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CSN5/Jab1 mutations affect axis formation in the *Drosophila* oocyte by activating a meiotic checkpoint

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SUMMARY

The COP9 signalosome (CSN) is linked to signaling pathways and ubiquitin-dependent protein degradation in yeast, plant and mammalian cells, but its roles in *Drosophila* development are just beginning to be understood. We show that during oogenesis CSN5/JAB1, one subunit of the CSN, is required for meiotic progression and for establishment of both the AP and DV axes of the *Drosophila* oocyte. The EGFR ligand Gurken is essential for both axes, and our results show that *CSN5* mutations block the accumulation of Gurken protein in the oocyte. CSN5 mutations also cause the modification of Vasa, which is known to be required for Gurken translation. This *CSN5* phenotype – defective axis formation, reduced Gurken accumulation and modification of Vasa – is very similar to the phenotype of the *spindle*-class genes that are required for the repair of meiotic recombination-induced, DNA double-strand breaks. When these breaks are not repaired, a DNA damage checkpoint mediated by *mei-41* is activated. Accordingly, the CSN5 phenotype is suppressed by mutations in *mei-41* or by mutations in *mei-W68*, which is required for double strand break formation. These results suggest that, like the *spindle*-class genes, *CSN5* regulates axis formation by checkpoint-dependent, translational control of Gurken. They also reveal a link between DNA repair, axis formation and the COP9 signalosome, a protein complex that acts in multiple signaling pathways by regulating protein stability.

Key words: Oogenesis, Embryogenesis, Ubiquitin, CSN, Jab1, DNA repair

INTRODUCTION

Polarization of the anteroposterior (AP) axis of the *Drosophila* oocyte occurs early in oogenesis, while the presumptive oocyte is still in the germarium (Gonzalez-Reyes and St Johnston, 1998). The dorsoventral (DV) axis is set up much later and relies on transfer of the AP axis polarity from the oocyte to the somatic follicle cells at the posterior end of the oocyte. During stages 4-6 in wild-type egg chambers, *grk* RNA that is localized next to the nucleus at the posterior end of the oocyte is translated and signals through the EGFR pathway to establish the adjacent follicle cells as posterior (Gonzalez-Reyes et al., 1995). After these posterior follicle cells signal back to the oocyte, microtubule orientation in the oocyte is reversed, and the oocyte nucleus migrates along the microtubules to an anterior corner of the oocyte. During stages 8-9 this anterior corner is defined as dorsal by translation of *grk* RNA and activation of EGFR signaling in the overlying follicle cells (Gonzalez-Reyes et al., 1995; Neuman-Silberberg and Schupbach, 1994; Roth et al., 1995). Thus, *grk* signaling is required for elaboration of the AP axis and establishment of the DV axis.

Recent results have shown that establishment of both AP and DV axes also depends on the successful repair of DNA double strand breaks (DSBs) that are formed during meiotic recombination (Ghabrial et al., 1998; Ghabrial and Schupbach, 1999). Meiotic prophase begins in early region 2a of the germarium, and both recombination and repair are probably completed before oocyte determination occurs in region 2b (Huynh and St Johnston, 2000).

Meiosis and axis establishment are related to each other because the accumulation of Grk protein in the oocyte cytoplasm depends on the successful completion of meiotic recombination (Ghabrial et al., 1998; Ghabrial and Schupbach, 1999). Mutations in the *spindle*-class genes, *spindle-B* (*spn-B*), *spindle-C* (*spn-C*) and *okra* (*okr*), cause a delay in oocyte determination and a failure to accumulate Grk protein, leading to defects in AP and DV patterning in late oogenesis (Gonzalez-Reyes et al., 1997; Ghabrial and Schupbach, 1999). *spn-B* and *okr* encode *Drosophila* homologs of the *RAD51* and *RAD54* genes from yeast that are required for DSB repair (Ghabrial et al., 1998; Kooistra et al., 1997). Their effects on Grk appear to be mediated by a DNA damage checkpoint governed by Mei-41, a *Drosophila* member of the ATM/ATR family of kinases that are required for DNA damage and recombination checkpoints in yeast, worms and humans, as well as flies (reviewed by Melo and Toczysky, 2002; Weinert, 1998; Murakami and Nurse, 2000). Because they eliminate the checkpoint, *mei-41* mutations suppress the effects of *spn* or *okr* mutations. The *spn* and *okr* mutations can also be suppressed by mutations in *mei-W68*, which encodes the *Drosophila* homolog of yeast gene *SPO11*, a gene required for the

induction of DSBs during recombination (Ghabrial et al., 1998; Roeder, 1997) These results indicate that the *spn* or *okr* patterning defects result from activation of a meiotic checkpoint in response to the presence of unrepaired DSBs.

We show that, like the *spindle*-class genes, *CSN5* is required for the repair of recombination-induced DSBs during *Drosophila* oogenesis. The CSN5 protein (also known as Jab1), is a subunit of the eight protein COP9 signalosome complex (CSN) originally identified in plants and conserved from plant to mammalian cells (for reviews, see Seeger et al., 2001; Schwechheimer and Deng, 2001; Bech-Otschir et al., 2002). As the genes for the CSN subunits were identified, a striking similarity was noticed between them and the eight subunits of the regulatory lid of the proteasome, suggesting a common ancestry and related function (Glickman et al., 1998; Seeger, 1998; Wei et al., 1998). This similarity was intriguing because examinations of CSN function have shown that it regulates protein stability in pathways leading to ubiquitination and degradation by the proteasome (reviewed by Seeger et al., 2001; Schwechheimer and Deng, 2001; Kim et al., 2001).

The CSN has been implicated in many regulatory and signaling functions including activation of the Jun transcription factor, stabilization of nuclear hormone receptors and interactions with integrins. Most relevant here, the CSN or its subunits have been shown to regulate multiple steps in the mitotic cell cycle. For example, the CSN regulates the ubiquitination and degradation of the CDK inhibitor, $p27^{kip1}$, and either a small, CSN5-containing subcomplex or CSN5 alone promotes $p27^{kip1}$ nuclear export (Yang et al., 2002; Tomoda et al., 1999). In addition, a CSN-associated kinase activity promotes degradation of p53, thereby allowing cell cycle progression (Bech-Otschir et al., 2001).

In *Drosophila CSN5* is essential for development (Freilich et al., 1999) and was recently shown to be required in photoreceptor cells to induce glial cell migration (Suh et al., 2002). We report the first example of a *CSN5* effect on meiosis and on axis determination. We find that homozygous *CSN5*-mutant clones disrupt both the DV and AP axes of the oocyte as a result of decreased Grk protein. These effects on axis determination appear to be caused by activation of the meiotic recombination checkpoint.

MATERIALS AND METHODS

Fly strains

Canton S and w^{1118} were used as standard strains. *placW* insertion line l(3)L4032 (referred to here as $CSN5^{L4032}$) was obtained from the Berkeley *Drosophila* Genome Project (Spradling et al., 1999). grk^{2B}, grk^{HK36}, grk^{HF}, $mei\text{-}41^{D3}$, $mei\text{-}41^{D1}$ and $mei\text{-}41^{RT}$ lines were a gift from T. Schupbach (described by Neuman-Silberberg and Schupbach, 1993; Ghabrial and Schupbach, 1999). kek^{15A6} was obtained from N. Perrimon and described in Musacchio and Perrimon (Musacchio and Perrimon, 1996). Enhancer trap line PZ6256 (Liu and Montell, 1999) was a gift from D. Montell. Strains $mei\text{-}W68^{1}$, $mei\text{-}W68^{k05603}$, $EGFR^{f2}$, $EGFR^{E1}$, $EGFR^{t1}$, $slbo^{01310}$ ($slbo^{1}$) were obtained from the Bloomington *Drosophila* Stock Center. Standard conditions were used for raising flies. Crosses were performed at 25°C except as described in the text. Embryos were collected on molasses/agar plates. Flies carrying a GFP balancer were used to determine the lethal stage of development.

