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Pairing centers recruit a Polo-like kinase to orchestrate meiotic chromosome dynamics in *C. elegans*

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Summary

Faithful segregation of homologous chromosomes during meiosis requires pairing, synapsis, and crossing-over. In *C. elegans*, homolog pairing and synapsis depend on pairing centers (PCs), special regions near one end of each chromosome that interact with the nuclear envelope (NE) and cytoplasmic microtubules. Here we report that PCs are required for nuclear reorganization at the onset of meiosis. We demonstrate that PCs recruit the Polo-like kinase PLK-2 to induce NE remodeling, chromosome pairing, and synapsis. Recruitment of PLK-2 is also required to mediate a cell cycle delay and selective apoptosis of nuclei containing unsynapsed chromosomes, establishing a molecular link between these two quality control mechanisms. This work reveals unexpected functions for the conserved family of Polo-like kinases, and advances our understanding of how meiotic processes are properly coordinated to ensure transmission of genetic information from parents to progeny.

Introduction

Sexual reproduction requires meiosis, the cell division process by which a diploid genome is partitioned to generate haploid gametes. Early in meiotic prophase, each chromosome pairs with its homologous partner. In most eukaryotes, pairing between homologs is stabilized by the formation of a protein scaffold called the synaptonemal complex (SC). Crossover

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recombination creates physical linkages between homologs. At the end of meiotic prophase, the SC disassembles, chromosomes condense, and cohesion is lost in a stepwise fashion to enable two successive nuclear divisions. The details of these mechanisms are not well understood and vary among species, but it is likely a universal feature of meiosis that homologs must form stable pairwise interactions to separate faithfully.

In the nematode *C. elegans*, homolog pairing and synapsis occur throughout the "transition zone" region of the germline, which corresponds to the classically defined meiotic stages of leptonema and zygonema. This zone is marked by a distinctive nuclear morphology in which chromosomes cluster into a crescent-shaped mass within the nuclear volume. Special *cis*-acting chromosome regions known as pairing centers (PCs) are required for both pairing and synapsis between homologous chromosomes (MacQueen et al., 2005). A family of zinc finger (ZnF) proteins, ZIM-1, ZIM-2, ZIM-3, and HIM-8, bind to short sequences that are highly enriched in these regions, and mediate all known PC functions. HIM-8 is specific for the X-chromosome PC, ZIM-1 for Chromosomes II and III, ZIM-2 for Chromosome V, and ZIM-3 for Chromosomes I and IV (Phillips and Dernburg, 2006; Phillips et al., 2009b; Phillips et al., 2005). In the transition zone, PCs and their cognate ZnF binding proteins associate with the nuclear envelope (NE) proteins SUN-1 and ZYG-12 to connect chromosomes to the microtubules and dynein. This linkage requires the inner NE protein SUN-1 and the outer NE KASH-domain protein ZYG-12. These proteins concentrate in "patches" that move along the nuclear surface, along with the associated PCs, during early prophase. Previous work has indicated that these interactions both promote homolog pairing and also ensure that SC formation occurs only between properly paired, homologous chromosomes (Penkner et al., 2007; Penkner et al., 2009; Sato et al., 2009). This linkage of PCs to the cytoskeleton through the intact NE appears to be a variant form of the meiotic "bouquet," a widely conserved feature of meiosis that is more typically mediated by telomeres (reviewed in Scherthan, 2001).

A major unanswered question has been how chromosomes and the nuclear envelope are reorganized at the onset of meiotic prophase. The observation that SUN-1 phosphorylation upon meiotic entry is important for nuclear reorganization and homolog pairing (Penkner et al., 2009) indicates that one or more kinase(s) likely regulates these early prophase events. The serine/threonine kinase CHK-2 is required for nuclear reorganization and homolog interactions, but its targets are unknown (MacQueen and Villeneuve, 2001).

Our previous work has indicated that the PCs may be required for this reorganization, and this hypothesis is investigated here by engineering a deletion of all four of the PC-associated ZnF genes. We also demonstrate that the PC ZnF proteins recruit the Polo-like kinase PLK-2, which leads to SUN-1 phosphorylation and homolog pairing. We report additional roles for PLK-2 in the synapsis checkpoint and SC disassembly. Thus, PLK-2 shows dynamic association with meiotic chromosomes and regulates several key aspects of interhomolog interactions.

