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A Dynamic population of prophase CENP-C is required for meiotic chromosome segregation

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Summary

The centromere is an epigenetic mark that is a loading site for the kinetochore during meiosis and mitosis. This mark is characterized by the H3 variant CENP-A, known as CID in *Drosophila*, which replaces canonical H3 at the centromeres. In *Drosophila*, CENP-C is critical for maintaining CID at the centromeres and directly recruits outer kinetochore proteins after nuclear envelope break down. It is not clear, however, if these two functions require the same population of CENP-C. In *Drosophila* and many other metazoan oocytes, centromere maintenance and kinetochore assembly are separated by an extended prophase. We used RNAi knockdown, mutants, and transgenes to study the dynamics and function of CENP-C in meiosis. CENP-C that is incorporated into cells prior to the onset of meiosis is involved in centromere maintenance and CID recruitment. We found this is not sufficient for the other functions of CENP-C. Indeed, CENP-C is loaded during meiotic prophase, while CID and the chaperone CAL1 are not. CENP-C prophase loading is required for meiotic functions at two different times. In early meiotic prophase, CENP-C loading is required for sister centromere cohesion and centromere clustering. In late meiotic prophase, CENP-C loading is required to recruit kinetochore proteins. Thus, CENP-C is one of the few proteins that links the function of the centromeres and kinetochores through the long prophase pause in oocytes.

INTRODUCTION:

The chromatin of the centromere is characterized by an H3 variant known as CENP-A rather than a specific sequence [1, 2]. One of its main functions is to recruit kinetochore proteins for meiosis and mitosis [3]. Failure to maintain the centromere after DNA replication or failure to recruit an effective kinetochore during M-phase can result in chromosome segregation defects, which is a feature of many cancers, infertility, and developmental disorders. In *Drosophila*, CENP-A is encoded by the *cid* gene [4].

CENP-A interacts with the “constitutive centromere associated network” (CCAN) complex in a wide variety of organisms such as humans and yeast [3]. The CCAN contains many proteins that form multiple links between the inner centromere and the outer kinetochore. In many vertebrate cells, two pathways defined by the components CENP-T and CENP-C provide the link between the centromere and outer kinetochore [5]. In some cases, one of these pathways has been lost during evolution. *C. elegans* and *Drosophila* are examples where most or all CCAN proteins have been lost and CENP-C forms the only linkage between the inner centromere and outer kinetochore.

Centromeres are maintained by loading new CENP-A once per cell cycle, but unlike other centromeric histones, this does not occur during S-phase. Thus, CENP-A levels are reduced during S-phase and return to normal levels once during the cell cycle. The timing of CENP-A loading varies in different species and possibly cell types, but typically occurs once per cell cycle, usually during M or G1 [6-8]. In yeast and mammals, maintenance of centromeric CENP-A depends on CENP-C, the Mis18 complex, and HJURP [9-15]. In *Drosophila*, the Mis18 complex and HJURP are absent, but CAL1 takes on a similar role by interacting with both CENP-C and CID [16-18]. CID,

71 CENP-C, and CAL1 rely on each other for loading to maintain centromere identity [10,
72 16, 18, 19].

73 While CENP-C is required for centromere maintenance, it also has a direct role in
74 kinetochore assembly by recruiting MIS12 in mammals [20-22] and *Drosophila* [23-25].
75 In many organisms, including *Drosophila* and mammals, the kinetochore is loaded once
76 the nuclear envelope breaks down, and is only present during cell division. Although
77 considered part of the inner kinetochore, CENP-C is present throughout the cell cycle.
78 In *Drosophila,* no other kinetochore proteins except for MIS12 exhibit this behavior [26].
79 CENP-C recruits MIS12 [24, 25], and they directly interact to form an inner kinetochore
80 complex that is present throughout the cell cycle [21, 23, 27].

81 Following the pachytene stage of meiosis, mammalian and *Drosophila* oocytes
82 enter a long pause in prophase. If centromere proteins are loaded once per cell cycle,
83 they would have to be maintained for a long time before kinetochores are assembled
84 and cell division commences [28]. Cohesins, for example, are loaded during S-phase
85 and deteriorate with age, causing aneuploidy in older mothers [29, 30]. However,
86 CENP-A nucleosomes in the mouse are stable and do not need to be maintained during
87 meiotic prophase [28]. Furthermore, CENP-C becomes immobilized at metaphase [31].
88 Thus, it is possible that the CENP-C loaded along with CENP-A in G1 is sufficient for
89 kinetochore assembly. However, the stability of CENP-C during meiotic prophase has
90 not been tested. How age affects proteins like CENP-C that are loaded before or during
91 the prophase pause is poorly understood and important for oocytes health [32].

92 In this study, we investigated the meiotic prophase dynamics of CENP-C, CAL1,
93 and CID. As with CENP-A in mouse oocytes [28], we found that CID is stable

94 throughout *Drosophila* oocyte meiotic prophase, and there was little addition of new
95 subunits. CAL1 is gradually lost during prophase and is absent in metaphase I oocytes.
96 CENP-C is uniquely loaded at centromeres using an exchange mechanism during
97 prophase, and this is required for assembly of the meiotic kinetochore. Thus, CENP-C is
98 loaded independently of centromere maintenance in a process that is required for
99 kinetochore assembly.
100

101 # RESULTS

102 **CENP-C, but not CAL1 or CID, loads onto centromeres during prophase I**

103 The analysis of centromere protein dynamics during prophase is facilitated by the
104 organization of the *Drosophila* ovary. Each of *Drosophila's* two ovaries contains several
105 strings, or ovarioles, of developing oocytes (Figure 1A). At the anterior end of each
106 ovariole is the germarium, which includes mitotically dividing cells (region 1) and early
107 meiotic prophase (regions 2-3). Region 3 (stage 1) oocytes leave the germarium and
108 enter the vitellarium. During stages 1-12, meiosis remains in prophase while the oocyte
109 grows and matures. At the posterior end of the ovariole (stage 13-14), the oocyte enters
110 prometaphase. Meiosis arrests at metaphase I until passage through the oviduct and
111 fertilization occurs.

112 The dynamics of CENP-C during meiotic prophase was investigated along with
113 comparisons to its known centromeric partners, CAL1 and CID. Expression of *UASP*
114 regulated EGFP-tagged transgenes of *Cenp-C, cal1,* and *cid* were induced during
115 female meiotic prophase by crossing to one of three GAL4-expressing strains (Figure
116 1). *P{GAL4: :VP16-nos.UTR}CG6325*^{MVD1} (referred to as *MVD1*) initiates expression of
117 transgenes in the pre-meiotic cyst cells of the germline and continues throughout oocyte
118 maturation [33]. *P{GAL4-nos.NGT}A* (referred to as *NGTA*) expresses only during early
119 prophase (Figure S 1). *P{w[+mC]=matalpha4-GAL-VP16}V37* (referred to as *mata*)
120 expression begins late in prophase (region 2B/3) and continues through metaphase I in
121 the stage 14 oocyte [34, 35]. We expressed the EGFP-tagged transgenes using one of
122 these promoters to determine whether centromeric loading of each protein could occur
123 during prophase.

124 Experiments with *MVD1* showed CID, CAL1, and CENP-C localization in region
125 2a and throughout stages 1 to 5 of oogenesis (Figure 1B-D). These results may closely
126 resemble wild-type expression because *MVD1* promotes expression of UAS transgenes
127 through all stages of the germline. We also observed centromeric localization of EGFP-
128 tagged CID, CAL1, and CENP-C in region 2a and stages 1-5 when expressed with
129 *NGTA*. This is consistent with expression of *NGTA* in the early stages of the germarium,
130 including the mitotic divisions. Similar results were observed using an *HA-Cenp-C*
131 transgene with *NGTA* (Figure S 2A).
132 We did not observe centromeric localization of EGFP-tagged CID or CAL1 when
133 expressed during late prophase I using *mata* (Figure 1B, C). In contrast, centromeric
134 localization of CENP-C was observed in late prophase when expressed with *mata*
135 (Figure 1D, Figure S 2A,B). These data indicate that meiotic CENP-C dynamics are
136 different than CID and CAL1. CENP-C is loaded onto the centromeres during meiotic
137 prophase. In addition, heat shock was used to induce expression of *Cenp-C*, which also
138 showed evidence of prophase loading of CENP-C (Figure S 2C). Together, these
139 results suggest a prophase function for CENP-C that is independent of centromere
140 establishment or maintenance.
141 While EGFP-CENP-C under the control of *NGTA* resulted in centromeric GFP
142 localization in stages 1-5 (see above), it was absent in stage 14 oocytes (Figure 1D).
143 These results suggest that CENP-C is unloaded from the centromeres during meiotic
144 prophase. In contrast, CID was retained at the centromeres in stage 14 when expressed
145 using *NGTA* (Figure 1B), suggesting CID loaded prior to meiotic prophase is maintained
146 throughout late prophase of oogenesis. Surprisingly, CAL1 showed a different pattern

147 with *NGTA* and *MVD1*. CAL1 localized in early prophase oocytes similarly to CID but
148 was not detected in stage 14 oocytes (Figure 1C, Figure S 3). Thus, like CENP-C, CAL1
149 is unloaded during prophase, but unlike CENP-C, CAL1 is not loaded during this time.
150

151 **CENP-C regulates centromere clustering, recombination, and MIS12 loading in**
152 **early prophase I**

153 These results show there is a population of CENP-C loaded during meiotic
154 prophase that is distinct from the population required for the maintenance of CID at the
155 centromeres. To investigate *Cenp*-C function in early prophase, we knocked down the
156 expression by RNAi using an shRNA (*GL00409*) and the *NGTA* or *MVD1* promoters.
157 Females expressing *GL00409* with *MVD1* (*GL00409*/*MVD1* oocytes) were sterile and
158 agametic. *MVD1* expression initiates early in the primordial germ cells of the embryo
159 and a requirement for CENP-C during these mitotic divisions would explain the
160 agametic phenotype. Thus, the remaining early prophase experiments were performed
161 with females expressing *GL00409* with *NGTA* (*GL00409/NGTA* oocytes).
162

163 *GL00409/NGTA* females were fertile but displayed a high level of homologous
164 chromosome nondisjunction (7%) compared to controls (0.25%) (Table 1). A similar
165 result was observed with a different shRNA (*HMJ21500*). *NGTA* expression peaks
166 during early prophase, affecting *Cenp-C* expression for only a limited time. Therefore,
167 these results suggest CENP-C has an early meiotic function required for chromosome
168 segregation that is separable from centromere maintenance. To investigate the
169 mechanisms of nondisjunction caused by loss of CENP-C, we characterized processes

