of recombination and increase in nondisjunction that we observe in *okr* and *spnB* mutants reflect the requirement for these genes in the DSB repair step of meiotic recombination. Moreover, the striking similarity between the *spnB* and *spnD* mutant phenotypes suggests that *spnD* may also encode a component of the meiotic DSB repair pathway.

Several of the mutant phenotypes described for *okr*, *spnB*, and *spnD* can be accounted for by the requirement for these genes in meiotic recombination. For instance, the fact that *okr*, *spnB*, and *spnD* are female sterile loci and are not required for male fertility is consistent with the role for these genes in meiosis, because in *Drosophila*, meiotic recombination occurs only in females. Further, the production of defective gametes as a consequence of nondisjunction could be a contributing factor to the low frequency with which mutant eggs hatch. In addition, as meiotic recombination occurs within the oocyte nucleus, the defect we observe in karyosome formation may well reflect an earlier meiotic defect. Notably, it has been suggested that the abnormal DNA arrangements observed in the oocytes of early stage egg chambers (stages 4–7) mutant for any of the *spindle* genes resemble the appearance of chromosomes within wild-type pro-oocytes (A.T.C. Carpenter, as cited in González-Reyes 1997). This raises the possibility that the karyosome defect we observe may reflect a persistence of this early meiotic state into the later stages of oogenesis.

Homologs of several *RAD52* epistasis group members have been identified in higher eukaryotes and have been implicated in meiotic processes on the basis of their expression in meiotic tissues. Immunocytological work in a number of model systems has demonstrated that Rad51-like proteins are associated with meiotic chromo-

Table 3. Recombination frequencies in mutant females

A. Recombination frequencies in <i>spnB</i> and <i>spnD</i> mutant backgrounds					
Genotype	N ^a	Recombinants ^b		Frequency ^c	
		y-v	v-f	y-v	v-f
<i>y v f/+++; +/+</i>	532	145	137	0.27	0.26
<i>y v f/+++; spnB^{BU}/spnB^{BU}</i>	309	14	10	0.05	0.03
<i>y v f/+++; spnB^{BU}/spnB¹⁵³</i>	366	27	23	0.07	0.06
<i>y v f/+++; spnD³⁴⁹/spnD³⁴⁹</i>	362	24	21	0.07	0.06
B. Recombination frequencies in <i>okr</i> mutant backgrounds					
Genotype	N ^d	Recombinants		Frequency ^c	
		[y w] - f			
<i>y w f/+++; okr^{AO}/+</i>	1919	797		0.42	
<i>y w f/+++; okr^{AO}/okr^{AO}</i>	951	174		0.18	
<i>y w f/+++; okr^{AO}/okr^{AA}</i>	1127	225		0.20	

^a(N) The total number of progeny scored from a cross of *yvf/Y* males and females of the genotype listed.

^bRecombinants for the two intervals were scored independently; thus, the y-v column includes all recombinant progeny between the markers *yellow* and *vermillion* whether or not there was a second event in the v-f interval.

^cThe recombination frequency for each interval was calculated as $f = [\text{recombinants}]/N$.

^d(N) The total number of progeny scored from a cross of *y w f/Y* males and females of the genotype listed.

Table 4. X-chromosome nondisjunction in mutant females

A. X-chromosome nondisjunction in spB and spD mutant backgrounds						
Maternal genotype ^a	N	XY	XO	Percent nondisjunction ^b		
Oregon-R	11,413	11,407	6	0.1		
<i>spnB^{BU}/spnB^{BU}</i>	366	343	23	13		
<i>spnB^{BU}/spnB¹⁵³</i>	167	160	7	8		
<i>spnD³⁴⁹/spnD³⁴⁹</i>	395	374	21	11		
B. X-chromosome nondisjunction in okr mutant backgrounds						
Maternal genotype ^c	N	XY	XX	XO	XXY	Percent nondisjunction ^d
<i>b pr cn bw</i>	10,816	5,108	5,700	3	2	0.09
<i>okr^{AO}/okr^{AO}</i>	1,270	642	615	9	4	2

^aProgeny were scored from a cross of *yvf/Y* males to wild type or *+/+*; *spn/spn* females. Normal disjunction of the X chromosome in females gives rise to *+/Y* (XY) males; nondisjunction gives rise to exceptional *yvf/O* (XO) males that can be distinguished from their phenotypically wild-type siblings.

