Centromere proteins CENP-C and CAL1 functionally interact in meiosis for centromere clustering, pairing, and chromosome segregation

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Meiotic chromosome segregation involves pairing and segregation of homologous chromosomes in the first division and segregation of sister chromatids in the second division. Although it is known that the centromere and kinetochore are responsible for chromosome movement in meiosis as in mitosis, potential specialized meiotic functions are being uncovered. Centromere pairing early in meiosis I, even between nonhomologous chromosomes, and clustering of centromeres can promote proper homolog associations in meiosis I in yeast, plants, and *Drosophila*. It was not known, however, whether centromere proteins are required for this clustering. We exploited *Drosophila* mutants for the centromere proteins centromere protein-C (CENP-C) and chromosome alignment 1 (CAL1) to demonstrate that a functional centromere is needed for centromere clustering and pairing. The *cenp-C* and *cal1* mutations result in C-terminal truncations, removing the domains through which these two proteins interact. The mutants show striking genetic interactions, failing to complement as double heterozygotes, resulting in disrupted centromere clustering and meiotic nondisjunction. The cluster of meiotic centromeres localizes to the nucleolus, and this association requires centromere function. In *Drosophila*, synaptonemal complex (SC) formation can initiate from the centromere, and the SC is retained at the centromere after it disassembles from the chromosome arms. Although functional CENP-C and CAL1 are dispensable for assembly of the SC, they are required for subsequent retention of the SC at the centromere. These results show that integral centromere proteins are required for nuclear position and intercentromere associations in meiosis.

CENP-A | CID

Centromeres are the control centers for chromosomes, and thus are essential for accurate segregation in cell division. Centromeres are the DNA regions with a specialized chromatin structure upon which the kinetochore is built. The kinetochore is a complex of at least 100 proteins that contains the proteins to bind microtubules, motors to move on or destabilize microtubules, as well as checkpoint proteins monitoring kinetochore–microtubule attachment (1). The ability of the kinetochore to control microtubule binding and chromosome movement is essential for proper segregation in both mitosis and meiosis. In meiosis, additional constraints are placed on kinetochore function to ensure that homologs segregate in the first division and that segregation of sister chromatids is deferred until the second division (2). Recent studies indicate that in addition, the centromere itself may influence homolog segregation by controlling homolog pairing and formation of the synaptonemal complex (SC) (3).

In prophase of meiosis I, the homologs must pair and ultimately become attached, usually by recombination and crossing-over. By quantifying centromere number through prophase I, it has been observed that centromeres pair in yeast, plants, and *Drosophila* (3). Perhaps unexpectedly, this pairing can be between nonhomologous centromeres; in yeast, this has been proposed as a mechanism to prevent recombination around the centromere, as centromere pairing resolves from initially being nonhomologous to being homologous (4, 5). Homologous centromere

pairing may play a critical role in ensuring segregation of chromosomes that do not undergo crossing-over, possibly by affecting orientation of the kinetochores (3, 6–8).

The centromere also regulates synapsis via the formation of the SC. SC formation initiates at the centromere and sites of cross-over formation in yeast, and the centromere is the first site for SC formation in *Drosophila* prophase I (9, 10). In addition, the SC persists at the centromere in yeast and *Drosophila* after the SC present along the chromosome arms has disassembled late in prophase I (7, 9, 11). Although SC assembly does not begin at centromeres in mouse meiosis, it persists at the centromeres and appears to promote proper segregation (12, 13).

Another centromere property has been observed in *Drosophila* oocytes. In most organisms, the centromeres are clustered together at one site at the onset of meiosis, likely a remnant of their configuration in mitosis, but this clustering breaks down as centromeres arrange in pairs (3, 4). In *Drosophila*, however, the centromeres remain clustered until exit from prophase I at oocyte maturation (9, 10, 14). Although an essential role for centromere clustering has not been demonstrated, it may facilitate homolog pairing, synapsis, or accurate segregation, particularly given that the homologous telomeres do not pair into a bouquet formation in *Drosophila* meiosis (15, 16). Components of the SC are necessary for centromere clustering, as is the cohesion protein ORD (9, 14).

The studies on centromere pairing and clustering define centromere geography within the meiotic nucleus, but they did not test whether centromere structure or function was involved. Centromeres have specialized nucleosomes with a histone H3 variant, centromere protein-A (CENP-A) (17). Incorporation of CENP-A into centromere chromatin is regulated precisely,

Significance

The centromere is essential for chromosome segregation because it is the site at which the kinetochore assembles to control microtubule attachment and chromosome movement. In meiosis, the homologous chromosomes pair, synapse by forming the synaptonemal complex (SC), and segregate. This is associated with the centromeres clustering in the nucleus. Little is known about the function of the integral centromere proteins in these processes because they are essential, and mutations in the genes are lethal. Here, we exploit allelic combinations to show the centromere proteins CENP-C and CAL1 are required in *Drosophila* meiosis for centromere pairing and clustering, and we find this occurs at the nucleolus. Defects in these proteins affect the SC at the centromere and cause meiosis segregation errors.