170 that occur early in meiotic prophase such as centromere assembly, meiotic
171 recombination, centromere pairing and synaptonemal complex (SC) formation.
172 The intensity CENP-C or CID at the centromeres was not significantly decreased
173 in early prophase nuclei of *GL00409*/*NGTA* oocytes (Figure 2A - C). The absence of a
174 reduction in CID suggests that inhibition of CENP-C occurred after CID was loaded and
175 did not affect centromere maintenance. The failure to observe a significant reduction in
176 CENP-C may have been due to the abundance of protein required for CID
177 maintenance. For another measure of CENP-C function in prophase, we examined
178 MIS12. In mitotic cells, CENP-C promotes kinetochore assembly by recruiting MIS12
179 [23]. MIS12-GFP localization was observed in the same prophase stages as CENP-C in
180 control oocytes, starting in region 2a of the germarium (Figure 2D). The intensity of
181 centromeric MIS12-GFP was significantly reduced in *GL00409/NGTA* females (Figure
182 2D, E) indicating that CENP-C promotes recruitment of MIS12 during meiotic prophase.
183 These results show that MIS12 localizes to the centromeres in meiotic prophase and
184 depends on CENP-C.
185 Increased frequencies of nondisjunction can be associated with loss of SC and/
186 or a reduction in crossing over (CO) [36]. SC assembly in *GL00409/NGTA* females was
187 found to be normal, indicated by the thread-like appearance of transverse filament
188 protein C(3)G [37] (Figure S 4). We genetically measured the frequency of COs in
189 *GL00409/NGTA* females and observed an increase in centromeric crossovers, but
190 overall, crossing over was not decreased (Table 1). In a separate experiment where the
191 centromeres were genetically marked (see Methods), we observed an increase in sister

192 chromatid nondisjunction. Thus, the meiotic nondisjunction may be occurring because
193 of loss of cohesion rather than crossing over.
194 A cohesion defect can be observed as an increase in the number of centromere
195 foci [38, 39]. Indeed, there was an increased number of CID foci in the germaria of
196 *GL00409/NGTA* ovaries, indicating a separation of centromeres (Figure 2F, G).
197 However, nonhomologous centromeres normally cluster in early prophase. Therefore,
198 we used chromosome-specific FISH probes for the pericentromeric regions of the X,
199 2nd, and 3rd chromosomes to examine pairing of the centromeres in early prophase of
200 *GL00409/NGTA* oocytes. Clustering defects were defined as nuclei containing 3 or
201 more clearly separated probe foci (of any color) where control oocytes had 1-2 foci.
202 Pairing defects were defined as greater than one nuclear focus per probe. In control
203 oocytes, homologous centromeres were efficiently paired throughout all stages of
204 meiosis in the germarium (Figure 3A). In *GL00409/*NGTA oocytes, pairing was defective
205 (Figure 3B). Those centromeres that were able to successfully pair, still failed to cluster
206 (Figure 3C). These results suggest that there are defects in both centromere pairing and
207 clustering, consistent with a defect in sister centromere cohesion. We also tested if a
208 cohesin defect was persistent, and therefore would result in increased CID foci in
209 metaphase I oocytes. The variability in CID foci number was greater in the experimental
210 group, but the mean was not significantly different from the control (Figure 2H, I).
211 These data show that during early meiotic prophase, CENP-C is responsible for
212 recruiting MIS12 and for centromere clustering and pairing. As these defects were
213 observed in the presence of constant centromeric CID levels, these CENP-C functions
214 appear to be distinct from centromere maintenance.

215

216 **Prophase CENP-C plays a critical role in maintenance of centromeres and**
217 **kinetochores.**

218 To test the function of CENP-C loaded during mid-late prophase, we used the
219 *mata* promoter to express *Cenp-C* shRNA (Figure 1, *GL00409/mata*). The
220 *GL00409/mata* females were sterile, although this could be due to the lack of CENP-C
221 in the early embryo. To investigate the effects on meiosis I, stage 14 *GL00409/mata*
222 oocytes were analyzed for meiotic phenotypes. The level of CENP-C at the centromeres
223 was reduced to approximately 20% of WT levels (Figure 4A, B). We also examined the
224 phenotype of a hypomorphic allele, *Cenp-C*$^{Z3\text{-}4375}$ [40]. When *Cenp-C*$^{Z3\text{-}4375}$ was
225 heterozygous to a null allele (*Cenp-C*^{IR35}), centromeric CENP-C levels in oocytes were
226 reduced, though not to the same levels as with *GL00409* (Figure 4A, B). The intensity of
227 CID at the centromeres, despite observing no significant changes when *GL00409* was
228 expressed in early stages of meiosis (Figure 2), was mildly reduced by 25% in
229 *GL00409/mata* oocytes compared to control levels (Figure 4A, C). A similar reduction in
230 centromeric CID was observed in the *Cenp-C*$^{Z3\text{-}4375}$/*Cenp-C*^{IR35} mutant oocytes. These
231 results suggest that CENP-C might play a role in stabilization of CID late in meiotic
232 prophase or in metaphase I.

233 To determine if the loss of CENP-C affects kinetochore assembly, we quantified
234 the intensity of MIS12 and SPC105R in mature oocytes. Consistent with the reduction in
235 CENP-C levels, we observed approximately a 75% reduction in MIS12 levels and a
236 90% reduction in SPC105R in *GL00409/mata* oocytes (Figure 4D-G). Consistent with
237 the loss of SPC105R recruitment, we observed an increase in the distance between

238 centromeres and microtubules (Figure S 5), which has previously been observed when
239 SPC105R is depleted [41]. These results suggest CENP-C recruits MIS-12, which then
240 recruits SPC105R.

241 To determine whether the loss of CENP-C in oocytes affected chromosome
242 segregation at meiosis I, we assayed for bi-orientation of homologous chromosomes
243 using FISH. We observed a dramatic increase in bi-orientation errors (primarily mono-
244 orientation) in *GL00409/mata* oocytes compared to the control (Figure 5). Bi-orientation
245 errors were also elevated in *Cenp-C*$^{Z3-4375}$ /*Cenp-C*^{IR35} mutant oocytes, though not to
246 the same extent as the shRNA-expressing oocytes. These spindle attachment errors
247 could indicate that CENP-C plays a direct role in error correction, but more likely has an
248 indirect effect due to the function of CENP-C in kinetochore assembly.

249

250 **Rescue of the CENP-C phenotype by late prophase expression of a wild-type**
251 **allele**

252 The defects observed in *GL00409*/*mata* females suggest that prophase CENP-C
253 expression is required for meiosis and fertility. To determine if prophase-expressed
254 CENP-C was functional in meiosis, we tested whether expression of a transgene in late
255 prophase could complement the *Cenp-C* RNAi or mutant phenotypes. Two *UASP* –
256 regulated transgenes expressing *Cenp-C* were used for these experiments, one GFP-
257 tagged and another HA-tagged. These two constructs differ at the 5'UTR, which is
258 present in *GFP-Cenp-C* and carries the target sequence for *GL00409*. In contrast, *HA-*
259 *Cenp-C* lacks the 5'UTR and is therefore resistant to knockdown by *GL00409.*

260 If prophase expression of CENP-C is functional, it should be able to rescue
261 phenotypes observed in *Cenp-C*$^{Z3-4375}$/*Cenp-C*^{IR35} females. The *HA-Cenp-C* transgene
262 was shown to be functional by co-expressing it in *GL00409*/*mata* oocytes. This resulted
263 in localization of HA-CENP-C to the centromeres and restoration of MIS12 and
264 SPC105R localization in *GL00409/mata* oocytes (Figure 4D-G). In addition, the
265 biorientation defects in *GL00409/mata* oocytes were rescued by expression of HA-
266 CENP-C (Figure 5).
267 Despite the rescue of the RNAi phenotypes, expression of HA-CENP-C using
268 *mata* caused sterility (Table 2). Using *P{w[+mC]=osk-GAL4::VP16}A11* (referred to as
269 *oskGal4*), which has an expression pattern similar to *matα* but at lower levels [42],
270 resulted in fertile females (Table 2). These results suggest overexpression of CENP-C
271 leads to loss of fertility. The females expressing HA-CENP-C had normal oocytes at
272 metaphase I, making it likely that the defect causing sterility is after meiosis I, possibly
273 during embryonic mitosis. Using *oskGal4*, we found that expression of HA-CENP-C or
274 GFP-CENP-C rescued the sterility of *Cenp-C*$^{Z3-4375}$/*Cenp-C*^{IR35} mutant females (Table
275 2). In addition, SPC105R localization was restored (Figure 4F-G) and the biorientation
276 defects in *Cenp-C*$^{Z3-4375}$/*Cenp-C*^{IR35} mutant females was rescued (Figure 5). These
277 results show that late prophase expression of CENP-C contributes to kinetochore
278 function in meiosis I.
279 The RNAi resistance of the *HA-Cenp-C* transgene can be used to express
280 mutant variants in the absence of wild-type CENP-C. Previous studies have shown that
281 the C-terminal domain of *Drosophila* CENP-C interacts with the centromere components
282 CID and CAL1 [17, 23]. To determine what domains of CENP-C are required for

283 prophase loading in oocytes, we generated two *Cenp-C* transgenes expressing either
284 the N-terminal (aa 1-788, *Cenp-C*^{N}) or C-terminal (789–1411, *Cenp-C*^{C}) domain of
285 CENP-C. The N-terminal domain was not detected by fluorescence microscopy,
286 suggesting it is either unstable or does not localize. In contrast, CENP-C^{C} was loaded
287 during prophase (Figure S 2), localized to the centromeres (Figure S 5), but failed to
288 recruit SPC105R or MIS12 (Figure 4D-G). These results support the conclusion that the
289 C-terminal domain of CENP-C is required for centromere localization, while the N-
290 terminal domain promotes recruitment of MIS12 and the rest of the kinetochore.

291 **Centromeric CENP-C exchanges during prophase I**

292 Our results have shown that CENP-C loading during prophase is required for
293 fertility. To test the relationship between the loading and unloading of CENP-C during
294 prophase, females were generated that expressed *GL00409* and *HA-Cenp-C* under the
295 control of *mata,* and *GFP-Cenp-C* under the control of its own promoter (Figure 6A).
296 GFP-CENP-C was expected to be expressed at all stages of the germline but, due to
297 containing the 5' UTR, was sensitive to the shRNA in meiotic prophase. In contrast,
298 expression of the HA-CENP-C would begin in early prophase and resistant to the co-
299 expressed shRNA because it lacks the 5'UTR. This setup allowed us to simultaneously
300 compare the unloading and loading of centromeric CENP-C during prophase. In
301 particular, is unloading of centromeric CENP-C dependent upon loading from the
302 cytoplasmic pool of CENP-C?

303 In females expressing GFP-CENP-C and *GL00409* but not HA-CENP-C, the
304 GFP intensity was reduced over time (Figure 6B). This is consistent with the results
305 using the CENP-C antibody (Figure 4A). In females expressing GFP-CENP-C and HA-

306 CENP-C, the GFP and HA signals were both maintained over time (Figure 6C). In
307 contrast, in females expressing GFP-CENP-C, *GL00409* and HA-CENP-C, the GFP
308 signal decreased and the HA signal increased (Figure 6D). The decrease in GFP signal
309 after induction of shRNA expression was greater when HA-CENP-C was expressed
310 than in females not expressing HA-CENP-C. This observation suggests the actions of
311 an exchange mechanism during prophase that is sensitive to cytoplasmic levels of
312 CENP-C. We propose that the unloading of centromeric CENP-C depends on the
313 availability of a replacement. Notably, while the GFP signal at the centromeres in
314 oocytes drops dramatically at the onset of HA and shRNA expression, low but
315 significant GFP signals were still observed in stage 14 oocytes (Figure 6D). These
316 results suggest that, although most CENP-C is exchanged during prophase, there is a
317 small pool of CENP-C that is stably maintained throughout meiosis. Similar results
318 were observed with the *HA-CenpC*^{C} mutant (Figure 6E), which suggests that exchange
319 of CENP-C is not dependent on the N-terminal domain of the protein.