^bNondisjunction was calculated as $(2 \times [\text{XO males}]/N) \times 100$. Only male progeny was counted in this experiment, and the number of XO males was multiplied by 2, to account for the YO products.

^cProgeny were scored from a cross of *yw/B^S-Y* males to *b pr cn bw* or *okr* females. Normal disjunction of the X chromosome in females gives rise to *+/B^S-Y* males; nondisjunction gives rise to exceptional *yw/O* (XO) males that can be distinguished from their *Bar-Stone* siblings. Similarly, in females, normal disjunction gives rise to *yw/+* (XX) females; non-disjunction gives rise to *+/+/B^S-Y* (XXY) females that can be distinguished from their phenotypically wild-type siblings.

^dNondisjunction was calculated as $(2 \times [\text{XO males} + \text{XXY females}]/N) \times 100$. The sum of XO males and XXY females was multiplied by 2 to account for YO and XXX products.

somes, suggesting that utilization of the *RAD52* recombinational repair pathway in meiosis is conserved in higher eukaryotes (for review, see Ashley and Plug 1998). However, it has yet to be shown in these organisms that any of the *RAD52* group genes are required for meiotic recombination. The unexpected lethality associated with the *rad51*-knockout mouse precludes addressing its function in meiosis until conditional mutants can be constructed (Lim and Hasty 1996; Tsuzuki et al. 1996), and although the *rad54* knockout mouse is viable, no meiotic phenotype has been reported (Essers et al. 1997). Thus, our findings provide the first functional data demonstrating the requirement for *RAD52* epistasis group genes in meiotic recombination in multicellular eukaryotes.

Our data indicate that *okr*, *spnB*, and *spnD* might not be absolutely essential for meiotic recombination, as females mutant for even the strongest allelic combinations of *spnB* or *spnD* still produce some recombinant progeny. The ability of mutant females to produce these escaper progeny may reflect partial redundancy for the proteins that function in the recombinational repair pathway. For instance, in yeast, the function of *RAD54* can be partially compensated for by the activities of other DNA helicases. Thus, the MMS sensitivity of null mutations in *RAD54* is enhanced by mutations in the *RAD54*-like gene, *RDH54* (Klein 1997; M. Shinohara et al. 1997). Similarly, it has been shown that a *rad54* null mutation is synthetically lethal with mutations in *SRS2*, another yeast DNA helicase, implying that the function of these two gene products is at least partially redundant (Palladino and Klein 1992). Given that *okr* is the *Drosophila* homolog of *RAD54*, it is possible that other *Dro-*

sophila helicases might be able to partially compensate for loss of *okr* function. Redundancy might also be present among the *RAD51*-like genes. For instance, the yeast *RAD51* and *DMC1* genes appear to have partially overlapping functions in meiotic DSB repair (A. Shinohara et al. 1997).

Mutations in okr, spnB, and spnD affect developmental patterning

Mutations in *okr*, *spnB*, and *spnD* cause a number of specific patterning defects. We have shown that many of the observed phenotypes reflect defects in the regulation or expression of *grk*. *grk* is affected at two levels: The mRNA is not always properly localized, presumably as a consequence of reduced levels of K10 within the oocyte, and the accumulation of Grk protein itself is reduced. The failure to accumulate wild-type levels of Grk and K10 in *okr*, *spnB*, and *spnD* mutants could reflect a defect in the translation or stability of these proteins. The effects of these mutations on protein accumulation, however, do not appear to be caused by a general defect in the translation or stability of all oocyte-specific proteins, as, for instance, levels of Orb protein do not appear to be altered (A. Ghabrial and T. Schüpbach, unpubl.).

The relationship between the meiotic and developmental phenotypes of okr, spnB, and spnD

We show here that the regulation of meiotic processes occurring within the oocyte nucleus affects accumulation of the nuclear protein K10, and in addition, also affects accumulation of Grk protein within the cyto-

plasm. These findings establish the existence of a connection between the regulation of meiotic progression in the oocyte nucleus, and the regulation of specific patterning processes in the oocyte cytoplasm.

One way of accounting for the patterning defects caused by mutations in *okr*, *spnB*, and *spnD* is that defects in DSB repair would lead to a general disorganization of the oocyte nucleus that would affect the organization of the oocyte as a whole. However, we did not observe general defects in the mutant egg chambers, characteristic of a global misorganization of the cytoskeleton.