Genetics

CSN5 homozygous-mutant germline clones were produced by using the dominant-female-sterile, FLP/FRT technique (Chou and Perrimon, 1992; Chou and Perrimon, 1996). Females of genotype *w; $CSN5^{L4032}$ P{neoFRT}82B/TM3B, Sb* or *w; $CSN5^{35ex}$ P{neoFRT}82B/TM3B, Sb* were mated with males of genotype *w hsFLP*; ovo^{D1} *FRT82B/TM3B, Sb*. Their progeny were heat shocked as third instar larvae or early pupae for two hours at 37°C for 2 consecutive days to induce *FLP* expression. Follicle cell mosaic clones were induced as described by Duffy et al. (Duffy et al., 1998): flies carrying *w; P{en2.4-GAL4}e22c P{UAS-FLP1.D}JD1/CyO*; *P{neoFRT}82B P{Ubi-GFP(S65T)nls}3R* were mated with *w; P{neoFRT}82B CSN5*/TM3B, Sb* flies. Eggs were collected and examined for several days after eclosion. Females were dissected to confirm the presence of homozygous-mutant follicle cells marked by the absence of GFP.

The original *CSN5* P element insertion l(3)L4032 was mobilized by introducing the *P[ry^{+}(Δ2-3)]99B* transposase source (Engels et al., 1987). Derivatives that had lost the w^{+} marker carried by the original insert were crossed back to $CSN5^{L4032}$ to identify imprecise excisions. The majority of new excision lines appear to be precise excisions of the original P-element insertion. They were fully viable and had no ovarian defects when homozygous or when heterozygous with $CSN5^{L4032}$. Several weak alleles of *CSN5* were also identified. They had poor viability and weak ovarian defects when heterozygous with $CSN5^{L4032}$. Finally, several lines failed to complement the lethality of $CSN5^{L4032}$, and produced, as germline clones, similar ovarian defects as did $CSN5^{L4032}$.

Staining procedures

The fixation and visualization of egg chambers and embryos was performed as described (Cant et al., 1994; Verheyen and Cooley, 1994). For immunostaining, the following antibodies were used: mouse anti-Grk (1:20), rat anti-Grk (1:500), rabbit anti-sperm-tail (1:500), rabbit anti-Vasa (1:1000) (gifts from T. Schupbach, R. Cohen, T. Karr and P. Lasko). To monitor *lacZ* expression of the P-*lacZ* insertion mutations, ovaries were treated according to Verheyen and Cooley (Verheyen and Cooley, 1994). For actin visualization, ovaries were stained with rhodamine-conjugated phalloidin (Molecular Probes). To visualize nuclei, tissues were stained with DAPI. High magnification fluorescent images were collected on a Zeiss 510 confocal microscope.

In situ hybridization

In situ hybridization using digoxigenin-labeled antisense RNA probes was carried out as described (Tautz and Pfeifle, 1989) with modifications (Harland, 1991). Hybridization signals were visualized by histochemical staining with alkaline phosphatase. Embryos and ovaries were mounted in 70% glycerol and viewed and photographed with Nomarski optics on a Leica DMRB microscope.

Western and northern blots

Protein extracts for western blot analysis were prepared as described by Sambrook et al. (Sambrook et al., 1989). *Drosophila* CSN5/JAB1 protein was detected using a mouse polyclonal, and three independent mouse monoclonal, anti-mouse Jab1 antibodies (GeneTex), or a rabbit polyclonal, anti-mouse Jab1 antibody (Santa Cruz Biotechnology). On a western blot, all of these antibodies recognized the same 37-38 kDa band, consistent with the predicted size of *Drosophila* CSN5. No other specific bands were detected. This band is strongly reduced in extracts from $CSN5^{L4032}$ germline clone ovaries and is reduced to different extents by the hypomorphic alleles derived from $CSN5^{L4032}$. Monoclonal antibody MS-JAB11-PXS (GeneTex) was used for the western blots in this paper. We used rat polyclonal and mouse monoclonal anti-Grk antibodies (gifts from T. Schupbach and R. Cohen), rabbit anti-Vasa (a gift from P. Lasko), or monoclonal anti-Actin (ICN). Secondary antibodies for signal detection were a goat

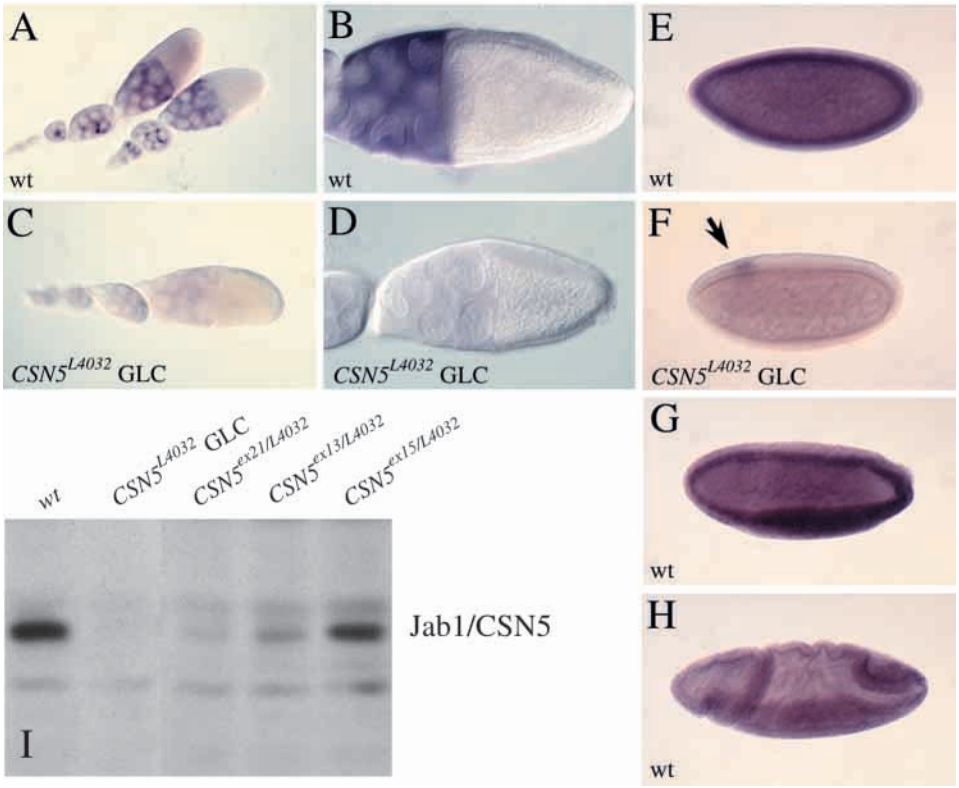

Fig. 1. *CSN5* expression in wild-type and *CSN5* mutants. (A,B) In wild-type ovaries, *CSN5* expression is detected in nurse cells beginning in the germarium. (C,D) In $CSN5^{L4032}$-mutant germline clones (GLC), expression of *CSN5* RNA is strongly reduced. Northern blot analysis of ovarian extracts show a similar reduction in *CSN5* GLC (data not shown). (E) Expression in early wild-type embryos indicates a strong maternal contribution. (F) In *CSN5* heterozygotes that lack maternal *CSN5* RNA, zygotic expression is detectable in an anterior stripe during cellular blastoderm (arrow). (G,H) During gastrulation *CSN5* RNA is most strongly expressed in the ventral furrow, the cephalic furrow, and both the anterior and posterior midgut invaginations. This expression pattern is absent in maternal and zygotic minus embryos (data not shown). All figures show anterior towards the left. (I) Western blot of wild-type and *CSN5*-mutant ovarian extracts. A single 37-38 kDa band seen in wild-type ovaries was strongly reduced in $CSN5^{L4032}$ GLC ovaries. Its size is consistent with the predicted size for *Drosophila* CSN5 (37 kDa) and no other specifically reduced bands were detected. Heteroallelic combinations of $CSN5^{L4032}$ with excision derivatives show a gradation of CSN5 protein that correlates with their allelic strength determined from viability, eggshell phenotypes and northern blots.

anti-rat or anti-mouse and a protein-A horseradish peroxidase conjugate (Molecular Probes; Santa Cruz Biotechnology). Proteins were visualized using chemiluminescent detection (NEN Life Science Products).