320 # DISCUSSION

321 **Prophase dynamics of centromere proteins**

322 In *Drosophila*, centromere maintenance involves three proteins, CID, CAL1, and
323 CENP-C, and occurs during late M or G1 (see Introduction). We show here that only
324 CENP-C is loaded during meiotic prophase. The prophase CENP-C population is
325 required for at least two meiotic functions: sister centromere cohesion and kinetochore
326 assembly. Reductions in CENP-C levels were not accompanied by similar decreases in
327 CID at the centromeres, showing that prophase-loaded CENP-C is not required to
328 maintain the centromeres.

329 We observed CENP-C unloading at a higher rate when there was a cytoplasmic
330 source of replacement CENP-C. This indicates that an exchange reaction may be
331 operating and without new sources of CENP-C, the dynamics are reduced. The C-
332 terminal domain of CENP-C had the same dynamic properties during prophase as the
333 full-length protein. This part of the protein also contains the elements required to interact
334 with CID and CAL1 for centromere maintenance [17, 18, 25]. Some evidence in
335 mammals, however, suggests CENP-C has both CENP-A-dependent and independent
336 localization mechanisms, although in most of these studies, loading during G2 is not
337 specifically addressed [43]. CENP-C loading occurs while CID is not loaded and CAL1
338 is unloaded. In addition, CAL1 was absent in metaphase I, in contrast to observations in
339 mitotic cells [7, 16]. Thus, prophase loading of CENP-C may involve a mechanism that
340 involve factors other than CAL1 and CID. For example, CDK1 has been implicated in
341 CENP-C dynamics in chicken and human cells [44].

342 We did not observe incorporation of CID during prophase, which is consistent
343 with studies on CENP-A in mouse oocytes [28]. However, it has been reported that CID
344 is incorporated during meiotic prophase of *Drosophila* females [45]. We suspect the
345 difference between our study and the prior study is how incorporation was measured.
346 The prior studies used changes in fluorescence intensity, which could be affected by
347 changes in the environment of the centromere as the oocyte develops (e.g. the oocyte
348 gets larger, the chromatin changes). We used pulsed expression, which allows for direct
349 observation of the incorporation of newly synthesized centromere proteins.

350 **Early prophase function: centromere clustering and cohesion**

351 When CENP-C was depleted from early prophase oocytes, we observed an
352 increased number of centromere foci. CID levels were not reduced by *Cenp-C* RNAi,
353 suggesting these phenotypes most likely result from early prophase loss of CENP-C.
354 Our results suggest the increased CID foci phenotype could be due to a loss of
355 centromeric cohesion because we also observed an increase in sister chromatid
356 nondisjunction. These observations are consistent with and extend prior studies on
357 *Cenp-C* [40]. These authors also found that *Cenp-C*$^{Z3\text{-}4375}$ mutant females have defects
358 in centromere clustering in pachytene oocytes (region 3 of the germarium). The
359 centromere clustering and pairing phenotypes are similar to a hypomorphic allele of
360 replication protein *mcm5* that was associated with loss of SMC1 at the centromeres
361 [46]. Thus, we propose that CENP-C is required to recruit or maintain cohesion. A
362 recruitment function of CENP-C could occur when cohesion is established in S-phase
363 [38]. Alternatively, our results show striking similarities to the known roles of mammalian

364 and yeast CENP-C in recruiting Moa1 or Meikin [47, 48], which are protectors of
365 centromeric cohesion and particularly important for meiosis I.

366 **Late prophase function: kinetochore assembly**

367 Depletion of prophase CENP-C resulted in phenotypes associated with loss of
368 the kinetochore. This includes loss of MIS12 and SPC105R when CENP-C was
369 depleted in prophase. These results show that CENP-C is required for most or all
370 kinetochore assembly, consistent with studies in *Drosophila* mitotic cells [19, 24, 25].
371 The C-terminal domain of CENP-C was recruited to the centromere but failed to
372 assemble a kinetochore. Thus, the N-terminal domain of CENP-C appears to interact
373 with MIS12 and recruit the rest of the kinetochore, consistent with previous studies in
374 *Drosophila* [23, 27]. These results also show that unlike CID/CENP-A, CENP-C that is
375 sufficient for centromere maintenance is not sufficient for kinetochore assembly. Studies
376 in vertebrate cells have shown that CENP-C is dynamic during interphase but stable in
377 metaphase [31, 49]. If the same is true here, then prophase loaded CENP-C provides a
378 platform for kinetochore assembly at the beginning of meiotic prometaphase in oocytes.

379 **Implications of CENP-C prophase loading**

380 CENP-A /CID is remarkably stable [50, 51]. Therefore, even though our results
381 show that CID/CENP-A is not loaded during extended prophase in oocytes, it appears
382 to be adapted for this by being extremely stable. CENP-C must be loaded during
383 prophase, making it among a small number of proteins that are required for kinetochore
384 function but are loaded prior to metaphase I. Why CENP-C but not CID/CENP-A must
385 be loaded during prophase is not known. It is possible that more CENP-C is needed for
386 kinetochore assembly than centromere maintenance. Loading CENP-C during

387 prophase could be a mechanism to establish a stable and invariant platform to regulate
388 assembly of a specific size of kinetochore in prometaphase. The requirement for CENP-
389 C prophase loading could affect the health of aging oocytes. If loading of CENP-C is
390 compromised in older oocytes that have spent more time in prophase, our results show
391 that both sister chromatid cohesion and kinetochore assembly could be affected.
392
393

394 **Acknowledgements**

395 We thank Marina Druzhinina for technical assistance, Christian Lehner for
396 providing Drosophila stocks and antibodies. We also thank Sarah Radford for early work
397 on this project. We thank the Bloomington *Drosophila* Stock Center (NIH
398 P40OD018537) and the TRiP project at Harvard Medical School for providing fly stocks
399 used in this study. J.F was supported by a NIH IRACDA post-doctoral Fellowship. This
400 work was supported by NIH grant GM101955 to K.S.M.
401
402

403 # METHODS

404 ## *Drosophila* strains and genetics

405 *Drosophila* crosses and stocks were kept at 25°C on standard medium. Fly
406 stocks were obtained from the Bloomington Stock Center or the Transgenic RNAi
407 Project at Harvard Medical School [TRiP, Boston, MA, USA, flyrnai.org], and information
408 about the genetic loci can be found on FlyBase [flybase.org]. *Drosophila* lines
409 expressing a short hairpin RNA (shRNA) were obtained from the Bloomington Stock
410 Center. Expression of the shRNA was controlled by the Upstream Activating Sequence
411 (UAS), which is activated by expression of GAL4 under the control of a tissue-specific
412 enhancer [1]. Five *Gal4* strains were used in this study, four of which were germline-
413 specific: *P{matalpha4-GAL-VP16}V37* and *P{w[+mC]=osk-GAL4::VP16}A11* promote
414 expression after zygotene, *P{GAL4-nos.NGT}A* promotes expression only in the
415 germarium (during prophase I), and *P{GAL4: :VP16-nos.UTR}CG6325*^{MVD1} promotes
416 expression throughout the whole germline. In addition, heat shock was used to induce
417 expression of transgenes using *P{GAL4-Hsp70,PB}89-2-1.*

418 The shRNA used to reduce *Cenp-C* expression, *GL00409*, is located on
419 chromosome II. To measure the extent of the *Cenp-C mRNA* knockdown, total RNA
420 was extracted from stage 14 oocytes expressing *GL00409* with *P{matalpha4-GAL-*
421 *VP16}V37* using the TRIzol Reagent (Life Technologies). cDNA was made using the
422 High-Capacity cDNA Reverse Transcription kit (Applied Biosystems). TaqMan Gene
423 Expression Assays (Life Technologies) were used to measure expression levels of
424 CENP-C. RT-qPCR was done on a StepOnePlus (Life Technologies) real-time PCR
425 system and results showed that the *Cenp-C* RNAi expressed only 4% of wild-type RNA.

426 In addition, CAL1 was knocked down using shRNA *GL01832* and CDK1 was knocked
427 down using shRNA *HMS01531*, both located on chromosome II.
428 *Cencp-C*$^{Z3-4375}$ is a homozygous-viable allele that has a missense mutation in the
429 CENP-C motif, changing a proline amino acid to a serine at position 1116. [40]. For the
430 analysis of *Cenp-C*$^{Z3-4375}$, trans heterozygous mutant strains were used, with one
431 chromosome carrying either the *Cenp-C*^{IR35} or *Cenp-C*^{pr141} mutations. The *Cenp-C*^{IR35}
432 mutation is a premature stop codon at position 858 before the CENP-C motif and Cupin
433 domain, resulting in a deletion of these regions [52]. The *Cenp-C*^{pr141} mutation is a
434 premature stop codon at position 1107 within the CENP-C motif [52], resulting in a
435 partial deletion of the CENP-C motif and the rest of the protein.

436 **HA- and GFP-tagged transgenes**

437 The coding region of cDNA clone FI18815 was PCR amplified and cloned in
438 frame into pENTR4 (HiFi assembly, NEB) and then into pPHW using Clonase (Life
439 Tech.). The target sequence for *GL00409* is located in the 5'UTR of *Cenp-C* and,
440 therefore, this transgene is RNAi resistant. The GFP-CENP-C is similar but contains the
441 5'UTR sequences upstream of the GFP sequence, and thus is RNAi sensitive. MIS12
442 localization was observed using a UASP regulated GFP-fusion transgene [53].
443
444 **Fertility trials, nondisjunction, and crossover assays**

445 The *GL00409 Cenp-C* shRNA was tested for fertility and chromosome
446 segregation errors by crossing females expressing the shRNA to *yw/ B*^{S} *Y* males. The
447 aneuploid genotypes that survive are *y/y/ B*^{S} *Y* (Bar-eyed females) and *y w / 0* (wild-type
448 males). Because half the aneuploid progeny have lethal genotypes, aneuploidy was

449 calculated by multiplying the number of aneuploid progeny by two and dividing by the
450 total number of progeny.
451 Sister chromatid aneuploidy was tested by crossing *y w/ Bwinscy* females
452 expressing the shRNA to *v f B ^ Y* males. If aneuploidy occurs among homologous
453 chromosomes, the expected genotypes that survive are *y w/Bwinscy* and *O/ v f B ^ Y*.
454 Aneuploidy among sister chromatids is detected by the genotypes *y w/y w* (non-bar-
455 eyed females) or *Bwinscy/Bwinscy* (Bar-eyed females). Because the O/ *v f B ^ Y*
456 genotype arises from both MI or MII nondisjunction, only the *y w/y w* and
457 *Bwinscy/Bwinscy* progeny were used to determine the sister chromatid aneuploidy
458 frequency. Therefore, the number of aneuploid progeny counted was multiplied by four
459 and divided by the total number of progeny in order to measure sister chromatid
460 aneuploidy.
461 In order to observe the role of CENP-C in crossing over, females were generated
462 that expressed the shRNA and were heterozygous for four genetic markers on
463 chromosome III: *st, cu, e* and *ca*. These females were crossed to a strain carrying all
464 the recessive traits and the frequency of the recombinants was scored.
465
466 **Cytology and immunofluorescence of early prophase oocytes**
467 For immunolocalization experiments in the germaria, mated females were aged
468 for 1-2 days at 25°C. In one well of a two well plate, 10-15 ovaries were dissected using
469 1x Robb's media and then were transferred to the second well containing fresh media.
470 A tungsten needle was used to break open the ovary sheath and tease the ovarioles
471 apart. The dissected ovaries were transferred to a 1.5 mL Eppendorf tube with 4%
472 formaldehyde in 500ul of Buffer A as described [54]. The ovaries were nutated at room

473 temperature for 10 minutes, then were washed four times before adding the primary
474 antibodies. The next day, following four washes, secondary antibodies were added and
475 incubated at room temperature for 4 hours.