Alternatively, a failure to repair DSBs could result in checkpoint-mediated arrest of meiotic progression, which, in turn, would block certain regulated processes in the cytoplasm. In yeast, mutations in the DSB repair genes, *RAD51* and *DMC1*, lead to a checkpoint-mediated arrest in pachytene (Bishop et al. 1992; Lydall et al. 1996; Xu et al. 1997). We have not investigated the nature of the nuclear defects in *okr*, *spnB*, and *spnD*, but, as discussed above, it is possible that these defects could be explained by an early arrest in meiosis. In yeast and multicellular eukaryotes, it is well established that mitotic and meiotic checkpoint proteins, in addition to their effect on genes involved in DNA metabolism, also regulate various cytoplasmic processes such as spindle assembly and nuclear envelope breakdown (for review, see Murray and Hunt 1993). It is therefore possible that in *Drosophila* the same factors that regulate meiotic cell cycle targets might also be used in parallel to regulate specific developmental targets. Such targets could include proteins that control translation of developmentally important proteins like Grk and K10.

Effectors for this kind of regulation might be found among the genes described above that produce mutant phenotypes similar to those of *okr*, *spnB*, and *spnD*. Such effectors could act downstream of the DSB-repair checkpoint and regulate translation in response to a signal from the oocyte nucleus. Whereas only two of these genes, *vasa* and *spnE*, have been cloned, they both encode RNA helicases and are implicated in the translational regulation of *grk* (Lasko and Ashburner 1988; Gillespie and Berg 1995; González-Reyes et al. 1997; Styhler et al. 1998; Tomancak et al. 1998). Regulation of genes required for the translation of developmentally important proteins, such as Grk, in response to the status of the oocyte nucleus, could serve to coordinate the timing of progression through meiosis with the developmental program. However, because *vasa* and *spnE* also produce nuclear defects, the pathway can not be unidirectional. Thus, information from the cytoplasm (e.g., factors required for chromosome condensation or karyosome formation) contributes to nuclear processes as well.

Meiotic prophase in *Drosophila* oogenesis occurs over an extended time period during which many different developmental events take place. The pachytene stage of meiotic prophase is believed to be achieved as early as region 2a of the germarium (for review, see Spradling 1993). Although the repair of DSBs in wild-type ovaries presumably occurs during pachytene, nevertheless, we

find that most of the events of oogenesis appear to proceed normally in these mutants, and only a few specific processes appear to be severely affected. This suggests that for *Drosophila*, only a subset of the processes occurring within the oocyte cytoplasm are dependent on normal meiotic progression within the oocyte nucleus. Perhaps the processes that are linked to progression through meiosis are those for which precise temporal regulation is of particular importance. It will be interesting to see whether similar effects will be associated with meiotic mutants in other developmental systems.

Materials and methods

Drosophila strains and manipulations

okra alleles Two EMS-induced alleles of *okr*, *okr^{RU}* and *okr^{WS}*, were identified previously (Schüpbach and Wieschaus 1991). We have mapped the gene to 5.6 cM on 2L. The locus is uncovered by *Df(2L)JS17* (23C1-2; 23E1-2), and falls between the proximal and distal breakpoints of *Df(2L)C144* (23A1-2; 23C3-5) and *Df(2L)JS7* (23C3-5; 23D3), respectively, placing it in 23C. To generate more *okr* alleles, we performed a standard F₃ female sterile mutagenesis. Thirty-eight hundred EMS-mutagenized chromosomes were screened, and five new alleles, *okr^{AA}*, *okr^{AB}*, *okr^{AG}*, *okr^{AK}*, and *okr^{AO}*, were isolated on the basis of their failure to complement *okr^{WS}*.

spnB and spnD alleles Two EMS-induced alleles of *spnB*, *spnB⁰⁵⁶*, and *spnB¹⁵³*, were identified previously, and the locus was deficiency mapped to the 88B interval on 3R between the distal breakpoints of *Df(3R)red-P52* (88A12-B1; 88B4-5) and *Df(3R)red-P93* (88A10-B1; 88C2-3) (Tearle and Nüsslein-Volhard 1987). For our experiments, we have used *Df(3R)trxE12* (88B1-88B3) as a standard deficiency for the locus. Two *spnD* alleles, *spnD³⁴⁹*, and *spnD¹⁵⁰*, were isolated from the same screen. *spnD* was meiotically mapped to 91 cM on 3R, and is uncovered by *Df(3R)TI-P* (97A1-10; 98A1-2). New alleles of both *spnB* and *spnD* were identified in an F₃ female sterile mutagenesis on the basis of their failure to complement either *spnB¹⁵³* or *spnB⁰⁵⁶* for the *spnB* alleles, or *spnD³⁴⁹* for the *spnD* alleles. Of 11,000 chromosomes scored, three *spnB* alleles, *spnB^{BC}*, *spnB^{BU}*, and *spnB^{CN}*, and one *spnD* allele, *spnD^{CX}*, were isolated.