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although it occurs at distinct cell cycle times in different cell types, varying between late mitosis and G1 (17). In vertebrates, a complex of 15 proteins, the constitutive centromere-associated network (CCAN), is present on the CENP-A chromatin throughout the cell cycle and is crucial for assembling kinetochore proteins (1). In *Drosophila*, the entire CCAN complex has not been identified, although the CENP-C protein is present (18). Another *Drosophila* protein, CAL1, binds to CENP-A (called CID in *Drosophila*) in a prenucleosomal complex, and CAL1 is required for loading CID (19–22). CAL1 interacts with both CID and CENP-C, and all three proteins show interdependency for centromere localization (21, 23).

Little is known about the activities of these centromere proteins in meiosis. In fission yeast, CENP-C has been demonstrated to be critical for kinetochore–microtubule binding in meiosis and also to control kinetochore orientation in meiosis I (24). The timing of assembly of kinetochore and centromere proteins onto meiotic chromosomes has been examined in mouse spermatocytes (25) and in *Drosophila* spermatocytes and sperm (26, 27). RNAi studies have shown that CAL1 and CENP-C (the latter to a lesser extent) are needed for CID localization in *Drosophila* male meiosis, with reduction in the levels of any of these three proteins being associated with meiotic segregation errors (26). *Drosophila* males differ from most organisms in not undergoing recombination or forming an SC, and centromere clustering does not occur (28). A question of particular interest that has yet to be addressed is whether centromere architecture and function are required for centromere clustering and pairing in meiosis.

Results

CENP-C Function Is Required for Centromere Clustering and Pairing. The essential function of centromeres has impeded analysis of requirements for centromere function in meiosis. We overcame this by exploiting the viable allele *cenp-C$^{Z3-4375}$* that we identified from a noncomplementation screen with a female-sterile mutant collection (29) (Fig. S1A). It is a missense mutation, but it appears to destabilize the protein because it results in decreased protein levels (Fig. S1 *C and D*). This allele was used in combination with homozygous lethal alleles of *cenp-C* and *cal1* we recovered from a screen for mutants with mitotic defects during *Drosophila* embryogenesis (30). These mutations cause C-terminal truncations in the proteins (Fig. S1). Although these genes are essential, we were able to analyze the role of CENP-C in meiosis by using transheterozygotes of the lethal and female-sterile alleles of *cenp-C*.

We tested whether a functional centromere structure is required for meiotic centromere clustering. Centromere clustering is detected as less than four CID foci, given that there are four homolog pairs in *Drosophila* (9, 14) (Fig. 1). The stages of meiosis can be distinguished in the *Drosophila* ovary by characteristic morphology of egg chambers (the egg chamber is the oocyte plus sister nurse cells and somatic follicle cells) and by staining with a marker for the SC, the transverse filament protein C(3)G (31). The germarium contains oocytes that have initiated meiosis: Region 2a of the germarium corresponds to zygotene and pachytene; from germarium region 2b through egg chamber stage 4, the oocyte remains in pachytene (9, 14). In stages 5–7, the SC disassembles (9, 32). Centromere clustering is not observed in mitotically dividing precursor cells in the germarium, and its initiation occurs in region 2a when meiosis initiates (9). From region 2a on, one or two CID foci are predominantly present, a consequence of centromere clustering (9, 14) (Fig. 1).