476 **Cytology and immunofluorescence of metaphase I oocytes**

477 For immunolocalization experiments in pro-metaphase I oocytes (stages 13-14),
478 we used the immunocytochemical protocol as described [55]. I brief, 100-200 females
479 were aged 2-3 days with males in yeasted vials [56]. Oocytes were collected by pulsing
480 the females in a blender and then separating the oocytes from the bulk fly tissues using
481 a mesh. Oocytes were fixed in 5% formaldehyde solution for 2.5 minutes, and then
482 equal amounts of heptane were added and the oocytes were vortexed for 30 seconds.
483 The membranes were removed by rolling the oocytes between a coverslip and the
484 frosted part of a glass slide. These oocytes were incubated in PBS/1% Triton X-100 for
485 two hours, then washed in PBS/0.05% Triton X-100. The oocytes were blocked in
486 PBS/0.1% Tween 20/0.5% BSA (PTB) for one hour, and then incubated with primary
487 antibodies overnight. Oocytes were washed the next day in PTB and incubated with
488 secondary antibodies for 4 hours at room temperature.

489 Tissues were mounted for confocal imaging using SlowFade Gold (Invitrogen). A
490 Leica TPS SP8 confocal microscope with a 63X, N.A. 1.4 lens was used to visualize
491 fluorescent tags using different colored lasers. Images were imaged by collecting
492 sections throughout the germarium or stage 14 oocyte spindle, using parameters
493 optimized by the Leica Confocal software based on the lens and wavelength. Images
494 were analyzed as image stacks and presented as maximum projections whole
495 germarium, cells or spindles.

496 **Antibodies**

497 An antibody against CENP-C made in guinea pig was made by generating a
498 clone expressing amino acids 502-939 (Genscript). This guinea pig anti-CENP-C was
499 used at 1:1000. Additional primary antibodies were rat anti-CID (Active motif, 1:100),
500 rabbit anti-CID (Active motif, 1:100), rabbit anti-SPC105R (1:4000) [57], rabbit anti-GFP
501 (Invitrogen, 1:400), rat anti-HA (Roche, 1:50), mouse anti-C(3)G (1:500) [58], two
502 mouse anti-ORB antibodies, 6H4 and 4H8 (1:100 for each) [59] and mouse anti-α
503 tubulin DM1A conjugated directly to FITC (Sigma, 1:50). The secondary antibodies that
504 were used were Cy3, Alexa 546, Alexa 633, or Alexa 647 from Jackson
505 Immunoresearch Laboratories, and Alexa 488 from Invitrogen. The oocytes were
506 stained with Hoechst 33342 at 1:10,000 (10 μg/ml). FISH probes were obtained from
507 IDT where the X359 repeat was labeled with Alexa 594, the dodeca repeat was labeled
508 with Cy5, and the AACAC repeat was labeled with Cy3.

509

510 **Quantification and statistical analysis**

511 Aneuploidy in flies expressing the *Cenp-C* shRNA with NGTA was compared to
512 that of the control group and a t-test was done to determine if the difference is
513 statistically significant (p-value < 0.05). Sister chromatid aneuploidy in flies expressing
514 the *Cenp-C* shRNA with NGTA was compared to that of the control group and a t-test
515 was used to determine whether the difference was statistically significant (p-value <
516 0.05). The percent of crossovers at each position was compared to that of the control
517 group using a t-test, and if the difference was statistically significant (p-value < 0.05),

518 then this indicated that elevated or reduced recombination occurred at that specific
519 location.

520 Quantification of CID foci and protein localization in both the germarium and in
521 stage 14 oocytes was measured using the Imaris Software. To quantify centromere foci,
522 the automated spots detection feature of Imaris was used. A spot with a XY diameter of
523 0.20 μm, a Z diameter of 1.00 μm, and a physical interaction with the DNA was counted
524 as a centromere. A t-test was done to compare the number of centromere foci in flies
525 expressing the knockdown to the number in the control group using a p-value < 0.05 to
526 indicate statistical significance.

527 To measure the localization of proteins to the centromere or the nucleus in *Cenp-*
528 *C* RNAi, intensity experiments were performed using Imaris. The Imaris software was
529 utilized to measure protein intensity at the centromere of the oocyte, using CID as the
530 centromere marker. Spots were chosen in random somatic cells to quantify the intensity
531 of the background. The intensity of the protein at the centromere was divided by the
532 background and a t-test was used to determine whether the centromere protein intensity
533 was reduced with a loss of CENP-C.

534 Intensity experiments were also performed to measure localization of MIS12,
535 SPC105R, or CENP-C to the centromere at stage 14 using a similar protocol. However,
536 background intensity was measured in the oocyte cytoplasm as opposed to measuring
537 the background in the somatic cells for germarium images. A t-test was used to
538 determine whether the intensity of the protein of interest at the centromere normalized
539 to the background was reduced with a loss of CENP-C using a p-value <0.05 to indicate
540 statistical significance.

543

544 **Table 1: Fertility, nondisjunction and crossing over in *Cenp-C* RNAi females**

545

	Fertility	Nondisjunction (NDJ)		Recombination (% of control)			
	Offspring per female (# of females)	Homologous Chromosome NDJ (# of offspring)	Sister Chromatid NDJ (# of offspring)	*st-cu (m.u.)*	*cu-e (m.u.)*	*e-ca (m.u.)*	# of progeny
Control	24.3 (105)	0.25% (1590)	0.5% (2696)	4.4 (100%)	2.2 (100 %)	29.4 (100 %)	2853
GL00409 / NGTA	10.0 (240)	7.0% (1658)	2.6% (1164)	9.4 (226%)	21.1 (101 %)	22.9 (81%)	1668
HMJ21500/ NGTA	3.3 (133)	7.6 (1213)	ND	ND	ND	ND	ND
GL00409 / MVD1	0.27 (105)	0% (54)	- [a]	-	-	-	-

546

547 [a] not enough progeny to accurately measure nondisjunction or crossing over

548

549

550 **Table 2: Fertility in *Cenp-C* RNAi, mutant and transgenic females**

551

Genotype	Offspring per female (# of females)
Control (*w-*)	22.93 (90)
HA-Cenp-C ; mata	0.58 (325)
HA-Cenp-C ; oskar	23.15 (125)
EGFP-Cenp-C ; mata	0.14 (150)
EGFP-Cenp-C ; oskar	18.11 (65)
Cenp-C^{Z}/+	11.5 (120)
Cenp-C^{IR35}/+	13.58 (160)
Cenp-C^{pr141}/+	19.82 (105)
Cenp-C^{Z}/Cenp-C^{Z}	1.46 (120)
Cenp-C^{Z}/ Cenp-C^{IR35}	0.06 (270)
Cenp-C^{Z}/ Cenp-C^{pr141}	0.25 (70)
Cenp-C^{Z}/ Cenp-C^{IR35}; HA-Cenp-C; mat a	1.358 (460)
Cenp-C^{Z}/ Cenp-C^{IR35}; HA-Cenp-C ; oskar	15.22 (180)
Cenp-C^{Z}/ Cenp-C^{pr141}; EGFP-Cenp-C; mata	0.21 (125)
Cenp-C^{Z}/ Cenp-C^{pr141}; EGFP-Cenp-C ; oskar	11.27 (130)
GL00409 ; mata	0 (120)
GL00409 ; oskar	10.81 (90)
GL00409; HA-Cenp-C; mat a	0.08 (430)
GL00409; HA-Cenp-C; oskar	26.72 (65)

552
553

554

555 **Supplementary Figures**

556

557 **Figure S 1: Expression pattern of NGTA. Related to Figure 1 and 2**

558 The expression pattern of **(A)** *P{GAL4-nos.NGT}* and **(B)** *P{GAL4::VP16-*
559 *nos.UTR}CG6325MVD1* using *UASP-β-galactosidase* as a reporter. Arrowheads
560 indicate anterior tip of the ovariole, where the germarium is located, and the blue stain
561 indicates where each GAL4 promotes expression.

562

563 **Figure S 2: Loading of centromere proteins during oocyte meiotic prophase.**
564 **Related to Figure 1 and 2**

565 In all images, HA-CENP-C is green, the centromeres are marked with CID (red), and
566 DNA is in blue. The scale bars represent 5 mm. **A)** Whole germarium with HA-tagged
567 CENP-C expressed using *NGTA* or *mata*. Enriched in the oocyte is the ORB protein in
568 white. **B)** HA-tagged CENP-C or CENP-C^C was expressed using *mata*. **C)** HA-CENP-C
569 was expressed using *hsp70-Gal4*. Oocytes were collected and fixed 6 hours after a 1-
570 hour incubation at 37°C.

571

572 **Figure S 3: CAL1 localization in stage 14 oocytes. Related to 1 and 4**

573 EGFP-tagged CENP-C or CAL1 (grey in single channel, green in merge) in stage 14
574 oocytes. The DNA is blue, microtubules in red, and the scale bars represent 5 mm. **A)**
575 Localization of GFP-tagged CENP-C expressed using the *MVD1* promoter. The
576 centromeres were detected using CID (white). **B)** Localization of GFP-tagged CAL1

using the *MVD1* promoter. The centromeres were detected using an antibody against CENP-C (white). **C)** Localization of GFP-tagged CAL1 using the endogenous *cal1* promoter. The centromeres were detected using an antibody against CENP-C (white).

Figure S 4: SC assembly in when CENP-C is depleted in prophase. Related to Figure 2

Confocal images of the germarium with *Cenp-C* RNAi (HMS01171) with (A) no *GAL4* and (A) *NGTA*. DNA is shown in blue, CENP-C is in red, and C(3)G is in green. The scale bar is 10 µm. CENP-C and C(3)G are shown in white in the single channel images. Region 1 of the germarium has been boxed to show increased centromeric C(3)G. The insets show single nuclei from region 1 in the germarium to show co-localization of CENP-C and C(3)G (Scale bar= 3 µm).

Figure S 5: Additional images of CENP-C localization in RNAi and transgenic oocytes. Related to Figure 4.

A) *Cenp-C* RNAi or *Cenp-C*^{Z} oocytes with CENP-C (green) and CID (red). **B)** *Cenp-C* RNAi or *Cenp-C*^{Z} oocytes expressing a *Cenp-C* transgene, with HA in red and SPC105R in green, DNA in blue, microtubules in white, and the scale bars represent 5 mm. **C)** Oocytes shown in panels A and B were assessed for KT-MT attachments. This was done by measuring the distance between each centromere and the nearest microtubule.