Total or polyA$^+$ RNA was isolated from ovaries as described (Sambrook et al., 1989). RNA was resolved on formaldehyde-agarose gels, transferred to nylon membranes, crosslinked and hybridized by standard procedures.

RESULTS

During oogenesis *CSN5* is expressed in nurse cells

Since most *CSN5* homozygotes die during larval or pupal development (this paper) (Freilich et al., 1999), it seemed likely that embryos receive a maternal contribution of *CSN5* RNA or protein. In situ hybridization confirmed this expectation, showing that *CSN5* RNA accumulates in the nurse cells beginning in the germarium and continuing through most of oogenesis (Fig. 1A-D). During stage 10, *CSN5* RNA is transferred to the oocyte along with the bulk of the nurse cell cytoplasm.

In embryos, uniformly distributed maternal RNA is evident until gastrulation begins. The earliest zygotic expression is in an anterior stripe during cellular blastoderm. During gastrulation, zygotic expression becomes evident in the ventral furrow, the cephalic furrow, and both the anterior and posterior midgut invaginations (Fig. 1E-H).

CSN5 is required for eggshell patterning

To enable an analysis of early embryonic requirements for *CSN5*, we induced homozygous, *CSN5*-mutant germline clones (Chou and Perrimon, 1992). These clones revealed requirements for *CSN5* during oogenesis as well as embryogenesis. In ovarian germline clones the level of *CSN5* RNA is dramatically reduced, but still detectable, indicating that the P-element-induced allele, $CSN5^{L4032}$, is hypomorphic (Fig. 1C,D). Depending on the paternal allele, embryos derived from the germline clones showed either a reduced amount of *CSN5* RNA in the zygotic pattern (Fig. 1F) or no detectable *CSN5* RNA (not shown).

Flies carrying *CSN5* germline clones laid eggs with a range of abnormal phenotypes that were affected by temperature (Fig. 2, Table 1). Flies grown at 25°C laid eggs with phenotypes closest to normal. The most frequent defects at 18°C were different from those at 29°C. At 18°C many of the defective eggs had fused dorsal appendages (Fig. 2B). At 29°C there was an increasing frequency of properly separated but short dorsal appendages (Fig. 2E). These results suggest that aberrations in patterning the follicular epithelium predominate at 18°C, while defects in follicle cell migration predominate at 29°C.

Because the eggshell phenotypes were only partially penetrant, it was possible that they were caused by somatic, rather than germline, *CSN5* clones. To test this possibility, we induced somatic clones in the ovary by using the follicle cell driver *E22c-GAL4* to induce expression of *UAS-FLP* (Duffy et al., 1998). Under these conditions, there were no eggshell defects at any temperature, indicating that this requirement for *CSN5* function is limited to the germline.

In addition to the eggshell defects, the viability of *CSN5*

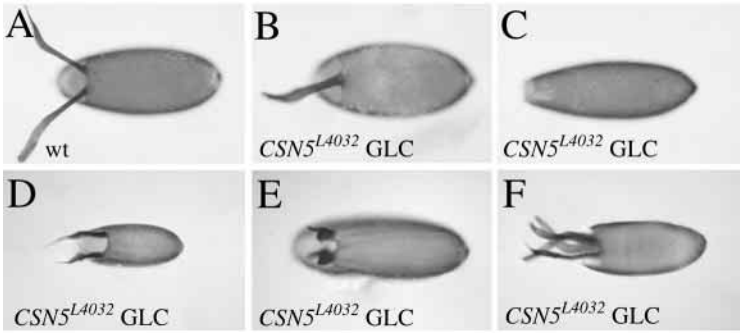

Fig. 2. Mutations in *CSN5* cause eggshell defects. (A) A wild-type eggshell. Note the length of the dorsal appendages (DA) and the separation at their bases. (B-F) Eggshells derived from females carrying *CSN5*-mutant, germline clones. (B,C) Fused or absent DA in weakly or strongly ventralized eggshells. (D) A partially dorsalized eggshell. Bases of the appendages are more widely separated than in wild type. (E) Short but normally spaced DA. (F) An unusual eggshell with duplicated DA. In addition to these eggshell defects, some eggs had an unusually weak eggshell. These eggs were sometimes destroyed during attempts to move them. This phenotype may be related to observations made during dechorionation or fixation that many eggs from *CSN5* GLC mothers fall into pieces.

mutants also depends on temperature. At 29°C the original P-element mutation is lethal during early development with fewer than 1% of the mutant larvae becoming prepupae. By contrast, at 18°C 90% of the mutant larvae pupariate and 1-2% escape as adults. Mobilization of the original P-element insertion confirmed that it was responsible, not only for lethality, but also for the eggshell defects; precise excisions were viable and had normal dorsal appendages.

Table 1. Eggshell defects in *CSN5* mutants depend on temperature

	Percentage of eggs		
Phenotype of eggs laid by $CSN5^{L4032}$ GLC females	18°C	25°C	29°C
Fused or partially fused DA	15-35	4-10	2-15
Strongly ventralized eggs	1-3	<1	<1
Dorsalized eggs	1-3	1-3	5-20
Short or absent DA	4-12	1-5	10-55
Multiple DA	1	<1	1
Soft chorion	5-10	5	40
Unhatched, mostly unfertilized embryos	8-10	2	95

Females carrying *CSN5*-mutant, germline clones (GLCs) frequently lay eggs with abnormal dorsal appendages (DA). The frequency of abnormal eggshells and the distribution among different classes of defective DA vary with temperature and fly age. 25°C is the most permissive temperature. At 18°C, more of the eggs were ventralized; at 29°C, the frequencies of short dorsal appendages and dorsalized eggs were higher. At 29°C, most *CSN5* GLC eggs remain unfertilized, as detected with anti-sperm tail antibody. Some fertilized mutant embryos (~5%) die during early embryonic development after a few nuclear divisions (revealed by DAPI staining). At all temperatures some eggs had an unusually soft chorion.

Maternal expression of *CSN5* is required for embryonic dorsal-ventral patterning

Some mutations that disrupt the DV patterning of the eggshell also affect the patterning of the embryo. To look for effects on the embryonic DV fate map, we used as markers the expression of three zygotic genes: *decapentaplegic* (*dpp*), *rhomboid* (*rho*) and *twist* (*twi*) (Fig. 3). *dpp* is expressed on the dorsal side of the embryo as well as its anterior and posterior ends (St Johnston and Gelbart, 1987). *rho* is expressed in two, eight-cell-wide ventrolateral domains and later also in a narrow stripe on the dorsal side of the embryo (Bier et al., 1990). *twi*, a marker for the mesoderm, is expressed ventrally in the embryo (Thisse et al., 1988).

For all three of the markers, many of the *CSN5*-mutant embryos appeared to be ventralized (Fig. 3B,E,H). In these embryos *dpp* expression on the dorsal side was reduced or absent. The dorsal *rho* stripe was reduced and the lateral stripes were moved dorsally. *twi* expression appeared to expand