Staining of ovaries from *cenp-C^{IR35}/cenp-C$^{Z3-4375}$* transheterozygous females with antibodies against CID revealed defects in centromere clustering. Although CENP-C is required for CID localization in mitosis (21, 23), CID staining was present in these oocytes, permitting us to use it as a centromere marker (Fig. S2). Quantification of the number of CID foci confirmed centromere clustering in WT and in *cenp-C$^{Z3-4375}$*/+ controls (Fig. 1). In contrast, there was a marked failure of centromere clustering in the *cenp-C* transheterozygous mutants, evident in pachytene in region
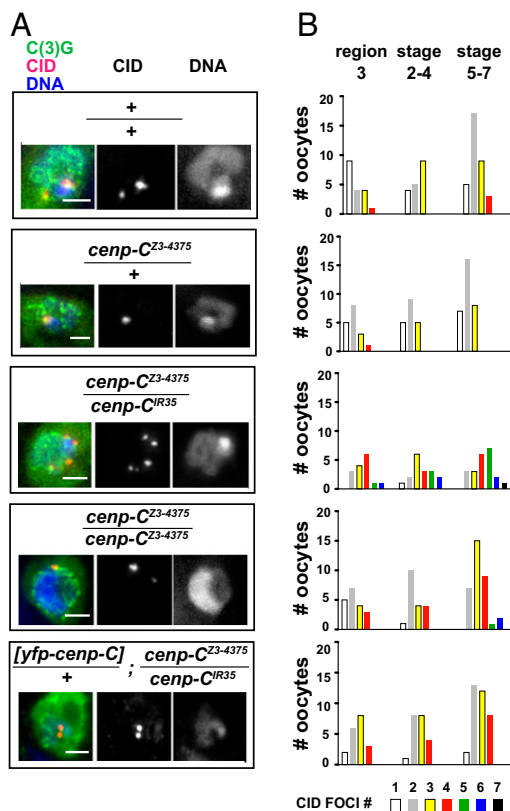
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Fig. 1. Centromere clustering and pairing require *cenp-C* function. (*A*) CID and DAPI staining of the oocyte from stage 5–7 egg chambers of WT (*Oregon R*), the indicated *cenp-C* mutants, and the [*yfp::cenp-C*]/+; *cenp-C$^{Z3-4375}$*/cenp-C^{IR35}* transgenic line. Increased CID foci are observed in the *cenp-C$^{Z3-4375}$*/cenp-C^{IR35}* mutants but not in the *cenp-C$^{Z3-4375}$* homozygote shown. The pronounced *cenp-C$^{Z3-4375}$*/cenp-C^{IR35}* defect is rescued by the [*yfp::cenp-C*] transgene. C(3)G, green; CID, red; DAPI, blue. (*Right*) Separate channels for CID and DAPI staining are shown. (Scale bars, 2 μm.) (*B*) Quantification of CID foci number in region 3, stages 2–4, and in stages 5–7 for the respective genotypes. The quantification shows that both centromere clustering and centromere pairing are disrupted in *cenp-C$^{Z3-4375}$*/cenp-C^{IR35}*, whereas *cenp-C$^{Z3-4375}$* females are slightly defective in both. By the Wilcoxon rank sum test, the distribution in *cenp-C$^{Z3-4375}$*/cenp-C^{IR35}* is significantly different from WT in all stages (region 3, $P = 1.1e-04$; stages 2–4, $P = 1.9e-03$; stages 5–7, $P = 1.5e-07$). The *cenp-C$^{Z3-4375}$* females were significantly different from WT in stages 5–7 ($P = 4.4e-05$). The transgene strain was significantly different from *cenp-C$^{Z3-4375}$*/cenp-C^{IR35}* in all stages (region 3, $P = 0.02$; stages 2–4, $P = 0.03$; stages 5–7, $P = 2.5e-05$). The *cenp-C$^{Z3-4375}$*/+ heterozygote was not significantly different from WT.

3 (Fig. 1). The homozygous female-sterile allele also showed centromere clustering defects, although not as pronounced as in the transheterozygotes (Fig. 1). We showed that this effect was due to loss of CENP-C function, because a transgene expressing a functional fusion of YFP–CENP-C (21) restored centromere clustering (Fig. 1). These results demonstrate a requirement for a structurally normal centromere for clustering. Moreover, they highlight the role of CENP-C, given that CID is still detectably localized to centromeres in these cells but clustering is defective nevertheless.

In addition to clustering of the centromeres, the centromeres of each homolog have been demonstrated to be paired until metaphase I in *Drosophila* oocytes (6, 33). In the *cenp-C* mutants, we observed more than four CID foci (Fig. 1). This reveals that alteration of centromere function affects centromere pairing as well as centromere clustering. Although this could possibly arise from a complete loss of sister-chromatid cohesion, we think this is unlikely,

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given that we do not observe the meiotic segregation errors that accompany loss of sister-chromatid cohesion (see below).

We tested whether CENP-C is required for the establishment of centromere clustering by examining earlier meiotic stages by staining with CID and an antibody that recognizes the fusome, a cytoskeletal structure that permits identification of the oocyte (34) (Fig. S3A). In contrast to later stages, centromere clustering was normal in zygotene oocytes of *cenp-C* transheterozygous females (Fig. S3B). Thus, either CENP-C and proper centromere structure are essential only for maintenance but not establishment of centromere clustering or there is sufficient CENP-C function in the transheterozygous mutant to set up but not to maintain clustering.

cenp-C and *cal1* Show Allele-Specific Interactions Causing Defects in Centromere Clustering and Pairing.