Characterization of alleles Characterization of the phenotypes produced by *okr*, *spnB*, and *spnD* mutant females is complicated by the variability of the phenotype with regard to genetic background, the age of the females, and the conditions in which they are raised. To control for differences in genetic background, homozygous viable recombinants for each allele were generated in which the majority of the mutagenized chromosome was replaced with the parental *b pr cn bw* or *st e* chromosome. The phenotypes of *okr^{AB}*, *okr^{AK}*, *okr^{RU}*, and *okr^{WS}* homozygotes are more severe than the corresponding hemizygotes, and thus, by genetic criteria, these alleles behave as recessive antimorphs. *okr^{AO}* also behaves as a recessive antimorph, but its phenotype is significantly weaker than that produced by the other antimorphic alleles. *okr^{AA}* and *okr^{AG}*, though null by molecular criteria, show slightly different phenotypes as homozygotes and hemizygotes, possibly reflecting effects of genetic background. All of these alleles are viable in *trans* to deficiency, indicating that the locus is not essential for viability. For *spnB*, *spnB^{BU}*, *spnB^{BC}*, and *spnB^{CN}* all behave as

recessive antimorphs. We have not been able to assess the phenotype of *spnB*¹⁵⁶ homozygotes because despite repeated outcrossing, we have not been able to recover a vigorous homozygous viable chromosome. *spnB*¹⁵³ is unique in showing extraordinary sensitivity to genetic background, making assessment of its phenotype difficult. *spnD*¹⁵⁰ and *spnD*³⁴⁹ both behave as loss-of-function mutations in genetic tests, however, the fecundity of the homozygous females when compared with the hemizygous females is significantly reduced. All alleles of *spnB* and *spnD* are viable in *trans* to deficiency, thus, these genes are also not essential for viability. Even though many of the alleles test as recessive antimorphs, even the most severe antimorphic alleles do not produce qualitatively different phenotypes than the more straightforward loss-of-function alleles, rather, the antimorphic character merely affects the frequency with which certain phenotypes are observed.

Antibody stainings and in situ hybridizations

Antibody staining in ovaries Immunolocalization of *grk* protein was performed as described previously (Neuman-Silberberg and Schüpbach 1996). The secondary antibody, biotin-anti-Rat (Vector), was used at a dilution of 1:1000 in a 1 hr incubation at room temperature, and this was followed by a tertiary detection step using Cy3-conjugated Streptavidin (Cy3-SA, Jackson) at a dilution of 1:1000 also for 1 hr at room temperature. The same protocol for fixation and labeling was used for immunolocalization of K10 protein with the Rat-anti-K10 antibody (Cohen and Serano 1995), with the addition of a 1 hr permeabilization step in PBS + 0.3% Triton-X100 (Sigma) prior to blocking. The K10 antibody was used at a dilution of 1:1600. Cell outlines were visualized by staining cortical actin with OregonGreen488- or Rhodamine-conjugated phalloidin (Molecular probes), as per manufacturer's recommendation. To visualize nuclei, ovaries were incubated in a 1:5000 dilution of OliGreen (Molecular Probes) and 20 µg/ml of RNaseA for 1 hr at room temperature. Fluorescent images were examined with a Bio-Rad MRC 600 confocal microscope.

Antibody staining of embryos Eggs were collected in 4 hr intervals, fixed in 4% paraformaldehyde in PBS for 20 min, and devitellinized in methanol according to standard protocols. Fixed embryos were transferred to a silanized glass slide and cellular blastoderms were hand selected under a dissecting microscope. The selected embryos were blocked in PBS + 10% BSA + 0.1% Tween 20 (Sigma), and the primary rabbit-anti-Twist antibody (a gift of S. Roth, Max-Planck Institute for Developmental Biology, Tübingen, Germany) was used at a dilution of 1:2000 in PBS + 1% BSA + 0.1% Tween 20 in an overnight incubation at 4°C. A secondary antibody, biotin-anti-rabbit (Vector) was used at a dilution of 1:1000 in a 1 hr incubation at room temperature, and this was followed by detection with the horseradish peroxidase-streptavidin (HRP-SA) ABC kit (Vector) according to the supplier's recommendation.