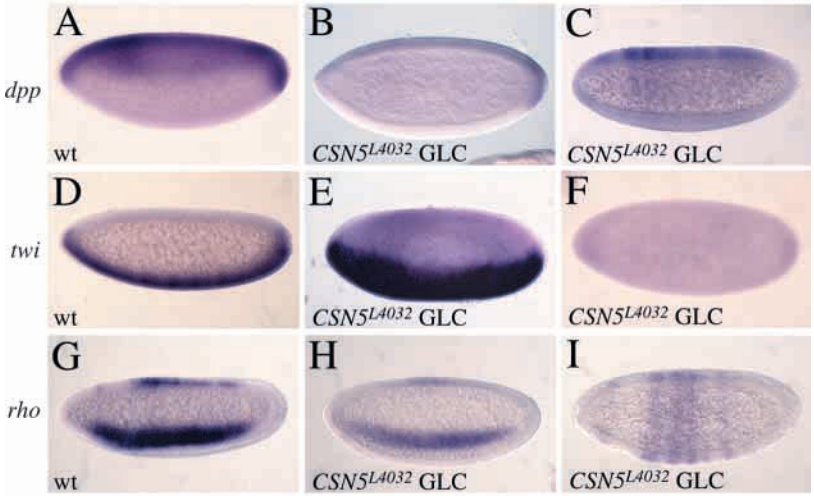

Fig. 3. *CSN5* is required for dorsoventral patterning of the embryo. (A) In wild-type embryos *dpp* RNA localizes to the dorsal side of the egg as well as the two poles. (B) In eggs from CSN5 GLC mothers, the *dpp*-expressing domain is often reduced, indicating that the embryo is ventralized. (C) In rare, dorsalized embryos, *dpp* RNA extends around the DV circumference. (D) In wild-type embryos, *twi* RNA is expressed in the ventral mesoderm and at both poles. (E,F) *twi* RNA in embryos derived from *CSN5* germline mothers. (E) In ventralized embryos, *twi* RNA is more broadly expressed ventrally and laterally. (F) In rare, strongly dorsalized embryos, *twi* RNA fails to accumulate in the mutant egg. (G) In wild-type embryos, *rho* RNA is expressed in ventrolateral stripes and a dorsal midline stripe. (H) In weakly ventralized eggs derived from *CSN5* GLC mothers, the lateral stripe of *rho* RNA was moved dorsally and the dorsal stripe was reduced. (I) In dorsalized embryos *rho* RNA accumulates in stripes that are probably derived from the dorsal stripe seen in wild-type embryos. Zygotic expression patterns could not be analyzed in the substantial fraction of embryos that were unfertilized or extremely fragile (see Table 1).

dorsally about halfway around the embryo. Some embryos showed stronger ventralization at their anterior or posterior ends (data not shown). There were also infrequent embryos that appeared to be dorsalized (Fig. 3C,F,I).

CSN5 is also required for anterior-posterior polarization

To characterize *CSN5* mutants further, we examined the spatial localization of the RNAs for two determinants of AP polarity, *bicoid* (*bcd*) and *oskar* (*osk*). The localization of *bcd* RNA to the anterior pole of the oocyte is crucial in the establishment of AP polarity (Nusslein-Volhard et al., 1987; Berleth et al., 1988; St Johnston et al., 1989). In *CSN5* mutant oocytes and embryos, *bcd* mRNA was abnormally expressed in 10-15% of oocytes (Fig. 4). In these abnormal oocytes, the *bcd* mRNA is diffusely distributed and sometimes accumulated near the center of the oocyte (Fig. 4B). In mutant embryos, the *bcd* RNA often shifted toward the dorsal side of the embryo (Fig. 4F).

The posterior pole of the egg chamber is defined by the tight, posterior localization of *osk* RNA (Ephrussi et al., 1991; Kim-Ha et al., 1991). Although most *CSN5*-mutant oocytes and embryos were nearly normal, *osk* RNA in 10-15% of mutant oocytes and embryos was reduced or mislocalized (Fig. 4). In the abnormal oocytes, the *osk* RNA was typically diffuse or concentrated in the center of the oocyte (Fig. 4D). Only small amounts were localized at the posterior pole. In the abnormal embryos only a small amount of *osk* RNA at the posterior pole remained. In these embryos the *osk* RNA appeared to be shifted slightly dorsally from its normal position at the extreme posterior end (Fig. 4H).

Since the localization of *osk* and *bcd* RNAs depends on polarization of the microtubule lattice, we used a reporter for the motor protein kinesin to examine microtubule organization in *CSN5* germline clones (Clark et al., 1994). Kinesin moves toward the plus ends of microtubules, and in stage 8-9 wild-type egg chambers kinesin-β-gal localizes to the posterior of the oocyte. However, in some *CSN5*-mutant oocytes kinesin-β-gal staining was diffuse or mislocalized (not shown).

CSN5 may also be required for proper pole cell organization

In addition to its role in determining the AP axis, *CSN5* may have a distinct role in pole cell development. In normal embryos, the pole cells form as a tight, contiguous cluster at the posterior end of the embryo (Fig. 4I). As gastrulation and germ band extension begin, somatic epithelial cells at the posterior end of the embryo form a shallow cup that will eventually become the posterior midgut invagination. The pole cells adhere to this cup and remain tightly clustered on its surface as they are conveyed over the dorsal side of the embryo and then into its interior. In *CSN5*-mutant embryos the number of pole cells is often reduced, as might be expected because of the inefficient localization of *oskar* RNA (Fig. 4J). In addition, the pole cells are occasionally found in a loose, non-contiguous group near, but not tightly associated with, the posterior end of the embryo (Fig. 4K). This is an unusual phenotype, not seen in other mutants that impair the formation of pole plasm. Thus, in addition to its role in *oskar* RNA localization, *CSN5* may have a separate role in organizing the pole cell cluster.

CSN5 is required for *grk* signaling

Since *CSN5* germline clones caused defects in both the AP and DV axes, it seemed possible that *grk* signaling was

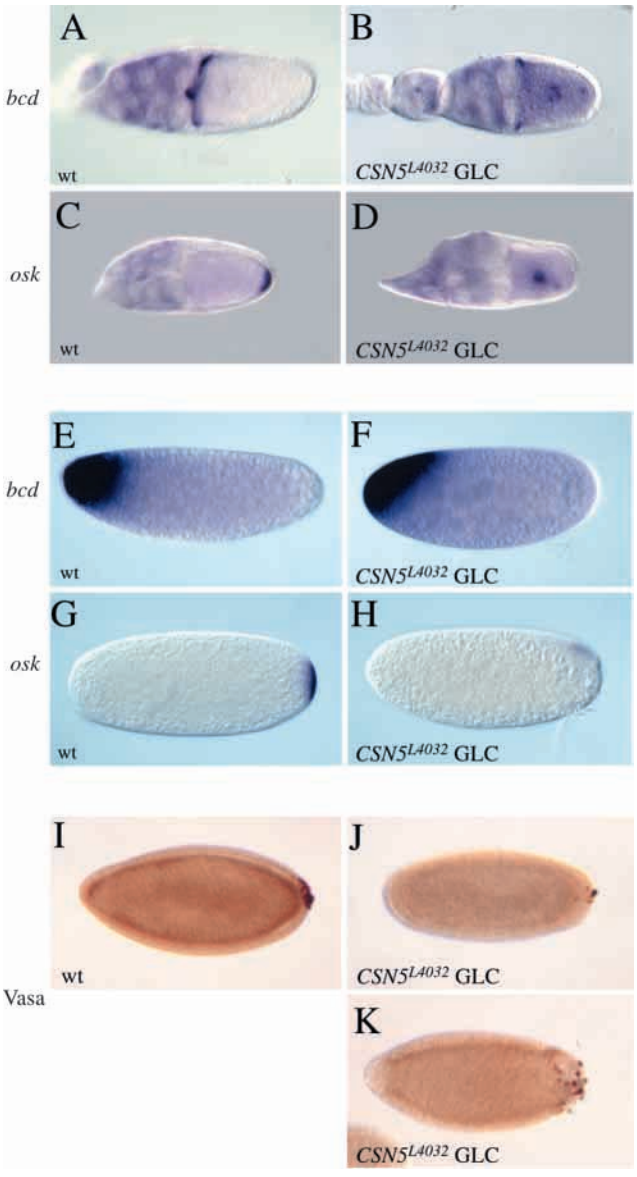

Fig. 4. Effects of *CSN5* mutations on the AP axis. (A-H) *CSN5* is required for proper localization of *bcd* and *osk* RNAs. (A,B) In *CSN5*-mutant oocytes, *bcd* RNA is often present diffusely throughout the oocyte. In addition, the amount of *bcd* RNA at the anterior end of the oocyte may be reduced and there may be central *bcd* RNA concentrations. (E,F) In embryos the effects of *CSN5* mutations on *bcd* RNA localization are less extreme. Some embryos do show dorsally displaced *bcd* RNA. (C,D) In *CSN5*-mutant oocytes, little *osk* RNA is localized at the posterior pole. Instead it is often present throughout the oocyte cytoplasm or in central concentrations. (G,H) In some mutant embryos, *osk* RNA is posteriorly localized, but is strongly reduced and sometimes shifted dorsally. (I-K) *CSN5* may affect localization and number of pole cells. In agreement with the *osk* RNA results, anti-Vasa staining often shows a reduced number of pole cells (J). In some cases the pole cells were not tightly clustered (K).