Failure of complementation between two mutations in different genes is diagnostic of a functional interaction between the gene products (35). This can result from a dosage effect, whereby decreasing the levels of the two products causes a phenotype, or it can reflect a disrupted physical interaction between the two proteins. The C terminus of CENP-C mediates its binding to CAL1 and its localization to centromeres (21). CAL1 binds to CENP-C via its C terminus and to CID via its N terminus (21). In mitosis, the three proteins are interdependent for their localization to the centromere (21, 23). Both the *cal1*[2k32] allele and the *cenp-C*[IR35] allele (36) truncate the C terminus of the proteins (Fig. S1); thus, they are predicted to weaken or eliminate binding between the two proteins. We wondered whether this would be sufficient to show a complementation failure. The double heterozygotes of these recessive alleles (*cal1*[2k32] +/+ *cenp-C*[IR35]) retain CID localization (Fig. S2). Remarkably, although *cal1*[2k32] alone had only slight defects in centromere clustering, the double heterozygotes (*cal1*[2k32] +/+ *cenp-C*[IR35]) were defective in centromere clustering (Fig. 2 A and C). In addition, many oocytes showed defective centromere pairing in the double heterozygote, but pairing defects were seen in only one or two oocytes of each of the single heterozygotes (Fig. 2 A–C).

We investigated whether the noncomplementation of *cenp-C* and *cal1* was specific for the C-terminal truncated forms of the proteins by examining each allele *in trans* to a deficiency for the other gene. These females had defects in centromere clustering, but the severity of the defect was considerably less than in the double heterozygotes with the truncation alleles (Fig. 2 D and E). The double heterozygotes of *cal1*[2k32] and *cenp-C*[Z3-4375] also had slight effects that were not as pronounced as in the truncation alleles (Fig. 2F). The *cenp-C*[Z3-4375] allele reduces CENP-C protein levels (Fig. S1D), and it exhibits the same extent of clustering defects as the deficiency when *in trans* to *cal1*[2k32]. Centromere pairing defects also occurred at lower frequencies in mutants in which *cal1*[2k32] was present *in trans* to a deficiency of *cenp-C* or the *cenp-C*[Z3-4375] allele (Fig. 2 D–F).

These results further emphasize the critical requirement for centromere structure in clustering and pairing. The striking noncomplementation between the *cal1*[2k32] allele and the *cenp-C*[IR35] allele appears to result from either reduced interaction or a poisonous interaction between the two proteins caused by the C-terminal truncations. There is sensitivity to dosage of the two proteins, however, because a deletion of either gene causes slight centromere clustering defects and rare pairing defects *in trans* to the C-terminal truncation alleles.

Meiotic Centromere Clustering Is Associated with the Nucleolus.

CAL1 has been demonstrated to localize to the nucleolus as well as the centromere in interphase mitotic cells (19), and it physically associates with the nucleolar protein Modulo, a nucleoplasmin homolog (37). In the absence of CAL1, CENP-C is present in the nucleolus in interphase cells and in meiotic spermatocytes (19, 26). A role for the nucleolus in mitotic centromere formation has been implicated by the observation that RNAi depletion of Modulo diminishes CAL1 at the centromere (37). In addition, depletion of Modulo causes loss of mitotic centromere clustering, changes in heterochromatin, and mitotic segregation defects (38). Given these results and the effect of the *cal1*[2k32] +/+ *cenp-C*[IR35] transheterozygote on centromere clustering, we investigated whether the nucleolus could be associated with centromere clustering in meiosis.

We examined the position of the centromere cluster relative to the nucleolus by staining ovaries with antibodies against CID and the nucleosomal Fibrillarin protein. The distance between the centromeres and the nucleolar surface was measured through 3D reconstruction by Imaris imaging software (www.bitplane.com). Strikingly, in WT, the centromere cluster always was adjacent to the nucleolus (Fig. 3A, quantified in Fig. S4). In contrast, in both the *cenp-C*[IR35]/*cenp-C*[Z3-4375] and *cal1*[2k32] +/+ *cenp-C*[IR35] mutants, the centromeres were separate from the nucleolus (Fig. 3 B and C and Fig. S4).

These intriguing observations raise the possibility that in meiosis, as in mitosis, the nucleolus is linked to centromere clustering. Furthermore, they suggest that proper centromere structure is required for nucleosomal association.

Centromere Function Affects SC Structure.

The site of centromere clustering in *Drosophila* is where synapsis and SC formation initiate in the oocyte (9, 10). Thus, we next tested whether SC formation was affected in the centromere protein mutants that disrupt centromere clustering. SC structure was analyzed by staining with an antibody against the transverse filament protein C(3)G (31). C(3)G was detected in continuous ribbon structures in region 2a of the germaria, reflective of synapsed homologs, in WT, *cenp-C*[IR35]/*cenp-C*[Z3-4375], and *cal1*[2k32] +/+ *cenp-C*[IR35] (Fig. S5). This indicates that failure of centromere clustering does not impair assembly of the SC.