Results

Loss of *plk-2* results in defects in meiotic chromosome pairing and synapsis

Mutation of *C. elegans plk-1*, which encodes an ortholog of mammalian Plk1, results in inviability due to an essential role in mitosis (Chase et al., 2000). Interestingly, we found that animals homozygous for either of two deletion alleles of *plk*-2, which encodes a closely related paralog of *plk-1*, were viable and fertile. Both alleles are predicted to be null mutations (Figure 1A and Materials and Methods), and gave identical results in all assays reported here. Although homozygous animals developed normally, their self-progeny showed reduced viability and many survivors were males (XO) (Table S1). This High incidence of males (Him) phenotype is indicative of meiotic segregation defects (Hodgkin et al., 1979). At diakinesis, only 25% of *plk-2* oocytes displayed the 6 bivalents normally seen in wild-type animals, while 75% contained 7–10 DAPI-staining bodies, indicating that one or more pair(s) of chromosomes had failed to undergo crossing-over (Figures 1B and 1C). Nondisjunction of these univalent chromosomes can account for the observed inviable eggs and male progeny.

To undergo crossing-over and segregate accurately during Meiosis I, each chromosome must first pair with its homolog and undergo synapsis, defined as assembly of the central region of the synaptonemal complex (SC) between paired chromosome axes. In wild-type animals, pairing and synapsis occur in the transition zone region, and are completed by entry into pachytene. We observed a unique defect in *plk-2* mutants: although there was a short region of nuclei with transition-zone-like chromosome morphology, most nuclei exited this region with extensive asynapsis. Suprisingly, the extent of synapsis continued to increase throughout the pachytene region of the germline (Figure 1D). This gradual increase was also seen in *plk-2; ced-4* double mutants, which lack germline apoptosis, indicating that it was not a consequence of culling of nuclei with unsynapsed chromosomes (data not shown). By the end of the pachytene region, most chromosomes were fully synapsed with their proper partners (Figure 1D). This delayed but fairly robust homologous synapsis is consistent with our observation of only limited numbers of univalent chromosomes at diakinesis. We tested whether the delay in synapsis reflects a delay in homolog pairing, and found that in *plk-2* mutants, pairing at both ends of chromosomes V and X continued to rise throughout pachytene, in contrast to wild-type animals, which achieved nearly complete homolog pairing by early pachytene (Figures 1E and 1F).

plk-2 mutant animals were clearly proficient for meiotic recombination, since most chromosome pairs were bivalent at diakinesis. We observed that DNA recombination intermediates, as detected by RAD-51 immunofluorescence, accumulated to higher levels and persisted later in *plk-2* mutants than in wild-type hermaphrodites (Figures S1A and S1B). Abundant and persistent RAD-51 foci are often seen in mutants with defective synapsis, since crossovers cannot be completed on unsynapsed chromosomes (Colaiacovo et al., 2003) and homolog-independent repair is suppressed until late in the pachytene stage (Hayashi et al., 2007).

In addition to early prophase defects in pairing and synapsis, chromosomes in diplotene *plk-2* mutant oocytes were more diffuse than in wild type, and asymmetric SC disassembly

was perturbed (Figure S1C). Asymmetric SC disassembly is thought to facilitate meiotic chromosome segregation by specifying a region of the bivalent that will release cohesion at the first meiotic division and a reciprocal region that will retain cohesion until the second division (Martinez-Perez et al., 2008; Nabeshima et al., 2005). Although we observed an apparent delay in asymmetric disassembly, the most mature (or "−1") oocyte in each gonad displayed asymmetric localization of LAB-1 (de Carvalho et al., 2008) and reciprocal patterns of the cohesins REC-8 and COH-3 (Severson et al., 2009) (Figure S1D and data not shown), indicating eventual differentiation of the two arms of the bivalent. Fertilized *plk-2* embryos underwent two oocyte meiotic divisions, extruded two polar bodies, and commenced mitotic division (data not shown), indicating that PLK-2 does not play an essential role in nuclear envelope breakdown in oocytes or the separation of meiotic chromosomes, in contrast to PLK-1 (Chase et al., 2000).