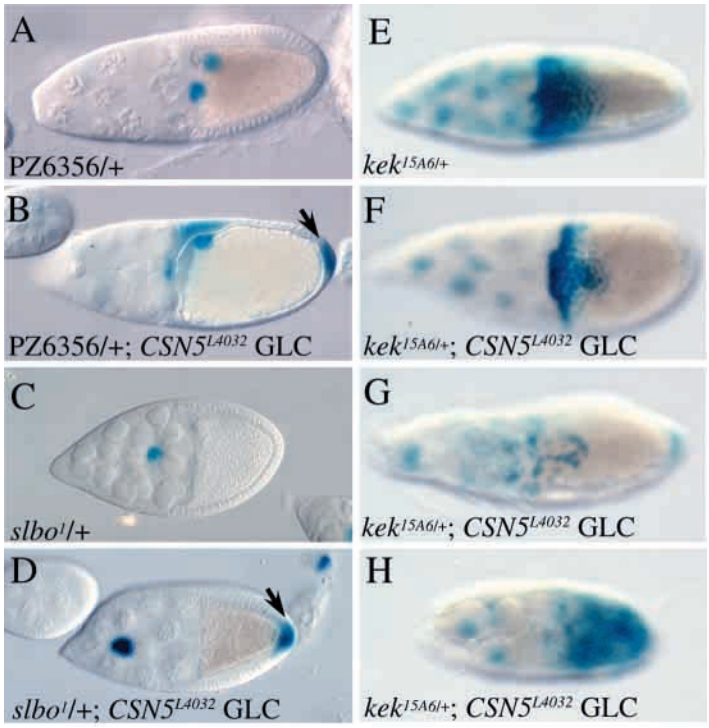

Fig. 5. Germline *CSN5* mutations affect follicle cell patterning. (A-D) Reduction of *CSN5* function disrupts the specification of the terminal follicle cells. In wild-type egg chambers, the PZ6356 enhancer trap is expressed in border cell and oocyte nuclei (A), while the *slbo* enhancer trap is expressed only in border cells (C). In *CSN5* mutant egg chambers, both enhancer traps are also expressed in posterior follicle cells (arrows, B,D), suggesting that they have taken on anterior fate. (E-H) *CSN5* GLCs affect patterning of the dorsoanterior follicle cells. In *CSN5*-mutant egg chambers, expression of the *kekkon* enhancer trap is often reduced (F,G) or in rare cases undetectable (not shown). In a small number of egg chambers, *kekkon* is expressed in a larger than normal patch of dorsal follicle cells (H).

compromised (Gonzalez-Reyes et al., 1995; Roth et al., 1995). As described in the introduction, *grk* is unusual among axis-determining genes in being required for both axes.

To assess the role of *CSN5* in *grk* signaling, we used reporters for either the posterior or the dorsal Grk signal. In the absence of the posterior Grk signal, the posterior follicle cells appear to adopt the anterior follicle cell fate and express markers that are characteristic of the border cells (Gonzalez-Reyes et al., 1995; Roth et al., 1995). We used two such markers, an enhancer trap called PZ6356 (Fig. 5A) and a *slbo-lacZ* enhancer trap (Fig. 5C) (Montell et al., 1992; Tinker et al., 1998; Liu and Montell, 1999), to monitor whether *CSN5* is required for the early Grk signal. For both markers, loss of *CSN5* from the germline caused *lacZ* expression in the posterior follicle cells of many egg chambers, suggesting a reduction in Grk signaling (Fig. 5B,D). To monitor *EGFR* signaling to the dorsal follicle cells at stages 9 and 10, we used a *kekkon* (*kek*)-*lacZ* reporter construct (Fig. 5E). Because the *kek* gene acts downstream of the *EGFR* pathway in the follicle cells, it can serve as a sensitive indicator of *grk* activity coming from the oocyte (Musacchio and Perrimon, 1996; Sapir et al., 1998). We found that at 18°C *kek* expression is abnormal in about a third of *CSN5*-mutant egg chambers at stage 10 (but only 3-4% at 25°C). In most of these egg chambers, expression in the dorsal anterior follicle cells over the oocyte was reduced or, rarely, absent (Fig. 5F,G). A small number of egg chambers show broader expression of *kek* in the follicle cells, probably reflecting the small number of dorsalized embryos arising from these mutant egg chambers (Fig. 5H). We conclude that in most egg chambers both posterior and dorsal Grk signaling are impaired in *CSN5*-mutant germline clones.

Further evidence that *CSN5* affects Grk signaling came from testing for genetic interactions between *CSN5* and either *grk* or *EGFR*. Females heterozygous for strong *grk* alleles lay eggs with fused or partially fused dorsal appendages (Table 2). This dominant phenotype provides a sensitive background for detecting interactions. With the exception of a precise P-element excision, all *CSN5* alleles showed strong enhancement of the dominant *grk* phenotype (Table 2). In addition, $CSN5^{L4032}$ weakly enhanced the dominant eggshell phenotype of a loss of function *EGFR* allele, $EGFR^{f2}$.

These results suggested that production of *grk* RNA or protein might be affected in *CSN5* germline clones. In situ hybridization using a *grk* probe showed normal or nearly normal localization of *grk* RNA in most *CSN5*-mutant stage 10 oocytes (Fig. 6B). In some of these mutant oocytes the messenger was improperly localized, probably because the oocyte nucleus was no longer located at the dorsal corner of the oocyte (Fig. 6C). Interestingly, in these oocytes the 'dorsal' follicle cells were often columnar as though the nucleus had been properly localized at an earlier stage (Fig. 6C). A northern blot showed nearly normal amounts of *grk* mRNA in ovaries carrying *CSN5*-mutant germline clones, consistent with the strong signals seen by in situ hybridization in most oocytes (Fig. 6G).

Table 2. *CSN5* and *gurken* interact genetically

	% fused or partially fused dorsal appendages (eggs counted)		
Genotype	18°C	25°C	29°C
grk^{2B}/+	75 (266)	27.7 (3140)	13.8 (2622)
grk^{2B}/+; $CSN5^{ex27}$/+	Not determined	23.6 (1284)	13.2 (580)
grk^{2B}/+; $CSN5^{L4032}$/+	97.7(347)	55.3 (2068)	52 (3853)
grk^{2B}/+; $CSN5^{ex15}$/+	Not determined	31.7 (1298)	25.3 (953)
grk^{2B}/+; $CSN5^{ex9}$/+	Not determined	66 (1585)	48 (440)
grk^{2B}/+; $CSN5^{ex35}$/+	Not determined	70.5 (1380)	52.3 (965)
grk^{HF}/+	98 (200)	66.7 (2760)	34 (842)
grk^{HF}/+; CSN^{L4032}/+	98 (180)	86.3 (2302)	59.5 (1951)
$EGFR^{f2}$/+	27.8 (2652)	24.6 (1267)	51.4 (5010)
$EGFR^{f2}$/+; $CSN5^{L4032}$/+	31.1 (2983)	25.7 (1012)	60.9 (1474)

Several genes involved in *Grk-EGFR* signaling were tested for dominant genetic interactions with *CSN5*. Eggs laid by control or doubly heterozygous flies were examined at 18, 25 or 29°C. Two *grk* alleles showed strong genetic interactions with *CSN5*. $EGFR^{f2}$, a strong loss of function allele, showed a weak dominant interaction with $CSN5^{L4032}$. No dominant, eggshell-phenotype interactions were seen with mutations in *vasa*, *encore*, *squid*, *rolled*, *Ras1*, *fs(1)K10*, *capu*, *chic* or *spire*.

The *CSN5* alleles tested were: $CSN5^{ex27}$, a viable, precise excision allele; $CSN5^{ex15}$, a viable, weak allele; $CSN5^{ex9}$ and $CSN5^{ex35}$, lethal, strong alleles. By themselves, heterozygous *CSN5* alleles had no abnormal phenotypes.

Immunostaining of egg chambers using anti-Grk antibodies showed a more extreme effect. Grk protein was strongly reduced in *CSN5* mutants compared with controls, although the residual protein usually appeared to be properly localized (Fig. 6D-F). This reduction was confirmed by western blot analysis (Fig. 6H). There were also a few cases of Grk protein mislocalization, sometimes being present all along the anterior end of the oocyte (data not shown).