600 **Literature cited**

601 1. Nechemia-Arbely, Y., Fachinetti, D., and Cleveland, D.W. (2012). Replicating centromeric
602 chromatin: spatial and temporal control of CENP-A assembly. Exp Cell Res *318*, 1353-1360.
603 2. Swartz, S.Z., McKay, L.S., Su, K.C., Bury, L., Padeganeh, A., Maddox, P.S., Knouse, K.A., and
604 Cheeseman, I.M. (2019). Quiescent Cells Actively Replenish CENP-A Nucleosomes to Maintain
605 Centromere Identity and Proliferative Potential. Dev Cell *51*, 35-48.e37.
606 3. Kixmoeller, K., Allu, P.K., and Black, B.E. (2020). The centromere comes into focus: from CENP-A
607 nucleosomes to kinetochore connections with the spindle. Open Biol *10*, 200051.
608 4. Rosin, L.F., and Mellone, B.G. (2017). Centromeres Drive a Hard Bargain. Trends Genet *33*, 101-
609 117.
610 5. Sridhar, S., and Fukagawa, T. (2022). Kinetochore Architecture Employs Diverse Linker Strategies
611 Across Evolution. Front Cell Dev Biol *10*, 862637.
612 6. Jansen, L.E., Black, B.E., Foltz, D.R., and Cleveland, D.W. (2007). Propagation of centromeric
613 chromatin requires exit from mitosis. J Cell Biol *176*, 795-805.
614 7. Mellone, B.G., Grive, K.J., Shteyn, V., Bowers, S.R., Oderberg, I., and Karpen, G.H. (2011).
615 Assembly of Drosophila centromeric chromatin proteins during mitosis. PLoS Genet *7*,
616 e1002068.
617 8. Schuh, M., Lehner, C.F., and Heidmann, S. (2007). Incorporation of Drosophila CID/CENP-A and
618 CENP-C into centromeres during early embryonic anaphase. Curr Biol *17*, 237-243.
619 9. Camahort, R., Li, B., Florens, L., Swanson, S.K., Washburn, M.P., and Gerton, J.L. (2007). Scm3 is
620 essential to recruit the histone h3 variant cse4 to centromeres and to maintain a functional
621 kinetochore. Molecular cell *26*, 853-865.
622 10. Chen, C.C., Dechassa, M.L., Bettini, E., Ledoux, M.B., Belisario, C., Heun, P., Luger, K., and
623 Mellone, B.G. (2014). CAL1 is the Drosophila CENP-A assembly factor. J Cell Biol *204*, 313-329.
624 11. Foltz, D.R., Jansen, L.E., Bailey, A.O., Yates, J.R., 3rd, Bassett, E.A., Wood, S., Black, B.E., and
625 Cleveland, D.W. (2009). Centromere-specific assembly of CENP-a nucleosomes is mediated by
626 HJURP. Cell *137*, 472-484.
627 12. Williams, J.S., Hayashi, T., Yanagida, M., and Russell, P. (2009). Fission yeast Scm3 mediates
628 stable assembly of Cnp1/CENP-A into centromeric chromatin. Mol Cell *33*, 287-298.
629 13. Sanchez-Pulido, L., Pidoux, A.L., Ponting, C.P., and Allshire, R.C. (2009). Common ancestry of the
630 CENP-A chaperones Scm3 and HJURP. Cell *137*, 1173-1174.
631 14. Dunleavy, E.M., Roche, D., Tagami, H., Lacoste, N., Ray-Gallet, D., Nakamura, Y., Daigo, Y.,
632 Nakatani, Y., and Almouzni-Pettinotti, G. (2009). HJURP is a cell-cycle-dependent maintenance
633 and deposition factor of CENP-A at centromeres. Cell *137*, 485-497.
634 15. Pidoux, A.L., Choi, E.S., Abbott, J.K., Liu, X., Kagansky, A., Castillo, A.G., Hamilton, G.L.,
635 Richardson, W., Rappsilber, J., He, X., et al. (2009). Fission yeast Scm3: A CENP-A receptor
636 required for integrity of subkinetochore chromatin. Molecular cell *33*, 299-311.
637 16. Erhardt, S., Mellone, B.G., Betts, C.M., Zhang, W., Karpen, G.H., and Straight, A.F. (2008).
638 Genome-wide analysis reveals a cell cycle-dependent mechanism controlling centromere
639 propagation. J Cell Biol *183*, 805-818.
640 17. Medina-Pritchard, B., Lazou, V., Zou, J., Byron, O., Abad, M.A., Rappsilber, J., Heun, P., and
641 Jeyaprakash, A.A. (2020). Structural basis for centromere maintenance by Drosophila CENP-A
642 chaperone CAL1. EMBO J *39*, e103234.
643 18. Schittenhelm, R.B., Althoff, F., Heidmann, S., and Lehner, C.F. (2010). Detrimental incorporation
644 of excess Cenp-A/Cid and Cenp-C into Drosophila centromeres is prevented by limiting amounts
645 of the bridging factor Cal1. J Cell Sci *123*, 3768-3779.

19. Orr, B., and Sunkel, C.E. (2011). Drosophila CENP-C is essential for centromere identity. Chromosoma *120*, 83-96.
20. Screpanti, E., De Antoni, A., Alushin, G.M., Petrovic, A., Melis, T., Nogales, E., and Musacchio, A. (2011). Direct binding of Cenp-C to the Mis12 complex joins the inner and outer kinetochore. Curr Biol *21*, 391-398.
21. Petrovic, A., Keller, J., Liu, Y., Overlack, K., John, J., Dimitrova, Y.N., Jenni, S., van Gerwen, S., Stege, P., Wohlgemuth, S., et al. (2016). Structure of the MIS12 Complex and Molecular Basis of Its Interaction with CENP-C at Human Kinetochores. Cell *167*, 1028-1040 e1015.
22. Tanaka, K., Chang, H.L., Kagami, A., and Watanabe, Y. (2009). CENP-C functions as a scaffold for effectors with essential kinetochore functions in mitosis and meiosis. Dev Cell *17*, 334-343.
23. Liu, Y., Petrovic, A., Rombaut, P., Mosalaganti, S., Keller, J., Raunser, S., Herzog, F., and Musacchio, A. (2016). Insights from the reconstitution of the divergent outer kinetochore of Drosophila melanogaster. Open Biol *6*, 150236.
24. Przewloka, M.R., Zhang, W., Costa, P., Archambault, V., D'Avino, P.P., Lilley, K.S., Laue, E.D., McAinsh, A.D., and Glover, D.M. (2007). Molecular analysis of core kinetochore composition and assembly in Drosophila melanogaster. PloS one *2*, e478.
25. Przewloka, M.R., Venkei, Z., Bolanos-Garcia, V.M., Debski, J., Dadlez, M., and Glover, D.M. (2011). CENP-C is a structural platform for kinetochore assembly. Curr Biol *21*, 399-405.
26. Venkei, Z., Przewloka, M.R., and Glover, D.M. (2011). Drosophila Mis12 complex acts as a single functional unit essential for anaphase chromosome movement and a robust spindle assembly checkpoint. Genetics *187*, 131-140.
27. Richter, M.M., Poznanski, J., Zdziarska, A., Czarnocki-Cieciura, M., Lipinszki, Z., Dadlez, M., Glover, D.M., and Przewloka, M.R. (2016). Network of protein interactions within the Drosophila inner kinetochore. Open Biol *6*, 150238.
28. Smoak, E.M., Stein, P., Schultz, R.M., Lampson, M.A., and Black, B.E. (2016). Long-Term Retention of CENP-A Nucleosomes in Mammalian Oocytes Underpins Transgenerational Inheritance of Centromere Identity. Curr Biol *26*, 1110-1116.
29. Mihajlovic, A.I., and FitzHarris, G. (2018). Segregating Chromosomes in the Mammalian Oocyte. Curr Biol *28*, R895-R907.
30. Toth, A., and Jessberger, R. (2016). Oogenesis: Ageing Oocyte Chromosomes Rely on Amazing Protein Stability. Curr Biol *26*, R329-331.
31. Hemmerich, P., Weidtkamp-Peters, S., Hoischen, C., Schmiedeberg, L., Erliandri, I., and Diekmann, S. (2008). Dynamics of inner kinetochore assembly and maintenance in living cells. J Cell Biol *180*, 1101-1114.
32. Das, A., Black, B.E., and Lampson, M.A. (2020). Maternal inheritance of centromeres through the germline. Curr Top Dev Biol *140*, 35-54.
33. Rorth, P. (1998). Gal4 in the Drosophila female germline. Mech Dev *78*, 113-118.
34. Radford, S.J., Jang, J.K., and McKim, K.S. (2012). The Chromosomal Passenger Complex is required for Meiotic Acentrosomal Spindle Assembly and Chromosome Bi-orientation. Genetics *192*, 417-429.
35. Sugimura, I., and Lilly, M.A. (2006). Bruno inhibits the expression of mitotic cyclins during the prophase I meiotic arrest of Drosophila oocytes. Dev Cell *10*, 127-135.
36. Baker, B.S., and Hall, J.C. (1976). Meiotic mutants: genetic control of meiotic recombination and chromosome segregation. In The Genetics and Biology of Drosophila, Volume 1a, M. Ashburner and E. Novitski, eds. (New York: Academic Press), pp. 351-434.
37. Hughes, S.E., Hemenway, E., Guo, F., Yi, K., Yu, Z., and Hawley, R.S. (2019). The E3 ubiquitin ligase Sina regulates the assembly and disassembly of the synaptonemal complex in Drosophila females. PLoS Genet *15*, e1008161.