PLK-2 mediates two distinct responses to unsynapsed chromosomes

We found that *plk-2* mutants have elevated germline apoptosis relative to the baseline "physiological" apoptosis seen in wild-type hermaphrodites (Figure 2A). In previous work we have shown that unsynapsed chromosomes can trigger selective apoptosis of the affected oocyte nuclei near the end of meiotic prophase (Bhalla and Dernburg, 2005). This synapsis checkpoint is independent of persistent recombination intermediates that trigger the germline DNA damage checkpoint, but requires the presence of an unsynapsed, functional PC bound by its cognate ZnF protein. However, in *plk-2; spo-11* double mutants, which do not form double strand breaks (Dernburg et al., 1998), germline apoptosis was reduced to the wild-type level. This indicates that elevated apoptosis in *plk-2* animals is triggered by persistent DNA damage, rather than unsynapsed chromosomes, despite the defect in synapsis (Figure 2A). This result suggested that PLK-2 might be required for the synapsis checkpoint. To further investigate this possibility, we combined *plk-2(tm1395)* with *syp-1(me17)*, which activates both the synapsis checkpoint and the DNA damage checkpoint (Bhalla and Dernburg, 2005). Quantification of apoptosis confirmed that loss of PLK-2 specifically abrogates the synapsis checkpoint, but not the DNA damage checkpoint, in the absence of *syp-1* (Figure 2A).

Most mutations that inhibit SC formation on one or more chromosomes result in an elongated transition zone, defined cytologically as the region of the germline containing nuclei with clustered chromosomes and obvious patches of SUN-1 and ZYG-12 at the NE (reviewed in Colaiacovo, 2006). Extension of this region in response to asynapsis is thought to extend the time available for homology search. Since the N-terminus of SUN-1 becomes phosphorylated at several residues upon meiotic entry and concomitant with patch formation, we used SUN-1 pSer8 immunofluorescence (Penkner et al., 2009) and DAPI morphology to assay transition zone length. Despite the incomplete and delayed synapsis in *plk-2* mutants, the transition zone appeared to be even shorter than in wild-type hermaphrodites, and the SUN-1 patches appeared smaller and less distinct (Figures 2B and 2C). We combined *plk-2(tm1395)* with mutations in *him-8*, *zim-1*, *zim-2*, *zim-3* and *syp-1*, all of which normally cause extension of the transition zone (MacQueen et al., 2002; Phillips and Dernburg, 2006; Phillips et al., 2005). In each case, a short transition zone was observed, despite the persistence of unsynapsed chromosomes (Figures 2B and 2C; and data

not shown). This result indicates that PLK-2 is required to prolong the transition zone, in addition to its role in promoting apoptosis in response to asynapsis.

PLK-2 is recruited to pairing centers during early meiosis

Antibodies that specifically recognize PLK-2 revealed a dynamic localization pattern in the germline (Figures 3A, S2A and S2B). Premeiotic nuclei undergoing mitosis showed faint staining at centrosomes and kinetochores (Figure S2C). By contrast, transition zone nuclei showed strong patches of PLK-2 staining at the nuclear envelope that colocalized with all four PC ZnF proteins and the associated patches of SUN-1 and ZYG-12 (Figures 3B and 3C; data not shown). This localization is consistent with a role for PLK-2 in pairing and/or synapsis initiation, based on prior evidence that synapsis initiates at or near the PCs (Hayashi et al., 2010; MacQueen et al., 2005). By mid to late pachytene PLK-2 staining was no longer detected at PCs, but appeared along the SCs and disassembled with SC central region components at diplotene (Figures S2D and S2E). Our analysis of *plk-2* mutants, described above, indicates that the later PLK-2 localization to the SC may facilitate its proper disassembly.