The reduction in Grk protein appeared to be most extreme at early stages in oogenesis. Grk expression begins in region 2a in wild-type germaria. The signal appears in several cells per cyst in regions 2a and 2b and then becomes concentrated in the oocyte cytoplasm by region 3 (Fig. 7A). In viable, hypomorphic combinations such as $CSN5^{ex21}/CSN5^{L4032}$, we could not detect Grk expression in the

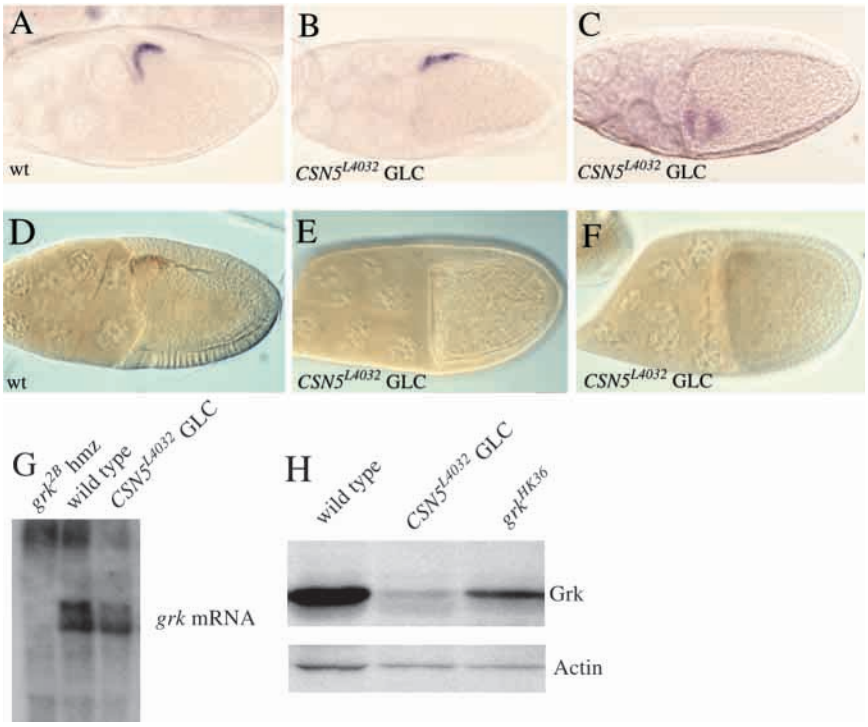

Fig. 6. *CSN5* is necessary for Grk protein expression, not for *grk* transcription. (A-C) In situ localization of *grk* RNA. At stage 10, *grk* transcript accumulates at the dorsoanterior corner of wild-type and most *CSN5*-mutant oocytes (A,B). Occasionally, *grk* RNA is mislocalized, probably because of mislocalization of the oocyte nucleus (C). (D-F) Grk antibody immunostaining. In *CSN5* GLC egg chambers, expression of Grk is reduced or more diffusely distributed than in wild type. (G) Northern blot analysis of *grk* mRNA levels. In *CSN5* GLC ovaries, the level of *grk* mRNA (extracted from an equal number of ovaries) is similar to wild type and significantly higher than in *grk* null-mutant ovaries. (H) Western blot analysis of Grk protein level. The Grk antibody recognizes a 46-47 kDa band that is reduced in ovaries homozygous for a hypomorphic *grk* allele, grk^{HK36}, and strongly reduced in ovaries from *CSN5* germline clones.

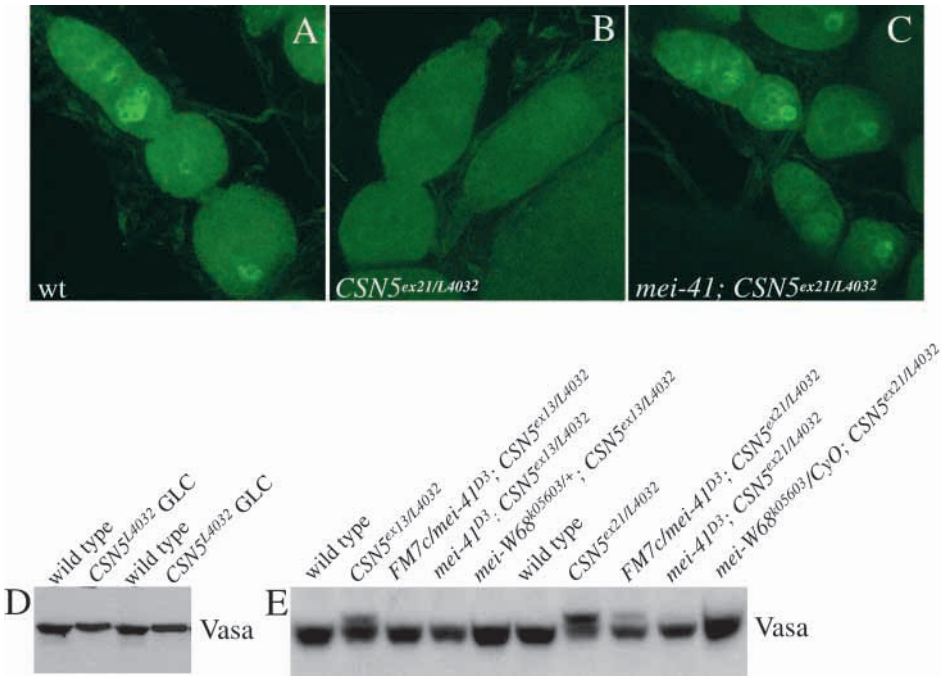

Fig. 7. *CSN5* mutations activate a *mei-41*-dependent meiotic checkpoint. (A) In wild type, Grk protein is detectable in region 2a of the germarium and is restricted to the oocyte from region 3 onwards. (B) In $CSN5^{ex21}/CSN5^{L4032}$ germaria, Grk is undetectable. (C) Loss of *mei-41* restores Grk expression in $CSN5^{ex21}/CSN5^{L4032}$ heterozygotes. (D,E) *CSN5* mutations affect the mobility of Vasa protein. (D) An anti-Vasa western blot of ovarian extracts shows retarded Vasa migration in $CSN5^{L4032}$ germline clones. (E) An anti-Vasa western blot of ovarian extracts from several *CSN5* allelic combinations. In wild type, Vasa migrates as a single 72 kDa band. Vasa migrates as two bands in $CSN5^{ex13}/CSN5^{L4032}$ and $CSN5^{ex21}/CSN5^{L4032}$. Notice, that in the stronger combination ($CSN5^{ex21}/CSN5^{L4032}$) more Vasa protein migrates slowly than in the weaker combination ($CSN5^{ex13}/CSN5^{L4032}$). Furthermore, in $CSN5^{ex13}/CSN5^{L4032}$, Vasa is fully restored to normal mobility by removal of one dose of *mei-41*, while $CSN5^{ex21}/CSN5^{L4032}$ requires homozygous *mei-41* for normal Vasa mobility. Removing one dose of *mei-W68* is sufficient to restore Vasa mobility in both hypomorphic, *CSN5*-mutant combinations. A second *mei-W68* allele ($mei\text{-}W68^{1}$) had a weaker effect. As a heterozygote with the *CSN5*-mutant combinations, it gave only a partial rescue of Vasa mobility, although the $mei\text{-}W68^{1}/mei\text{-}W68^{k05603}$ combination resulted in full rescue of Vasa mobility (data not shown).

germarium (Fig. 7B). With this combination Grk does become detectable from stage 2-3 onwards (data not shown), suggesting that a reduction in CSN5 causes a delay in the beginning of Grk accumulation (see Discussion). Taken together these results show that the major effect of *CSN5* mutations appears to be on *grk RNA* translation or on stability of the protein.