694 38. Gyuricza, M.R., Manheimer, K.B., Apte, V., Krishnan, B., Joyce, E.F., McKee, B.D., and McKim, K.S.
695 (2016). Dynamic and Stable Cohesins Regulate Synaptonemal Complex Assembly and
696 Chromosome Segregation. Curr Biol *26*, 1688-1698.
697 39. Tanneti, N.S., Landy, K., Joyce, E.F., and McKim, K.S. (2011). A Pathway for Synapsis Initiation
698 during Zygotene in Drosophila Oocytes. Curr Biol *21*, 1852-1857.
699 40. Unhavaithaya, Y., and Orr-Weaver, T.L. (2013). Centromere proteins CENP-C and CAL1
700 functionally interact in meiosis for centromere clustering, pairing, and chromosome segregation.
701 Proc Natl Acad Sci U S A *110*, 19878-19883.
702 41. Radford, S.J., Hoang, T.L., Głuszek, A.A., Ohkura, H., and McKim, K.S. (2015). Lateral and End-On
703 Kinetochore Attachments Are Coordinated to Achieve Bi-orientation in Drosophila Oocytes. PLoS
704 Genet *11*, e1005605.
705 42. Telley, I.A., Gaspar, I., Ephrussi, A., and Surrey, T. (2012). Aster migration determines the length
706 scale of nuclear separation in the Drosophila syncytial embryo. J Cell Biol *197*, 887-895.
707 43. Nagpal, H., Hori, T., Furukawa, A., Sugase, K., Osakabe, A., Kurumizaka, H., and Fukagawa, T.
708 (2015). Dynamic changes in CCAN organization through CENP-C during cell-cycle progression.
709 Mol Biol Cell *26*, 3768-3776.
710 44. Watanabe, R., Hara, M., Okumura, E.I., Herve, S., Fachinetti, D., Ariyoshi, M., and Fukagawa, T.
711 (2019). CDK1-mediated CENP-C phosphorylation modulates CENP-A binding and mitotic
712 kinetochore localization. J Cell Biol *218*, 4042-4062.
713 45. Dunleavy, E.M., Beier, N.L., Gorgescu, W., Tang, J., Costes, S.V., and Karpen, G.H. (2012). The cell
714 cycle timing of centromeric chromatin assembly in Drosophila meiosis is distinct from mitosis
715 yet requires CAL1 and CENP-C. PLoS Biol *10*, e1001460.
716 46. Hatkevich, T., Boudreau, V., Rubin, T., Maddox, P.S., Huynh, J.R., and Sekelsky, J. (2019).
717 Centromeric SMC1 promotes centromere clustering and stabilizes meiotic homolog pairing. PLoS
718 Genet *15*, e1008412.
719 47. Watanabe, Y. (2012). Geometry and force behind kinetochore orientation: lessons from meiosis.
720 Nat Rev Mol Cell Biol *13*, 370-382.
721 48. Nasmyth, K. (2015). A meiotic mystery: How sister kinetochores avoid being pulled in opposite
722 directions during the first division. Bioessays *37*, 657-665.
723 49. Watanabe, R., Hirano, Y., Hara, M., Hiraoka, Y., and Fukagawa, T. (2022). Mobility of kinetochore
724 proteins measured by FRAP analysis in living cells. Chromosome Res *30*, 43-57.
725 50. Bodor, D.L., Valente, L.P., Mata, J.F., Black, B.E., and Jansen, L.E. (2013). Assembly in G1 phase
726 and long-term stability are unique intrinsic features of CENP-A nucleosomes. Mol Biol Cell *24*,
727 923-932.
728 51. Das, A., Iwata-Otsubo, A., Destouni, A., Dawicki-McKenna, J.M., Boese, K.G., Black, B.E., and
729 Lampson, M.A. (2022). Epigenetic, genetic and maternal effects enable stable centromere
730 inheritance. Nat Cell Biol *24*, 748-756.
731 52. Heeger, S., Leismann, O., Schittenhelm, R., Schraidt, O., Heidmann, S., and Lehner, C.F. (2005).
732 Genetic interactions of separase regulatory subunits reveal the diverged Drosophila Cenp-C
733 homolog. Genes Dev *19*, 2041-2053.
734 53. Głuszek, A.A., Cullen, C.F., Li, W., Battaglia, R.A., Radford, S.J., Costa, M.F., McKim, K.S., Goshima,
735 G., and Ohkura, H. (2015). The microtubule catastrophe promoter Sentin delays stable
736 kinetochore-microtubule attachment in oocytes. J Cell Biol *211*, 1113-1120.
737 54. McKim, K.S., Joyce, E.F., and Jang, J.K. (2009). Cytological analysis of meiosis in fixed Drosophila
738 ovaries. Methods in molecular biology (Clifton, N.J *558*, 197-216.
739 55. Radford, S.J., and McKim, K.S. (2016). Techniques for Imaging Prometaphase and Metaphase of
740 Meiosis I in Fixed Drosophila Oocytes. Journal of visualized experiments : JoVE *116*, e54666.

741 56. Gilliland, W.D., Hughes, S.F., Vietti, D.R., and Hawley, R.S. (2009). Congression of achiasmate
742 chromosomes to the metaphase plate in Drosophila melanogaster oocytes. Dev Biol *325*, 122-
743 128.
744 57. Schittenhelm, R.B., Chaleckis, R., and Lehner, C.F. (2009). Intrakinetochore localization and
745 essential functional domains of Drosophila Spc105. EMBO J *28*, 2374-2386.
746 58. Page, S.L., and Hawley, R.S. (2001). c(3)G encodes a Drosophila synaptonemal complex protein.
747 Genes & development *15*, 3130-3143.
748 59. Lantz, V., Chang, J.S., Horabin, J.I., Bopp, D., and Schedl, P. (1994). The Drosophila ORB RNA-
749 binding protein is required for the formation of the egg chamber and establishment of polarity.
750 Genes & Dev. *8*, 598-613.
751 60. Spradling, A.C. (1993). Developmental genetics of oogenesis. In The Development of Drosophila
752 melanogaster, M. Bate and A.M. Arias, eds. (Cold Spring Harbor, NY: Cold Spring Harbor
753 Laboratory Press), pp. 1-70.
754 61. Jia, D., Xu, Q., Xie, Q., Mio, W., and Deng, W.M. (2016). Automatic stage identification of
755 Drosophila egg chamber based on DAPI images. Sci Rep *6*, 18850.
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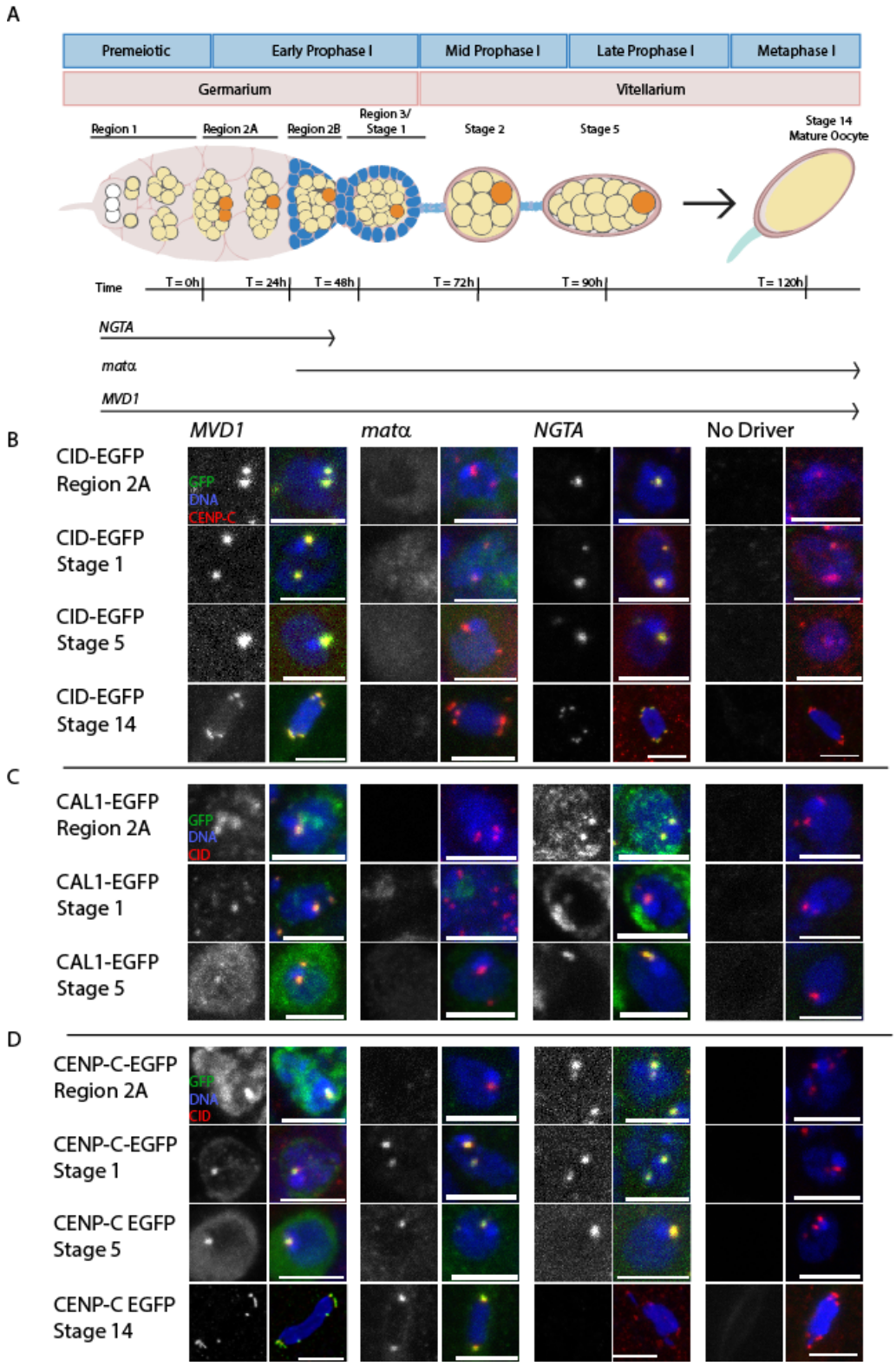
757