Although the SC disassembles after pachytene, in stages 5–7, a remnant of the SC persists at the centromere until stage 9, detectable as brightly staining C(3)G foci that overlap with CID foci (9) (Fig. 4 A–C). Despite the lack of detectable effects on SC assembly, we observed that even by region 3 of the germaria, centromere association of C(3)G frequently was absent in centromere protein mutants (Fig. 4 D–I). Thus, in addition to the demonstrated requirement for C(3)G for centromere clustering
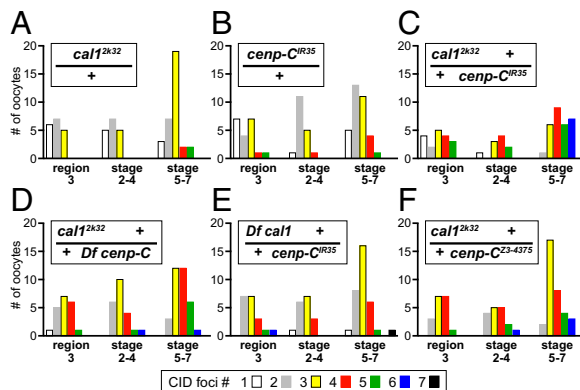

Fig. 2. Allele-specific noncomplementation between *cal1* and *cenp-C* causes defects in centromere clustering and pairing. Quantification of CID foci number at region 3, stages 2–4, and in stages 5–7 for the respective genotypes is shown. (*A* and *B*) *cal1*[2k32] causes a slight dominant defect (stages 5–7, P = 0.02), but the *cenp-C*[IR35] stop codon is not significantly different from WT (compare with Fig. 1B). (*C*) Double heterozygotes *cal1*[2k32] +/+ *cenp-C*[IR35] exhibit failure of both centromere clustering and pairing (a significant difference from WT region 3, P = 0.01; stages 2–4, P = 1.0e-03; stages 5–7, P = 8.8e-11). (*D–F*) These defects are more severe than in females bearing either truncation allele *in trans* to a deletion of the other gene or with *cal1*[2k32] *in trans* to the *cenp-C*[Z3-4375] allele (significant differences in stages 5–7 between *cal1*[2k32] +/+ *cenp-C*[IR35] and *cal1*[2k32] +/+ *Df cenp-C*, P = 0.02; *Df cal1* +/+ *cenp-C*[IR35], P = 7.9e-06; *cal1*[2k32] +/+ *cenp-C*[Z3-4375], P = 0.01). The deletions were *Df(3R)Exel6149* for *cenp-C* and *Df(3R)Exel6176* for *cal1*. *Df*, deficiency.
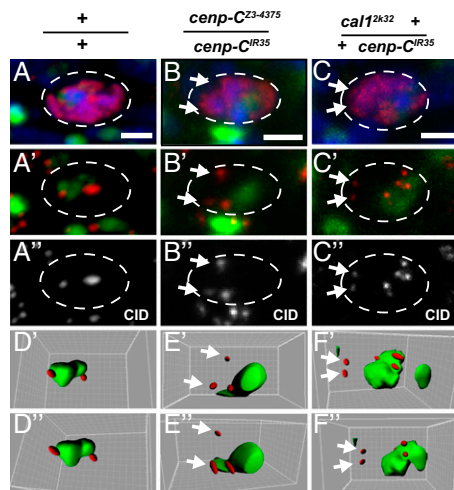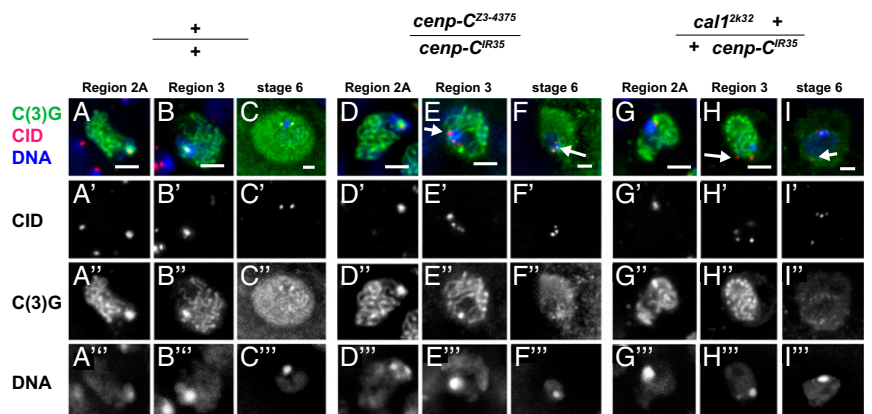
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Fig. 3. Centromere clustering is associated with the nucleolus, and this is disrupted in centromere protein mutants. Region 3 germaria of WT (A–A''), cenp-C $^{Z3-4375}$/cenp-C^{IR35} (B–B''), and cal1^{2k32} +/+ cenp-C^{IR35} (C–C'') mutants were examined for centromere–nucleolus association. (A–C) Merged images of C(3)G (red), Fibrillarin (green), and DAPI (blue) staining. C(3)G staining identifies the oocyte nucleus (dotted line). (A'–C') Merged images of CID (red) and Fibrillarin (green) staining. (A''–C'') Isolated CID channels of A'–C', respectively. (Scale bars, 2 μm.) (D–F') 3D reconstructions of A'–C', respectively, via IMARIS software. Centromere (CID, red) and nucleolus (Fibrillarin, green) are shown. The 3D reconstructions are displayed at two different off-center angles to convey positions between the centromere and the nucleolus better. White arrows indicate centromeres not in close proximity with the nucleolus. Quantification is shown in Fig. S4.