CSN5 mutations activate a meiotic checkpoint

Because of the similarity between the *CSN5* and *spindle*-class phenotypes, we tested for a connection between *CSN5* and the meiotic checkpoint mediated by *mei-41*. As mentioned above, the viable hypomorphic combination $CSN5^{ex21}/CSN5^{L4032}$ caused a reduction in Grk protein level, especially during the early stages of oogenesis (Fig. 7B). Five to fifteen percent of eggs laid by these transheterozygotes had fused dorsal appendages, indicating a partial reduction of Grk. When $CSN5^{ex21}/CSN5^{L4032}$ flies were also homozygous-mutant for *mei-41*, however, the normal Grk protein level was restored (Fig. 7C), and the eggshell phenotype was rescued (not shown).

Interestingly, checkpoint activation leads to modification of the Vasa protein, as shown by a slightly reduced mobility during SDS polyacrylamide gel electrophoresis (Ghabrial and Schupbach, 1999). This result is relevant to the *spindle*-class and *CSN5* phenotypes because Vasa regulates translation of Gurken and, as a consequence, axial patterning (Styhler et al., 1998; Tomancak et al., 1998). This Vasa modification is checkpoint dependent since it is present in *spn-B* mutants but absent in *mei-41 spn-B* double mutants (Ghabrial and Schupbach, 1999).

We detected a similar reduced mobility of Vasa protein in *CSN5* mutants (Fig. 7D). For viable *CSN5* mutants there were two Vasa bands: one corresponding to Vasa from wild-type ovaries and a second with lower mobility. In stronger mutant combinations, most of the Vasa protein was modified, while in weaker combinations most Vasa had normal mobility. The shift in Vasa mobility was suppressed by *mei-41* mutations. Interestingly, removal of one dose of *mei-41* completely restored normal Vasa mobility for a weak *CSN5* combination. For stronger *CSN5* mutants, full restoration of Vasa mobility required removal of both *mei-41* genes (Fig. 7E).

The gene *mei-W68* is required for the initiation of meiotic recombination in *Drosophila* ovaries and is likely to induce DNA double strand breaks (DSBs) as recombination begins (McKim and Hayashi-Hagihara, 1998). Mutations in *mei-W68* were shown to rescue *spindle*-class defects, including Grk protein accumulation, eggshell morphology and Vasa modification (Ghabrial and Schupbach, 1999). These results suggested that since DSBs were not formed in the absence of *mei-W68*, DNA repair by the *spindle*-class genes was not required. A similar interaction was seen between *mei-W68* and *CSN5*. Hetrerozygosity for *mei-W68* was sufficient to suppress the phenotypes of both strong and weak *CSN5* allelic combinations (Fig. 7E).

These data demonstrate that absence of CSN5 function during meiosis activates a DNA-damage checkpoint that is mediated by Mei-41. Because the reduction in DSBs in *mei-W68* heterozygotes removes the requirement for *CSN5*, it is likely that CSN5 promotes DNA repair, as do the *spindle*-class genes.

DISCUSSION

CSN5 participates in axis establishment

Establishment of both AP and DV polarity requires expression of the TGF-α homolog Gurken in the oocyte and activation of the EGF receptor and its downstream effectors in the adjacent follicle cells. Our results show that CSN5 is required in the germline for these critical signaling events. Several results tie *CSN5* to Grk-EGFR signaling. First, *CSN5* mutations affected both axes as shown by DV defects in the eggshell, mislocalization of *bcd* and *osk* RNAs in both the oocyte and embryo, and mislocalization of *dpp*, *rho* and *twi* expression in the embryo. Second, *CSN5* germline clones affected the expression of follicle cell reporters for Grk-EGFR signaling: *slbo* and the PZ6356 enhancer trap in the posterior follicle cells, *kek* expression in the dorsal anterior follicle cells. Third, *CSN5* alleles show strong genetic interactions with *grk* alleles. Finally, Grk protein is reduced in *CSN5* germline clones, starting in region 2a of the germarium but still evident in stage 10 egg chambers or in ovary extracts.

CSN5 mutations activate a *mei-41*-dependant meiotic checkpoint

Previous studies have shown that the accumulation of Grk protein can be affected by activation of a meiotic checkpoint in response to the persistence of DNA double-strand breaks (Ghabrial and Schupbach, 1999). Mutations in several genes that play a role in DNA repair (*okra*, *spn-B*, *spn-C* and *spn-D*) activate this meiotic checkpoint and disrupt axial patterning in the oocyte. There is a remarkable similarity between the *CSN5*-mutant phenotype and defects caused by mutations in these *spindle*-class genes (described by Gonzalez-Reyes et al., 1997; Ghabrial et al., 1998; Ghabrial and Schupbach, 1999). In both cases mutant females produced eggs with a variety of partially penetrant eggshell defects: mild or strongly ventralized, dorsalized, or small eggs or eggs with multiple dorsal appendages. Embryonic patterning was also disrupted, and both axes were affected. As had been seen in *spindle*-class mutants, the oocyte of some *CSN5*-mutant egg chambers was positioned laterally or at the anterior end, and some had defects in karyosome morphology (data not shown). There was also a similar, strong reduction in Grk protein, with one intriguing difference. At early stages of oogenesis in *CSN5* mutants, the level of Grk protein was always strongly reduced, both in germline clones of the strong $CSN5^{L4032}$ allele and in hypomorphic combinations of $CSN5^{L4032}$ with viable excision mutants (Fig. 7). Although Grk was also strongly reduced in $CSN5^{L4032}$ germline clones at later stages (Fig. 6), it often appeared to be present at higher levels than in the germarium. With the hypomorphic combinations, it was often difficult to detect any reduction in Grk protein at later stages. By contrast, in *spn-B* and *spn-D* mutants, Grk accumulates normally in early oogenesis but then declines and is often undetectable by stage 9-10 (Ghabrial et al., 1998). In *okr* mutants, the amount of Grk protein varies from one egg chamber to the next in a single ovariole, but a bias towards lower levels at early stages was not reported (Ghabrial et al., 1998). Thus, there seem to be three different patterns of Grk accumulation in these mutants. *CSN5* mutants appear to cause a more immediate response of Grk to DNA damage than do *spn-B* and *spn-D* mutants.

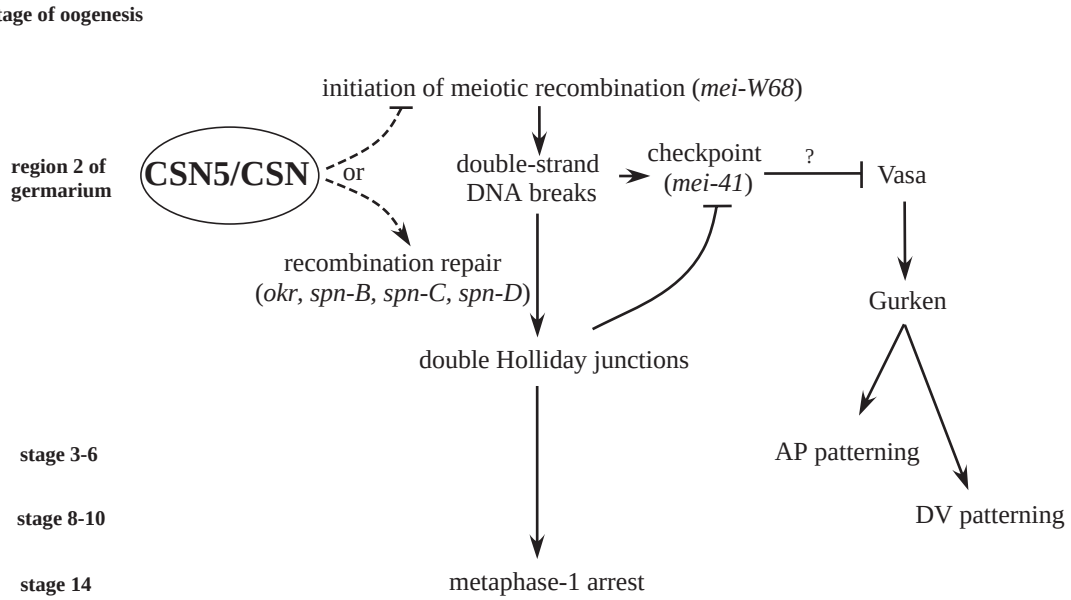

Fig. 8. Model of CSN5/CSN function during oogenesis (modified from Ghabrial and Schupbach, 1999). In region 2a of the germarium, meiotic recombination begins with the formation of DSBs under the control of *mei-W68* (McKim and Hayashi-Hagihara, 1998). These breaks are repaired by proteins of the recombination repair pathway, including the *spindle*-class genes. The progress of DSB repair is monitored by a *mei-41*-dependent checkpoint. If DNA damage persists, as it does in *CSN5* or *spindle*-class mutants, the checkpoint is activated and Vasa is modified to a form that prevents efficient translation of *grk* message. The ensuing underproduction of Grk protein leads to axial patterning defects (Ghabrial and Schupbach, 1999). We propose that the role of CSN5 is either to limit the production of DSBs, perhaps by promoting Mei-W68 turnover, or to stabilize one of the repair proteins, thereby promoting repair and bypass of the *mei-41*-dependent checkpoint.