Polo-like kinases (Plks) are defined by the presence of a Polo box domain (PBD), in addition to a serine/threonine kinase domain. The PBD often determines the substrate specificity of Plks by binding to phosphothreonine or phosphoserine, typically in the context of S-pS/T-P, in substrates or associated proteins (Archambault and Glover, 2009; Elia et al., 2003). The PC ZnF proteins and the nucleoplasmic N-terminus of SUN-1 all contain evolutionarily conserved STP motifs, and might therefore recruit PLK-2 to the NE during early prophase (Figures S3A and S3B). To investigate how PLK-2 is recruited to the NE patches, we examined PLK-2 localization in various mutants. *sun-1(gk199)* is a deletion allele that eliminates detectable SUN-1 protein in the germline, and results in profound mitotic and meiotic defects (Fridkin et al., 2004; Sato et al., 2009). Nevertheless, we found that PLK-2 colocalized transiently with HIM-8 and ZIM proteins in early meiotic nuclei of *sun-1(gk199)* hermaphrodites (Figure 3D and data not shown), indicating that SUN-1 is not required for PLK-2 association with PCs. In *chk-2* mutants, which fail to form NE patches or promote homolog interactions, PLK-2 staining was not detected at any of the PCs (Figure 3E).

The *him-8(me4)* allele is a missense (S85F) mutation in the N-terminal region of HIM-8, near the consensus PBD-binding motif (Figure S3A). This allele results in expression of a mutant protein that localizes to X chromosome PCs and the NE, but fails to associate with a SUN-1/ZYG-12 patch, and does not promote X chromosome pairing or synapsis (Phillips et al., 2005; Sato et al., 2009). We found that in *him-8(me4)* mutants, PLK-2 was not recruited to the unpaired HIM-8^{S85F} foci during early prophase, suggesting that this residue is important for PLK-2 recruitment and that PC function may rely on PLK-2 activity (Figure 4A). To test whether the putative Polo box binding domain of the PC ZnF proteins (Figure S3A) was required for PLK-2 recruitment, we created transgenic lines carrying single-copy insertions of the wild-type *him-8* gene or a missense allele changing threonine 64 within the STP motif to alanine. Both transgenes were crossed to *him-8(tm611)*, a deletion allele that eliminates HIM-8 binding to the X PC (Phillips et al., 2005). The wild-type transgene fully

complemented *him-8(tm611)* and showed normal HIM-8 foci that colocalized with patches positive for PLK-2, SUN-1, and ZYG-12 (Figure 4B and data not shown). By contrast, expression of HIM-8^{T64A} did not rescue the Him phenotype; the mutant protein localized to the X PCs and to the nuclear envelope but did not recruit PLK-2 (Figure 4C) or promote X chromosome pairing, similar to HIM-8^{S85F}.

To identify proteins that physically interact with PLK-2, we performed yeast two-hybrid (Y2H) screens. Both the full-length coding sequence and a C-terminal fragment containing the Polo box domain (PBD) were used as baits. The “prey” library was constructed from young adult hermaphrodites, and should therefore include cDNAs encoding meiotic components. Positive interactors identified in this screen included both *mex-5* and *mex-6* (Table S2A), which were previously shown to interact with *plk*-2 in pairwise Y2H assays (Nishi et al., 2008), and the uncharacterized *pqn-59* gene (Tables S2A and S2B), which was identified in a high-throughput screen as a PLK-1 interactor (Li et al., 2004). We also recovered multiple clones corresponding to the *him-8* gene with both bait clones, indicating that HIM-8 can physically associate with PLK-2 and that the PBD is sufficient for this interaction.

To further explore the interaction between PLK-2 and HIM-8, we carried out pairwise Y2H assays with the PBD fragment of PLK-2 and various fragments of HIM-8 (Figure 4D). We found that a HIM-8 fragment containing the N-terminal 112 amino acids, including the STP motif, was sufficient for the interaction with PLK-2, but a shorter fragment bearing the motif (aa 41–80) did not interact. Mutation of threonine 64 within the candidate PBD-binding motif to alanine abolished the Y2H interaction with PLK-2. We also found that the HIM-8^{S85F} mutant protein encoded by *him-8(me4)* was unable to interact with the PLK-2 PBD by Y2H, supporting the idea that that the pairing and synapsis defects observed in *him-8(me4)* mutants reflect an inability to recruit PLK-2 to the X PC. These findings, together with evidence that SUN-1 is not required for localization of PLK-2 to PCs, support the idea that the recruitment of PLK-2 to the PCs in early meiosis occurs through a direct interaction with the ZnF proteins.