758 **Figure 1**



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762 **Figure 1**

763 **Loading of centromere proteins during meiotic prophase in oocytes. A)** Oocytes
764 are generated and enter meiotic prophase in the germarium. Region 1 contains the
765 stem cell niche as well as the pre-meiotic cyst cells undergoing mitosis. Prophase I of
766 meiosis begins in region 2A. Time points are relative to the onset of meiosis [60, 61].
767 Region 3 is also known as vitellarium stage 1, and at this stage, a single oocyte
768 (orange) has been determined. *NGTA* begins expressing in the mitotic germline (region
769 1) and ends in region 3 or early in the vitellarium. *MVD1* expression begins earlier than
770 *NGTA,* being expressed in all the mitotic and meiotic stages of the germline, including
771 the stem cells. *Mata* begins expressing in region 2B or 3 and continues until stage 14,
772 the metaphase I-arrested oocyte [34]. **B)** Localization of GFP-tagged CID (green) and
773 centromeres detected using a CENP-C antibody (red). **C)** Localization of GFP-tagged
774 CAL1 (green) with the centromeres detected using a CID antibody (red). **(D)**
775 Localization of GFP-tagged CENP-C, with the centromeres detected using a CID
776 antibody (red). In all images, the DNA is blue and the scale bars are 5 mm.
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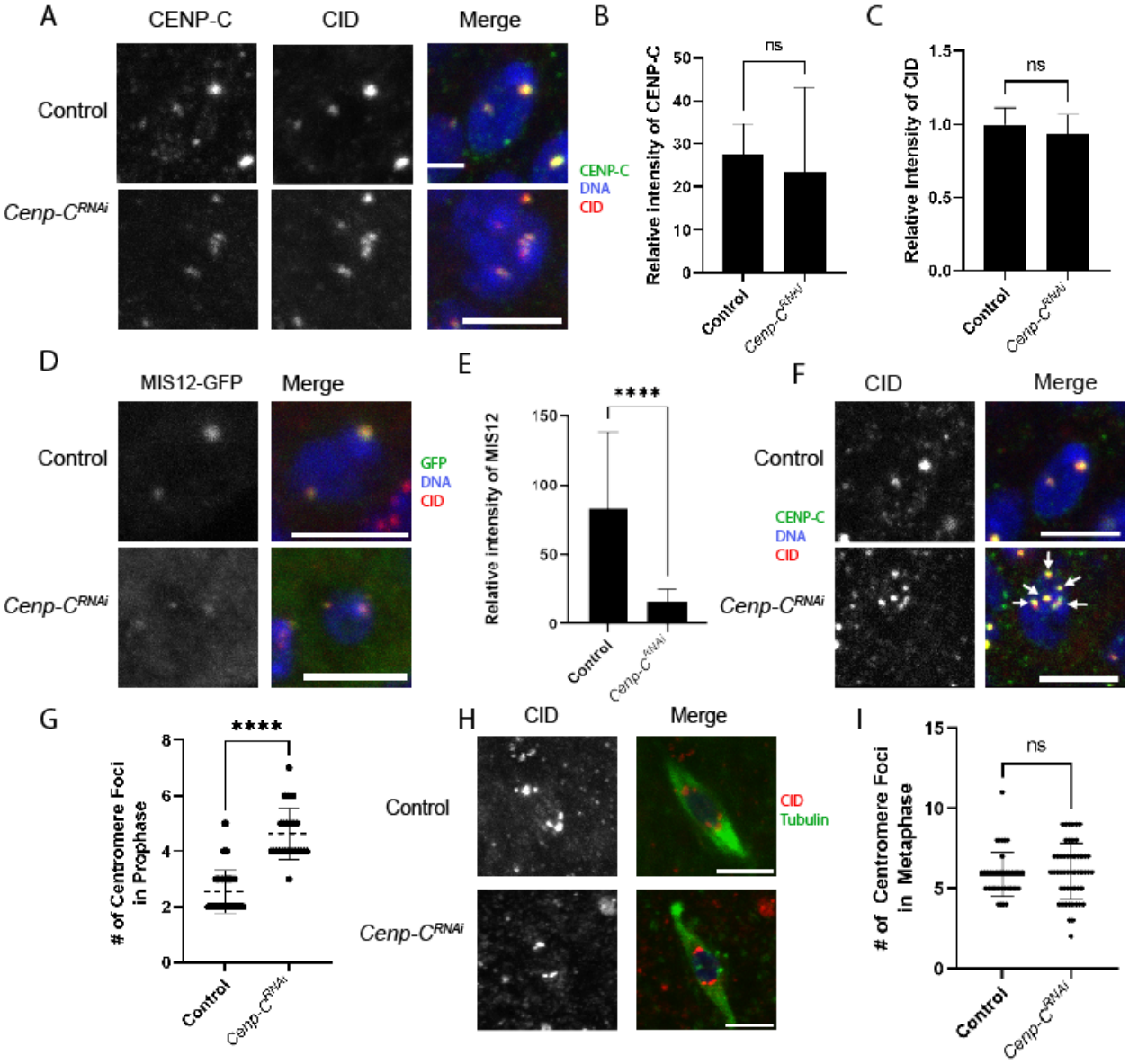
778

779 **Figure 2**



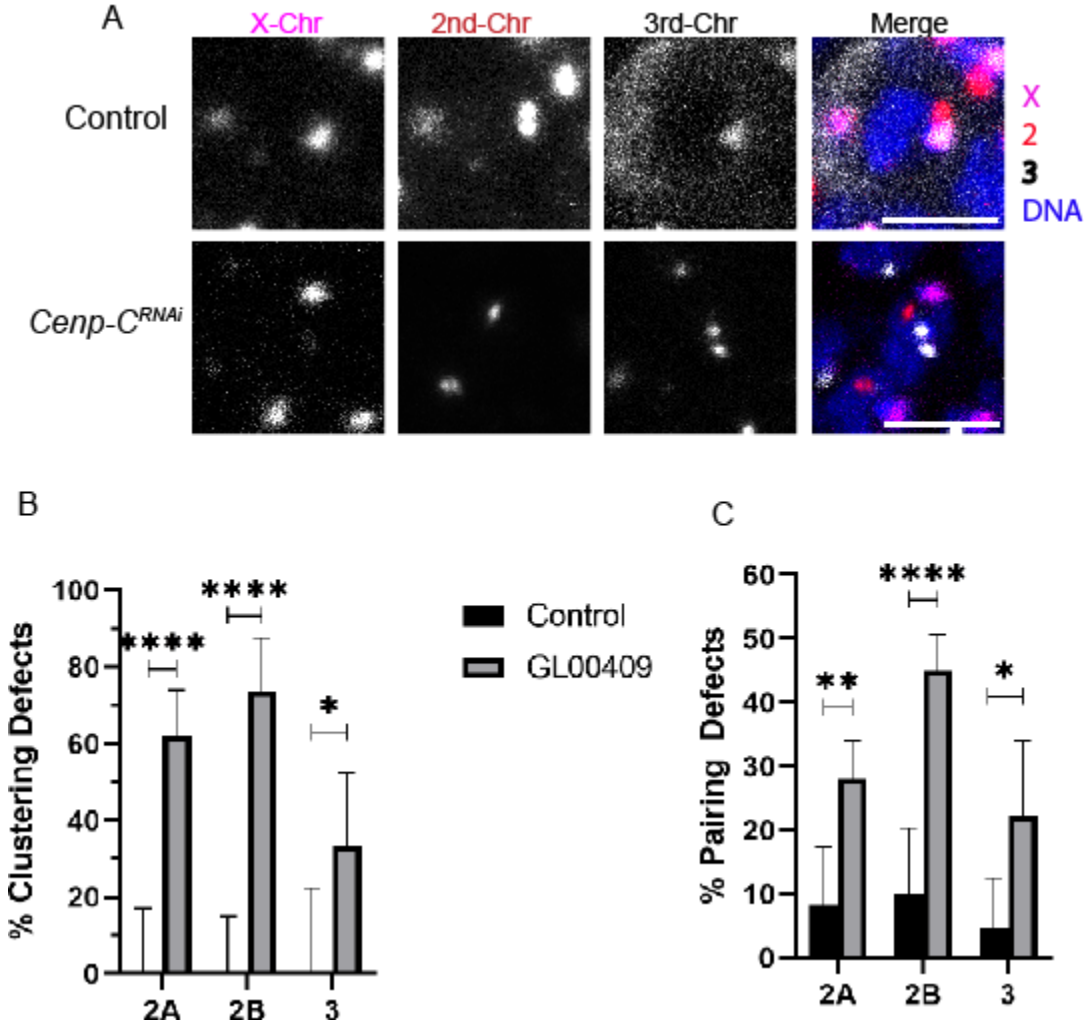
780

781 **Figure 2**

782 **Prophase I defects when CENP-C is depleted.** Immunocytology was performed on
783 *GL00409/NGTA* oocytes with CID (red), DNA (blue), and scale bars represent 5 mm. **A)**
784 Region 2 oocytes showing CENP-C in green. **B-C)** The intensities of CENP-C and CID
785 in region 2 were measured in *Cenp-C* RNAi and control oocytes (For B, n = 17 and 23;
786 for C, n = 11 and 29). **D)** Region 2 oocytes with MIS12-GFP in green. **E)** MIS12 intensity
787 was measured in region 2 (n = 9 and 30). **F)** CID foci in *Cenp-C* RNAi region 3 oocytes,
788 with CENP-C in green. **G)** Number of centromere foci was measured based on CID foci
789 in contact with the DNA (n = 84 and 27). **H)** tubulin (green) and CID foci (red) in *Cenp-C*
790 RNAi metaphase I oocytes. **I)** There was no increase in CID foci observed in stage 14
791 oocytes (n = 40 and 52). Error bars represent standard deviation from the mean. **p =
792 0.0037, ***p = 0.0001, ****p<0.0001
793

794

795 **Figure 3**

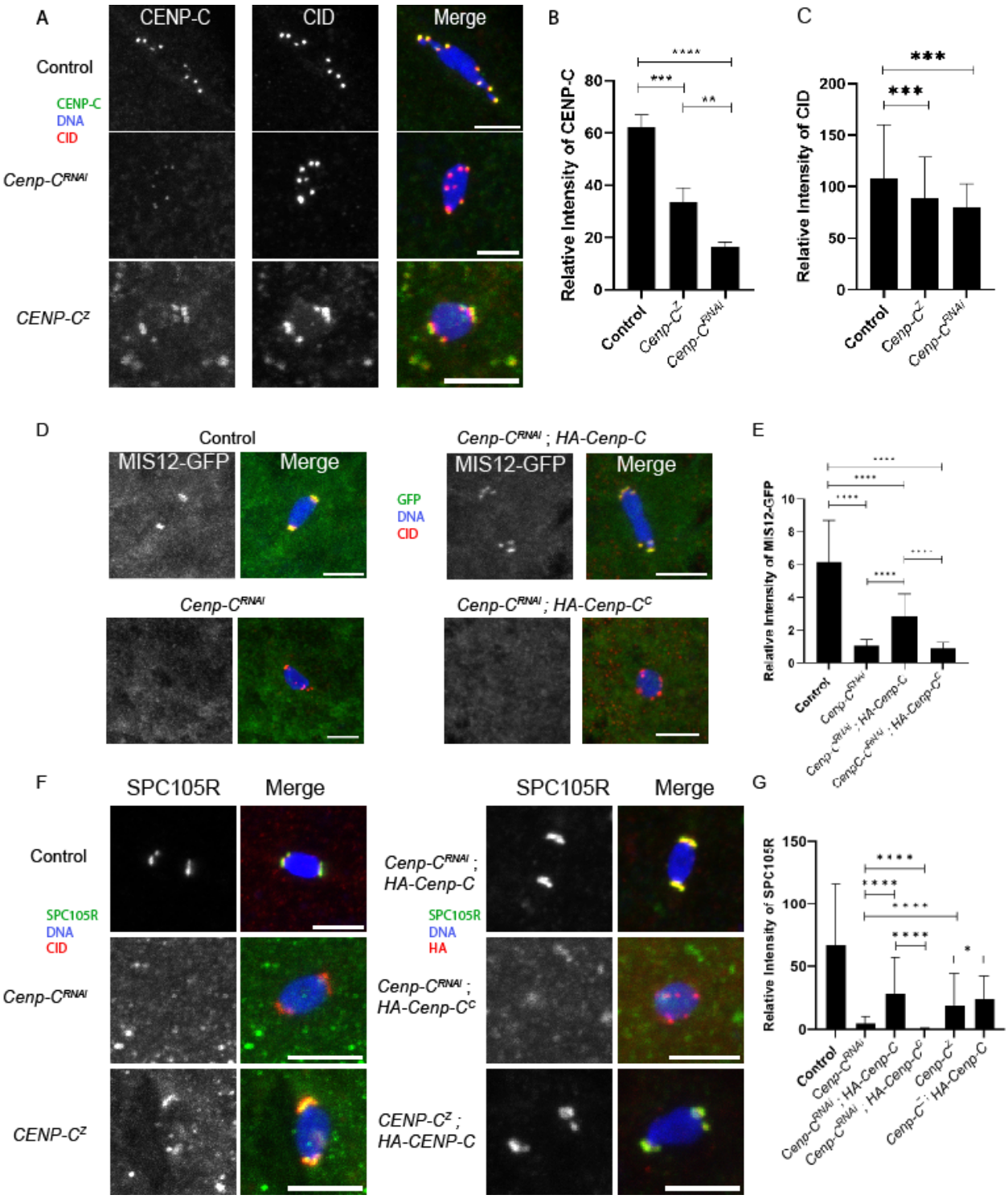


796

797 **Figure 3**

798 **CENP-C is required for centromere pairing and clustering. A)** FISH probes were
799 used to detect the pericentromeric regions of chromosome 2 (red), 3 (grey), and X
800 (magenta) in the germarium of *GL00409/NGTA* or control oocytes. DNA is in blue. **B)**
801 Clustering defects were defined as nuclei with greater than 2 of any centromere foci
802 (control n = 16, 20, 14; RNAi n = 42, 38, 18). **C)** Pairing defects were defined as oocytes
803 with greater than one focus for a chromosome in a nucleus. Scale bars represent 5mm.
804 * = 0.0196>p>0.0238, ** = 0.0045>p>0.0081, ****p<0.0001