(9), proper centromere structure is needed for retention of C(3) G at the centromere.

Noncomplementation of cenp-C and cal1 Affects Meiotic Chromosome Segregation. The cenp-C^{IR35}/cenp-C$^{Z3-4375}$ females are sterile, but the cal1^{2k32} +/+ cenp-C^{IR35} flies are fertile, permitting us to investigate whether the centromere defects result in meiotic segregation errors. Segregation of the sex chromosomes in females can be quantified in crosses to attached XY males, and the segregation of the 4th chromosome can be assessed by crossing to males with an attached 4th chromosome (39). This permits recovery of exceptional progeny arising from segregation errors, as well as distinction between gametes aberrantly bearing two copies of the chromosome (diplo exceptions) vs. none (nullo exceptions).

We simultaneously measured X and 4th chromosome segregation in WT control, cal1^{2k32}/+, cenp-C^{IR35}/+, and cal1^{2k32} +/+ cenp-C^{IR35} females by crossing to males with attached XY and attached 4 chromosomes. These genetic experiments revealed that both the cal1^{2k32} and cenp-C^{IR35} alleles are semidominant for X chromosome nondisjunction and cal1^{2k32} is semidominant for 4th chromosome nondisjunction (Table 1). There was a notable noncomplementation and genetic interaction between cal1^{2k32} and cenp-C^{IR35}. Females double-heterozygous for these mutants showed missegregation of both the X and 4th chromosomes. The effects on the two chromosomes were independent; the gamete classes arising from nondisjunction of the X chromosome together with the 4th chromosome were not elevated compared with gametes with missegregation of one of these chromosomes (Table 1). The X chromosome used for these tests was heterozygous for the centromere-linked car mutation. Failure to recover homozygous car mutant diplo exceptional (XX) progeny shows that the nondisjunction events occurred in meiosis I.

To confirm that these genetic interactions were the consequence of mutation of the CAL1 and CENP-C proteins, we tested whether a transgene expressing a functional YFP fusion to CENP-C or a GFP fusion to CAL1 (21) could rescue the nondisjunction phenotype. For each transgene, two copies significantly rescued the nondisjunction resulting from noncomplementation in cal1^{2k32} +/+ cenp-C^{IR35} (Table S1).

Given that the centromere clustering and pairing defects resulting from the centromere protein mutants showed allele specificity, we explored whether the meiotic nondisjunction was a consequence of genetic interaction between the two truncation alleles. Either a deletion of cal1 in trans to cenp-C^{IR35} or a deletion of cenp-C in trans to cal1^{2k32} did not cause X chromosome nondisjunction (Table S2). Thus, simply reducing the levels of one of the proteins in the presence of a truncated form of the other is insufficient to cause meiotic segregation defects.

We also analyzed whether cal1^{2k32} +/+ cenp-C^{IR35} compromised sex chromosome segregation in males by crossing to females with attached X chromosomes. Although there was a significant increase in XY segregation errors (Wilcoxon rank sum probability <0.01), the effect was considerably reduced compared with the defects observed in females (Table S3). Interestingly, the diplo exceptional sperm carried XX rather than XY chromosomes. Thus, meiosis II rather than meiosis I nondisjunction occurred, suggesting that in males, meiosis II segregation may be particularly vulnerable to compromised centromere function. Given the distinct mechanisms used in Drosophila male meiosis (absence of recombination and SC, specific homolog pairing sites and proteins) (28), the effects of the cal1 and cenp-C mutations in males may differ from those observed in females.

Fig. 4. cal1 and cenp-C function to maintain the SC at the centromere. CID (red), C(3)G (green), and DAPI (blue) staining of oocyte nuclei from region 2A and region 3 germaria and stage 6 egg chambers from WT (A–C), cenp-C$^{Z3-4375}$/cenp-C^{IR35} (D–F), and cal1^{2k32} +/+ cenp-C^{IR35} (G–I) mutant females are shown. The split single channels are indicated. CID foci colocalize with strong SC staining in region 2A of WT and the centromere protein mutants. In region 3, centromere SC signal is observed in the WT [only 2 of 48 region 3 oocytes have CID foci without coincident C(3)G staining], but centromere SC is not maintained in the centromere protein mutants [15 of 27 region 3 oocytes have CID foci without C(3)G staining in cenp-C$^{Z3-4375}$/cenp-C^{IR35}, and 8 of 16 have CID foci without C(3)G staining in cal1^{2k32} +/+ cenp-C^{IR35}]. By Fisher's exact test, the mutants are significantly different from WT with P = 6.8e-07 for cenp-C$^{Z3-4375}$/cenp-C^{IR35} and P = 1.0e-04 for cal1^{2k32} +/+ cenp-C^{IR35}. Although the SC persists at WT centromeres in stage 6, it does not localize to centromeres in stage 6 centromere protein mutants. CID foci devoid of C(3)G staining in the mutants are indicated by arrows. (Scale bars, 2 μm.)