We did not recover clones corresponding to any of the *zim* genes in Y2H screens with PLK-2 or its PBD. We tested whether full-length ZIM-3 can interact with the PBD of PLK-2 in a pairwise Y2H assay, and obtained negative results. Nevertheless, the localization of PLK-2, and the defects associated with its absence, indicate that it interacts with all PCs in *C. elegans*. It is possible that HIM-8, which is somewhat diverged from the three ZIM proteins (Phillips and Dernburg, 2006), is more effectively phosphorylated by a priming kinase in yeast cells. There are some interesting differences in the behavior of HIM-8 and the ZIM proteins in *C. elegans*: HIM-8 foci are detected earlier, in premeiotic nuclei, and disappear much later in prophase than the ZIM proteins, which only form prominent PC-associated foci in the transition zone. Further studies are needed to explain these differences in regulation.

Pairing centers are required for chromosome reorganization during early meiosis

In the absence of individual PC ZnF proteins, the corresponding chromosomes fail to pair, synapse and recombine with their partners (Phillips and Dernburg, 2006; Phillips et al.,

2005). Consequently they undergo frequent missegregation during the first meiotic division. We have speculated that PCs might nucleate the NE patches observed in the transition zone, since patches are observed only in association with functional PCs, defined as the *cis*-acting chromosomal region bound by its cognate ZnF protein (Sato et al., 2009). Additionally, high-copy arrays carrying binding sites for the ZnF proteins associate with large NE patches (Phillips et al., 2009b). We found that such arrays can also recruit PLK-2 (data not shown).

To directly test whether PC ZnF proteins are strictly required for PLK-2 localization and NE patch nucleation, we deleted the four ZnF genes, *zim-1, zim-2, zim-3,* and *him-8* (Figure 5A). Because these genes are adjacent to each other, we engineered a 20-kb genomic deletion that excised all four coding sequences using MosDel (Frokjaer-Jensen et al., 2010). Animals homozygous for the resulting deletion, designated *ieDf2*, were viable and fertile, but most (97.5%) of their embryos died before hatching (Table S1). Among the survivors, a high fraction (17%) were males (Table S1). In mutant hermaphrodites, we observed 12 univalent chromosomes in oocyte nuclei at diakinesis (Figure 5B), indicating that no functional crossovers occurred, consistent with the Him phenotype and high embryonic lethality.

In support of the idea that NE patches are nucleated by PCs, SUN-1 and ZYG-12 both remained distributed throughout the NE in early prophase nuclei in *ieDf2* hermaphrodites (Figure 5C and data not shown). In addition, these nuclei lacked any apparent chromosome clustering based on DAPI staining, and no pairing of homologous chromosomes was detected by FISH (Figures S4A, 5E and data not shown). Although the lack of NE patches and chromosome clustering indicate that PCs are required to establish a normal transition zone, we observed linear tracks of SC axial element staining in the early meiotic region of the germline (Figure 5D), indicating that some aspects of meiotic chromosome reorganization occurred on schedule.

Somewhat unexpectedly, central region proteins of the SC loaded robustly along all chromosomes in *ieDf2* homozygotes, despite the absence of homolog pairing. Many transition zone nuclei contained one or two bright, thick, chromosome-associated stretches of SYP-1 (Figures 5D and S4D). In situ hybridization analysis indicated that this structure was not preferentially associated with any particular chromosome (data not shown). By the late pachytene region of the gonad, SC central region proteins were distributed along all chromosome axes despite homolog pairing levels remaining at background levels (Figure 5D and Figure S4B). Detailed analysis revealed that this loading reflected fold-back synapsis of the twelve unpaired chromosomes (Figures S4B –D). Absence of individual PC proteins usually results in asynapsis of the corresponding homolog pair(s) (Phillips and Dernburg, 2006; Phillips et al., 2005). It was therefore surprising that deletion of all PC proteins gives rise to fold-back synapsis. However, this result is consistent with prior evidence that connections between the PCs and SUN-1/ZYG-12 inhibit aberrant loading of SC proteins (Penkner et al., 2007; Sato et al., 2009).

We considered the possibility that homolog pairing in the absence of functional PCs might be inhibited by premature, aberrant loading of SC. To test this, we blocked SC assembly by RNAi of *syp-2* (Colaiacovo et al., 2003). This did not restore homolog associations, even when synapsis was fully abrogated (Figure 5E). Thus, PCs are required for homolog pairing,

consistent with previous analysis (MacQueen et al., 2005; Phillips and Dernburg, 2006; Phillips et al., 2005).