In situ hybridizations RNA in situ hybridizations on ovaries and embryos were done according to standard procedures (Roth and Schüpbach 1994) with minor modifications. Antisense RNA probes were made by use of linearized cDNA templates with the Genius RNA Labeling Kit (Boehringer) according to the manufacturer's protocol. Hybridizations were performed at 55°C.

Molecular cloning

okra The locus falls in the region between the proximal break-

point of *Df(2L)C144* and the distal breakpoint of *Df(2L)JS17* which contains three previously characterized genes, *RNA binding protein 9 (Rbp9)* (Kim and Baker 1993), *Recombination repair protein 1 (Rrp1)* (Sander et al. 1991), and a γ -tubulin isoform (γ Tub23C) (Sunkel et al. 1995). Southern mapping of the deficiencies in the region indicated that the *Rbp9* gene spanned the distal breakpoint of *Df(2L)C144*, and we used this as an entry point to initiate a genomic walk in the region. Phage were isolated from a *dp cn bw* λ DASH genomic library (R. Padgett, unpubl.) that spanned the interval between *Rbp9* and the distal break of *Df(2L)JS7*. Subclones from this walk were used to probe Northern blots of ovarian poly(A)⁺ RNA to screen for candidate transcripts in the region. In addition to the transcripts corresponding to *Rrp1* and γ Tub23C, four other fragments were found to hybridize to ovarian RNAs. A single copy of the rescue construct pRra54E4.7w+, which includes a complete 2.7-kb ovarian transcription unit (see Fig. 3), rescues the *okr* mutant phenotype, indicating that the 2.7-kb RNA corresponds to the *okr* gene. A single cDNA corresponding to the 2.7-kb RNA was isolated from a poly(dT) primed ovarian cDNA library (Stroumbakis et al. 1994), and this cDNA and the entire 4.7-kb genomic fragment were sequenced. The other two transcription units in the fragment were identified as expressed sequence tags (ESTs) in the Berkeley Drosophila Genome Project Database, the 4.3-kb transcript corresponding to sequences LD23852 and LD24692, and the 1.4-kb transcript corresponding to GM04879. The cDNA corresponding to the latter EST was sequenced (GenBank accession no. AF 069781) and it was found to overlap with the last exon of the 2.7-kb transcript, reading off the opposite strand (see Fig. 4). The GenBank accession number for the *okr* genomic rescue fragment is: AF069779, the accession number for the partial *okr* cDNA is AF069780.

spindleB *spnB* was mapped to the 88B interval on 3R based on its inclusion in *Df(3R)redP93* and not in *Df(3R)redP52*. This region was included in an existing genomic walk in the region (kindly provided by R. Kelley, Baylor College of Medicine, Houston, TX), and the interval between the distal breakpoints of the two deficiencies included 12 kb of DNA that was contained in a single cosmid (cos144) of this walk. Subclones of this cosmid were used to probe Northern blots of ovarian poly(A)⁺ RNA to identify candidate transcripts in the region. Four transcripts were identified, and subclones including each of these transcripts were cloned into Casper4 to generate rescue constructs, pR144Xb14w+, pR144NS9.5w+, pR144E5.9w+, and pRE5.9BE3.5w+ (see Fig. 7). Of these, only the last two rescue the *spnB* mutant phenotype, indicating that the 1.35-kb transcript corresponded to the *spnB* gene. Multiple cDNAs corresponding to the 550-nucleotide (GenBank accession no. AF069530) and 1.35-kb transcripts were isolated from a poly(dT) primed ovarian cDNA library (Stroumbakis et al. 1994), and two cDNAs for each gene, and the entire genomic region, were sequenced. The GenBank accession number for the *spnB* cDNA is AF069531.