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Table 1. Nondisjunction tests in centromere protein mutant females

Female genotype	+/+	cal1^{2K32}/+	cenp-C^{IR35}/+	cal1^{2K32} +/ + cenp-C^{IR35}
Ova				
X; 4	2,214	1,650	2,226	692
X; 0	0	18	0	14
X; 44	1	24	11	7
0; 4	0	5	1	25
0; 0	0	1	2	1
0; 44	0	0	0	3
XX; 4	0	1	2	17
XX; 0	0	0	0	1
XX; 44	0	0	0	0
Total progeny	2,215	1,700	2,242	760
Adjusted total	2,215	1,707	2,247	807
X exceptions, %	**0**	**0.8***	**0.44****	**11.6****
4 exceptions, %	**0.05**	**2.64*****	**0.58**	**3.4******

The percentage nondisjunction for the X and 4th chromosomes are shown in bold. Significantly different from WT, as measured by the X non-disjunction significance test of Zeng et al. (46) (*P = 8.0e-03; **P = 0.025; ***P < 1.0e-04). Significantly different from WT by the Kruskal–Wallis test (****P = 1.5e-03; *****P = 4.9e-03).

Discussion

Localization studies demonstrated centromere pairing in yeast, *Drosophila*, and plants, and it showed that the centromeres cluster together in *Drosophila* meiosis I. Here, we establish that centromere function is required for both pairing and clustering. Thus, centromeres are integrally involved in these two processes and not brought together solely by external factors. Because these events occur before assembly of the kinetochore, it is likely that the chromatin and associated proteins at the centromere are critical. The mutations in *cenp-C* reveal that functional CENP-C is necessary at a minimum for maintenance of centromere pairing and clustering in *Drosophila* oocytes. The noncomplementation between truncated CENP-C and CAL1 protein forms implicates CAL1 as also being crucial for centromere pairing and clustering. Given the role of CENP-C in recruiting proteins to the centromere (40), the requirement for this protein could reflect a direct role in centromere pairing and clustering or the need for a protein whose localization is dependent on CENP-C and/or CAL1. In the *cenp-C* mutant and the *cenp-C cal1* double-heterozygous mutant, CID is still localized to the centromere, as evidenced by its presence at brightly DAPI-stained heterochromatin at levels that, by immunofluorescence, are not significantly lower than WT (Fig. S2). Thus, CID presence is insufficient for centromere clustering and pairing. The reduced level of CID staining in the double-heterozygous mutant is nearly significant, however; thus, we do not exclude the possibility that reduced CID levels contribute to the mutant defects.

The proteins at the centromere may interact with nuclear structures to promote centromere clustering. This study identifies the nucleolus as a likely candidate. We find that the centromere clusters are associated with the nucleolus in WT oocytes, and this association requires *cenp-C* and *cal1* function. In *Drosophila* female meiosis, the nucleolus may serve as an anchor site for centromeres throughout prophase I.

The SC also may cluster centromeres. Clustering has been shown to be disrupted in mutants for the SC transverse and central elements (9). Our observation that the SC protein C(3)G fails to be retained at the centromere in *cenp-C* and *cal1* mutants raises the possibility that the failure of clustering in these centromere protein mutants is a consequence of the absence of the SC. The hypothesis of this causality is consistent with the timing of defects; as early as pachytene, both centromere SC and clustering are absent. It remains to be determined how the SC, a structure contained between pairs of homologs, could gather centromeres into a cluster. In *c(3)g* mutants, more than four CID foci can be observed, indicating that both centromere pairing and clustering can be affected (9). Thus, failure of centromere retention of the SC also could account for the pairing defects in the centromere protein mutants.