805 **Figure 4**



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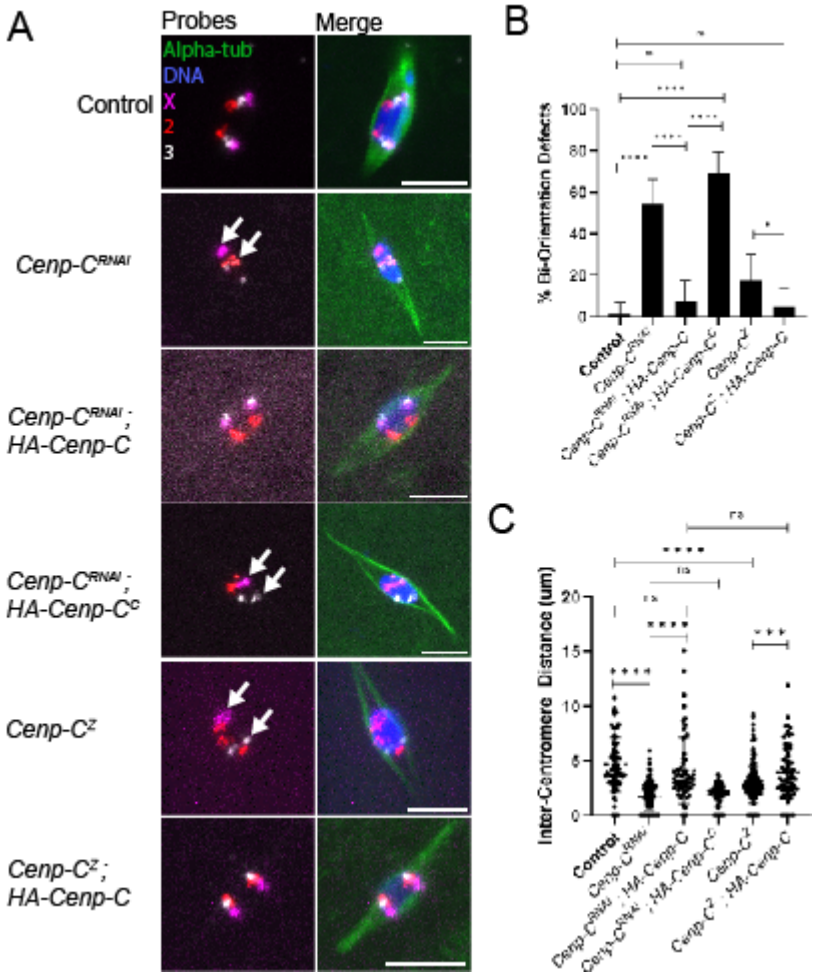
809 **Figure 4**

810 **CENP-C is required for kinetochore assembly and chromosome segregation.**
811 Immunocytology was performed on stage 14 oocytes heterozygous for *Cenp-C*
812 mutations (*Cenp-C*$^{Z3-4375}$/*Cenp-C*^{IR35} referred to as "*Cenp-C*^{Z}") or expressing the
813 *GL00409* with *mata* (referred to as *Cenp-C*^{RNAi}). HA-tagged *Cenp-C* transgenes were
814 coexpressed using the *mata* promoter in RNAi experiments or *osk-Gal4* in *Cenp-C*^{Z}
815 experiments. **A)** Oocytes with CENP-C (green) and CID (red). **B)** The intensity of
816 CENP-C was measured relative to background (n = 55, 67, 54). **C)** The intensity of CID
817 was measured relative to background (n = 186, 221, 54). **D)** Oocytes expressing MIS12-
818 GFP (green) and CID (red). **E)** Mis12-GFP localization was measured relative to the
819 background (n = 57, 41, 47, 67). **F)** Oocytes with SPC105R (green) and CID (red). **G)**
820 Intensity of SPC105R was measured relative to background (n = 108, 136, 115, 132,
821 245, 150). *p = 0.0379, **p = 0.0086, *** = 0.0002>p>0.0001, ****p<0.0001
822

823

824

825 **Figure 5**

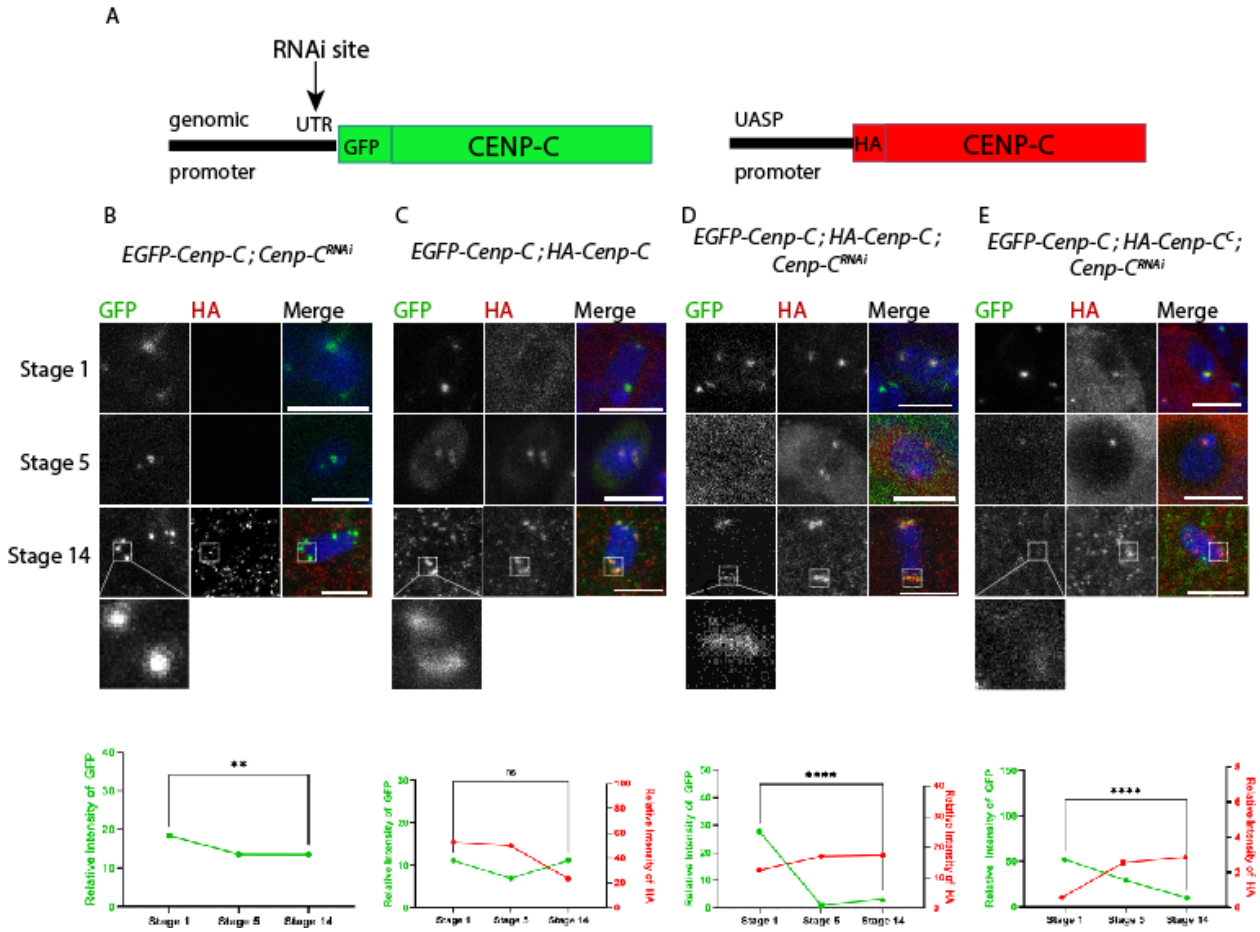


826

827 **Figure 5**

828 **Prophase-loaded CENP-C is required for bi-orientation in meiosis I. A)** FISH
829 probes were used to detect the pericentromeric regions of chromosome 2 (red), 3
830 (grey), and X (magenta). Stage 14 oocytes were *Cenp-C*$^{Z3\text{-}4375}$/*Cenp-C*^{IR35} (*Cenp-C*^{Z}) or
831 *GL00409/mata* (*Cenp-C*^{RNAi}), in some cases expressing *HA-Cenp-C* using *mata* in
832 *Cenp-C*^{RNAi} experiments or *oskGal4* in *Cenp-C*^{Z} experiments. **B)** Bi-orientation defects
833 were defined as two foci of the same probe that were on the same side of the spindle
834 midzone or only a single focus, indicating mono-orientation (n = 75, 59, 53, 58, 56, 60).
835 **C)** The distance between the center mass of two foci of the same probe was measured
836 as a readout for the microtubule tension across the kinetochore (n = 90, 156, 60, 60,
837 192, 72). All scale bars represent 5 mm and error bars represent standard deviation
838 from the mean. * = 0.0473>p>0.0387, **p = 0.0024, *** = 0.0002 >p> 0.001,
839 ****p<0.001.
840

841 **Figure 6**



842

843 **Figure 6**

844 **CENP-C is exchanged at the centromeres during meiotic prophase. A)**
845 Immunocytology was performed on oocytes ubiquitously expressing GFP-CENP-C
846 (green), plus or minus *GL00409* (*Cenp-C*^{RNAi}) and HA-CENP-C (red), using the *mata*
847 promoter. *GL00409* recognizes a sequence in the 5' UTR, and, therefore, degrades
848 *GFP-Cenp-C* but not *HA-Cenp-C*. All scale bars represent 5 mm and DNA is blue. **B)**
849 With *GFP-Cenp-C* and *Cenp-C*^{RNAi} expression, the GFP levels decrease moderately
850 over time. **C)** With expression of *GFP-Cenp-C* and *HA-Cenp-C*, both GFP and HA foci
851 are observed. **D)** With expression of *GFP-Cenp-C*, *Cenp-C*^{RNAi}, and *HA-Cenp-C*, the
852 loss of GFP is associated with an increase in HA signal. **E)** Similar results were
853 observed with expression of *GFP-Cenp-C*, *Cenp-C*^{RNAi}, and *HA-Cenp-C*^{C}. Below each
854 image is a graph showing the relative centromeric intensity of GFP-CENP-C (green line)
855 and HA-CENP-C (red) for each genotype (n = 70, 23, 104; 117, 19, 156; 28, 19, 50; 67,
856 98, 90). For each genotype, the intensity between stage 1 and stage 14 was compared
857 using an unpaired t-test. ** $p = 0.0016$; ****$p<0.0001$.
858