Sequencing of mutant alleles Genomic DNA was prepared from flies of the genotypes *okr*^{*}/*okr*^{*} or *spnB*^{*}/*Df(3R)trxE12* according to standard procedures (Sambrook et al. 1989). By use of primers flanking the coding region of the gene in question, the mutant locus was amplified by PCR with the KlenTaq high fidelity polymerase (Clontech) by the two-step cycle program recommended by the manufacturer. The PCR product was verified by gel electrophoresis, purified by Wizard PCR Prep (Promega), and sequenced with a Perkin-Elmer ABI Prism 377 DNA Sequencer. Sequences were assembled with the AssemblyLign

program (Kodak/IBI) and compared with the wild-type genomic sequence by use of the MacVector (Kodak/IBI) or Genetics Computer Group (Devereux et al. 1984) programs. In all cases, a single unique nucleotide change was found to be associated with each mutant chromosome.

Tests of DSB repair in mitosis and meiosis

MMS sensitivity To test for a requirement in mitotic double-strand break repair, *okr*, *spnB*, and *spnD* homozygotes were exposed during larval development to the mutagen MMS (Sigma). Crosses of appropriate genotypes were made in pairs, one of each pair to be treated with the mutagen, and the other to serve as a control. For *okr*, *okr^{AA}/S^{2N}cn bw sp* or *okr^{WS}/S^{2N}cn bw sp* males were mated to *Df(2L)JS17/CyO* females, and the crosses were transferred daily. Two days after the transfer (at about the second larval instar), 250 μ l of 0.08% MMS (in water) was added to one of each pair of vials. After eclosion, the number of *okr^{*}/Df* and *S/Df* progeny was determined, and the percent of expected calculated as $[(N_{okr/Df}/N_{S/Df}) \times 100]$. Sensitivity to MMS was expressed as the fraction of percent expected in the treated vial to that in the control: $[(N_{okr/Df}/N_{S/Df})_{MMS}/(N_{okr/Df}/N_{S/Df})_{CONT}]$. For *spnB*, *spnB⁰⁵⁶, ru st e ca/ri red e*, or *spnB^{BU}, st e/ri red e* males were mated to *spnB¹⁵³, ru h th st ri roe p^e e^s ca/TM3, Sb* females, and the crosses were treated as described above. To determine the percent of expected progeny, the number of *spnB^{*}/spnB¹⁵³* progeny was compared with the number of *spnB¹⁵³/ri red e* which are distinguished by recessive markers unique to the two genotypes. Sensitivity to MMS was expressed as the fraction of percent expected in the treated vial to that in the control. For *spnD*, *spnD³⁴⁹/TM3, Sb* males were mated to *spnD¹⁵⁰/TM3, Sb* females, treated as described above, and the percent of expected was determined as the number of *spnD¹⁵⁰/spnD³⁴⁹* progeny divided by half the number of *spnD^{*}/TM3, Sb* progeny.

Recombination frequency To determine the recombination frequency in *spnB* mutants, females of the genotypes *yvf/+++;spnB^{BU}/spnB^{BU}* or *yvf/+++;spnB^{BU}/spnB¹⁵³* were mated to *yvf/Y* males and the progeny were scored for recombination events in the *y-v* and *v-f* intervals independently. A cross of *yvf/+++* females by *yvf/Y* males was used as a control. For *okr*, the recombination frequency was determined in females of the genotypes *ywf/+++;okr^{AO}/okr^{AO}* and *ywf/+++;okr^{AO}/okr^{AA}*. These females were mated to *ywf/Y* males and the progeny scored for recombination events in the *w-f* interval. A cross of *ywf/+++;okr^{AO}/+* females by *ywf/Y* males was used as a control.

Nondisjunction To assess the frequency of nondisjunction in *spnB* mutant females, *y v f/Y* males were crossed to *+/+;spnB^{BU}/spnB^{BU}* or *+/+;spnB^{BU}/spnB¹⁵³* females, and male progeny were scored for exceptional XO males of the genotype *yvf/O* that can be distinguished from their *+++/Y* brothers. For *okr*, *yw/B^S-Y* males were crossed to *+/+;okr^{AO}/okr^{AO}* females, and both male and female progeny were scored. Exceptional XO male progeny of the genotype *yw/O* can be distinguished from their *+/+Y* brothers, and exceptional XXY female progeny of the genotype *+/+/B^S-Y* can be distinguished from their *yw/+* sisters.

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***okra* and *spindle-B* encode components of the *RAD52* DNA repair pathway and affect meiosis and patterning in *Drosophila* oogenesis**

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