The allele-specific noncomplementation [type I second-site noncomplementation (35)] between the mutations causing C-terminal truncations of CENP-C and CAL1 is unusual and informative. Such mutations that alter protein structure rather than simply reducing protein levels provide the opportunity to investigate genetic interactions. This allele-specific noncomplementation affects all the processes we analyzed: centromere pairing, centromere clustering and nucleolar association, SC retention at the centromere, and meiotic segregation. The antagonistic genetic interaction requires the truncated protein forms, because deficiencies for each of the genes complement the truncation allele of the other for meiotic segregation and cause only slight defects in centromere pairing and clustering. This is also true for the *cenp-C*$^{Z3-4375}$ allele that reduces protein levels. Thus, simply decreasing the levels of the proteins does not perturb these processes. The C-terminal region of CAL1 binds to CENP-C, whereas the N terminus binds to CID; thus, the truncated form could have a dominant negative effect by binding CID and blocking its link to CENP-C (21). The C terminus of CENP-C is required for its localization to the centromere as well as binding to CAL1, whereas it binds the KNL-1/Mis12 complex/Ndc80 complex (KMN) kinetochore network via its N terminus (41). Thus, C-terminal truncated CENP-C also could act as a dominant negative to uncouple the KMN complex from a functional centromere association, particularly given that the N terminus alone can bind to kinetochore proteins but not to the centromere (41, 42). Expression of the N terminus alone also can disrupt the spindle assembly checkpoint (43). The truncation alleles of *cenp-C* and *cal1* each alone have slight semidominant effects on centromere pairing, clustering, and meiotic segregation, consistent with dominant negative activities. The combination of the two dominant negative effects could account for perturbation of the meiotic processes. We cannot exclude, however, the possibility that these truncation alleles act as recessive neomorphs, conferring novel properties on the proteins.

A critical question is whether centromere clustering is required for proper meiotic segregation. It remains to be determined whether the meiotic nondisjunction that occurs in these centromere protein mutants is linked to the failure of centromere clustering and/or centromere pairing. The meiotic segregation errors in oocytes affect both the *X* chromosome, which undergoes recombination, and the *4th* chromosome, which is achiasmate and lacks SC (44, 45). One way that meiotic segregation of both types of chromosomes could be dependent on clustering would be if association with the nucleolus is necessary for proper assembly of the kinetochore later in prophase I. It is notable, however, that the meiotic segregation errors in oocytes assayed for the *X* chromosome occurred exclusively in meiosis I; thus, a defect in kinetochore function necessary for both meiosis I and II was not evident. There are known meiosis I-specific requirements of the kinetochore, such as the need for the two sister kinetochores to co-orient in meiosis I, and establishment of these may require centromere clustering and/or nucleolar association. This proposal is consistent with the demonstrated effects of *cenp-C* mutants in meiosis in *Saccharomyces pombe* (24).

An alternative possibility is that the centromere mutations have independent effects on centromere clustering and subsequent segregation. For example, the centromere clustering defects could result from failure to retain the SC at the centromere and the meiotic nondisjunction could be an independent consequence of improperly assembled kinetochores later in meiosis I. The centromere mutations clearly can affect meiotic segregation independent of centromere pairing and clustering, given the meiotic nondisjunction in males double-heterozygous for the *cenp-C* and *cal1* alleles. In *Drosophila* male meiosis, centromere clustering, SC formation, and recombination do not occur.

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Although observed in yeast, plants, and *Drosophila*, a role for intrinsic centromere function in the nuclear localization of centromeres and associations between centromeres in meiosis has not yet been defined. The demonstration that proper centromere architecture is necessary for these interactions opens a path to define the molecular basis of centromere pairing and clustering across these species in meiosis.

Materials and Methods

Ovary Immunostaining. Fixation and staining protocols are provided in *SI Materials and Methods*. Antibodies used were rat anti-CID (Claudio Sunkel, Instituto de Biologia Molecular e Celular, Porto, Portugal) at 1:1,000, rabbit anti-C(3)G (Mary Lilly, National Institutes of Health, Bethesda, MD) at 1:1,000, mouse anti-1B1 (Developmental Studies Hybridoma Bank) at 1:5, and mouse anti-Fibrillarin (Cytoskeleton, Inc.) at 1:200. Signals were detected using Alexa Fluor dye-conjugated secondary antibodies (1:400; Molecular Probes) and stained with 0.5 μg/mL DAPI.

Microscopy. The images in Figs. 1 and 4 were taken with a Nikon Eclipse Ti microscope with a Hamamatsu camera and a Nikon Apo TIRF 100× oil objective. Confocal images were acquired with a Zeiss Axio LSM 700 or LSM710 microscope and a Zeiss LSM-TPMT camera. Objectives used for confocal images were a Zeiss 25× apochromat and a Zeiss 40× Plan-apochromat. Wide-field fluorescence image acquisition and deconvolution were carried out using NIS elements software (Nikon Instruments). Confocal image acquisition was carried out using Zen software (Carl Zeiss). IMARIS software (BITPLANE Scientific Solutions) was used to measure the distance between the nucleolus and CID foci in the oocyte from images acquired on a Zeiss LSM 710 confocal microscope.

Nondisjunction Tests. Nondisjunction tests for the *X* and *4th* chromosomes in the female and the sex chromosomes in the male were carried out, and the frequency of nondisjunction was calculated as in the study by Kerrebrock et al. (39).

Three tests were used to compare nondisjunction frequencies between genotypes statistically: the nonparametric Kruskal–Wallis test, the Wilcoxon rank sum test, and the method detailed by Zeng et al. (46) for the *X* chromosome frequencies.

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