Because of the similarities between the phenotypes and because at least two of the *spindle*-class genes, *okr* and *spn-B*, encode components of the RAD52 DNA repair pathway, it seems likely that *CSN5* directly or indirectly regulates DSB repair. The fact that *mei-41* and *mei-W68* mutations can suppress the *CSN5* phenotypes reinforces this conclusion. Kinases in the ATM/ATR subfamily that includes Mei-41 play a central role in checkpoint-mediated responses to DNA damage (for reviews, see Melo and Toczysky, 2002; Weinert, 1998; Murakami and Nurse, 2000). These checkpoint kinases are thought to act as sensors of DNA damage, becoming activated on binding damaged DNA. Phosphorylation of several downstream effectors, including the Chk1 and Chk2 kinases and p53, then restrains cell cycle progression until the DNA damage is repaired and the checkpoint kinases dissociate from the DNA. In *Drosophila mei-41* mutants, the checkpoint cannot be activated, and oocytes with damaged DNA, such as those mutant for *spindle*-class genes, can proceed through oogenesis. Suppression of *CSN5* phenotypes by *mei-41* mutations demonstrates that the *CSN5*-mutant lesion acts upstream of the DNA damage checkpoint and suggests that DSBs arising during meiotic recombination cannot be efficiently repaired in *CSN5*-mutant cells (Fig. 7).

Suppression by *mei-W68* restricts the possible role of CSN5 further. *mei-W68* encodes a topoisomerase II-like protein homologous to *S. cerevisiae* Spo11 and has been proposed to create the DSBs needed to initiate meiotic recombination (McKim and Hayashi-Hagihara, 1998). In flies mutant for *mei-W68*, DSBs are absent and meiotic recombination is eliminated. In double mutants of *mei-W68* with either *okr*, *spn-B* or *spn-C*, Grk protein accumulation and eggshell patterning are normal and other *spindle*-class defects are suppressed (Ghabrial and Schupbach, 1999). We found that heterozygosity for *mei-W68* was sufficient to suppress hypomorphic *CSN5*-mutant phenotypes (Fig. 7). Combination of this result with the *mei-41* suppression result indicates that CSN5 acts in the recombination pathway to regulate the formation of DSBs or their successful repair (Fig. 8).

vasa mutants show similar effects on axis determination and Grk protein accumulation as do *spindle* mutants and *CSN5* GLCs (Styhler et al., 1998). However, the *vasa* phenotypes are not suppressed by *mei-41* or *mei-W68* mutations, indicating that Vasa acts downstream of the meiotic checkpoint (Ghabrial and Schupbach, 1999). Indeed, Vasa is one of the targets of Mei-41 activity as Vasa electrophoretic mobility is changed in *spn-B* mutants but restored in *mei-41 spn-B* double mutants (Ghabrial and Schupbach, 1999). Since Vasa protein binds to *grk* mRNA and is required for both its localization in the oocyte and its translation, it seems likely that the checkpoint effects on Grk accumulation are directly mediated by Vasa, although other Mei-41 targets cannot be excluded (Fig. 8). Our results that show effects of *CSN5* mutants on Vasa mobility are entirely consistent with the previous *spn-B* results, as would be expected if both types of mutants activate the same checkpoint.

We propose that in *CSN5*-mutant oocytes DSBs created by Mei-W68 during meiotic recombination are repaired more slowly than in wild type. Accumulation of unrepaired DNA breaks would then activate the *mei-41*-dependent checkpoint leading to a block in the progression of meiotic prophase (Fig. 8). Since activated Mei-41 is an ATR-related kinase, it might modify Vasa directly or through downstream kinases such as Chk1 or Chk2. Modified Vasa would then prevent efficient Grk translation. Because *CSN5* mutants are likely to affect the stability rather than the presence or absence of repair proteins, the DNA DSBs might be slowly repaired during the checkpoint-induced delay, thereby allowing cell cycle progression to resume. Delayed repair might explain why the early *CSN5* effects on Grk expression are stronger than at later times. It might also explain why *CSN5*-mutant phenotypes are weaker and less penetrant than in *okra* and *spn-B* mutants, in which repair proteins are absent and DNA probably remains unrepaired.

CSN5 and DNA repair

How might CSN5 regulate DNA repair? Two mechanisms of CSN activity have been reported, and either might affect the activity or stability of proteins involved in DNA repair. In addition, since there is an excess of CSN5 relative to other CSN subunits in many cells (Yang et al., 2002), CSN5 might regulate DNA repair independent of the large CSN complex.

The best-documented mechanism for CSN activity works through regulation of the SCF (Skp1/cullin-1/F-box) ubiquitin ligases (Lyapina et al., 2001; Yang et al., 2002). This pathway is attractive here because SCF-dependent ubiquitination mediates the degradation of many cell-cycle regulators, including not only $p27^{kip1}$, but also cyclins E, A and B, CDK inhibitor p21, E2F1, β-catenin and IκBα (Michel and Xiong, 1998; Russell et al., 1999; Yu et al., 1998; Carrano et al., 1999; Marti et al., 1999; Kitagawa et al., 1999; Hatakeyama et al., 1999). Recently, a connection has been made in *C. elegans* between the SCF complex and the regulation of meiosis. Members of the Skp1-related (*skr*) gene family in *C. elegans* are required for the restraint of cell proliferation, progression through the pachytene stage of meiosis, and formation of bivalent chromosomes at diakinesis (Nayak et al., 2002).

The CSN regulates SCF activity by removing the ubiquitin-like protein Nedd8 from the cullin subunit of SCF (Lyapina et al., 2001). Nedd8/Rub1 is covalently attached to target proteins through an enzymatic cascade analogous to ubiquitination (Lammer et al., 1998; Liakopoulos et al., 1998; Osaka et al., 2000). It is ligated to all cullin family proteins, and so far cullins are the only known targets for neddylation (Hori et al., 1999; Read et al., 2000). Nedd8 modification enhances the ubiquitinating activity of the SCF complex in vitro and is required in vivo for embryogenesis in both mice and nematodes (Kawakami et al., 2001; Tateishi et al., 2001; Jones et al., 2002). As the CSN mediates cleavage of the Nedd8 conjugate, it can antagonize SCF-dependent protein degradation. For example CSN inhibits ubiquitination and degradation of $p27^{kip1}$ in vitro and injection of the purified complex inhibited the G1-S transition in cultured cells (Yang et al., 2002).

Although this deneddylation activity of the CSN would explain our results, the kinase activity associated with the CSN might also be important. This kinase activity co-purifies with the CSN complex though it is uncertain whether it is intrinsic to one of the CSN subunits (Bech-Otschir et al., 2002). It phosphorylates and stabilizes the Jun transcription factor against proteasomal degradation (Musti et al., 1997). Conversely, it sensitizes p53 degradation by the SCF-ubiquitin pathway (Bech-Otschir et al., 2001).

Although the DNA repair-related targets of *CSN5* or the CSN remain unclear, proteins encoded by the *spindle*-class genes or by *mei-W68* are strong candidates (see Fig. 8). The deneddylation activity of the CSN might protect a DNA repair protein from SCF-dependent degradation. Alternatively, the kinase activity might promote Mei-W68 turnover, thereby limiting the production of DSBs. Further investigation may help to distinguish among these and other hypotheses and find direct targets for *CSN5* in oogenesis.

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