To assess whether PCs are required for double-strand break (DSB) formation, we stained *ieDf2* animals with RAD-51 antibodies (Figure 5F). We observed foci that rose in abundance from the transition zone through the pachytene region, indicating that DSBs occur independently of PC activity. Like most other known mutants with defects in homologous synapsis, including *plk-2*, RAD-51 foci accumulated to higher levels than observed in wild-type animals due to the absence of a homolog available for repair. These unrepaired recombination intermediates resulted in elevated germline apoptosis through the *hus-1*-dependent DNA damage checkpoint (Figure S4E). Despite aberrant synapsis in *ieDf2* mutants, germline apoptosis was not induced through the synapsis checkpoint (Figure S4E), consistent with prior evidence that this pathway requires functional PCs (Bhalla and Dernburg, 2005).

Consistent with the evidence presented above that PLK-2 is recruited to PCs by direct interaction with the ZnF proteins, PLK-2 was not detected at the nuclear envelope or chromosomal foci in *ieDf2* homozygotes. However, we observed PLK-2 associated with stretches of aberrantly loaded SC central region proteins (Figure 5G). Together with the localization pattern in wild-type animals, this suggests that PLK-2 associates with SC central region proteins upon its release from the ZnF proteins, which normally occurs as nuclei progress into pachytene.

Concomitant with the appearance of NE patches in early prophase, SUN-1 is phosphorylated at multiple serine residues within its nucleoplasmic N-terminus (Penkner et al., 2009). Phosphorylation of SUN-1 at Ser8 and Ser12 requires the CHK-2 kinase (Penkner et al., 2009). In the absence of CHK-2, SUN-1 and ZYG-12 fail to aggregate and homolog pairing is abolished (MacQueen and Villeneuve, 2001; Penkner et al., 2009; Sato et al., 2009). Interestingly, in *ieDf2* homozygotes we found that SUN-1 was phosphorylated at Ser8, but not at Ser12 (Figure 5H). This indicates that these two modifications are mediated by different activities, one of which requires PCs. The observed restriction of SUN-1 pSer12 to NE patches contrasts with pSer8, which is detected throughout the NE (Penkner et al., 2009 and this work), further supporting the notion that phosphorylation at Ser12 is a direct consequence of PC association. Together with prior data, our analysis of *ieDf2* reveals that PCs are necessary to establish homolog interactions, and that this likely occurs through their role in nucleation of the NE links to microtubules. Intriguingly, the dependence of SUN-1 Ser12 phosphorylation on PCs suggests that recruitment of PLK-2 may facilitate NE patch formation.

PLK activity is required for NE patch formation and homolog pairing

The pairing and synapsis defects we observed in *plk-2* mutants, described above, were less severe than those observed in the absence of all four PC ZnF proteins. This indicated that PLK-2 contributes to, but is not absolutely required for PC function. *C. elegans* has three Polo-like kinases, PLK-1, PLK-2 and PLK-3. Previous work has shown that PLK-2 is partially redundant with the closely related paralog, PLK-1, for establishment of polarity in

the *C. elegans* embryo (Nishi et al., 2008). We therefore tested the hypothesis that PLK-1 might be providing some function during meiotic prophase in the absence of PLK-2.

Because PLK-1 is required for embryonic viability and germline proliferation (Chase et al., 2000), we depleted *plk-1* in *plk-2* mutants by RNAi. This produced severe meiotic defects: No chromosome clustering was observed in early prophase, HIM-8 foci were consistently unpaired (Figure 6B) and even in the pachytene region, homologous pairing at other loci was reduced to levels typical of premeiotic nuclei (for VL: 9.7%, VR: 12.6%, XR: 16.9%, XL: 15.5%; compare to Figure 1F). Loading of SC central region components was markedly reduced and presumably occurred between nonhomologous regions (Figure 6A and 6B). Abundant RAD-51 foci were detected on meiotic chromosomes in the absence of *plk-1* and *plk-2*, indicating that these kinases are not required for DSB formation or resection (Figure 6C). At diakinesis, chromosome condensation was delayed, but mature oocytes with condensed chromosomes contained 12 univalents, as expected based on the failure of homolog pairing and synapsis (Figure 6D). Fertilized eggs were produced by *plk-2; plk-1(RNAi)* hermaphrodites, but were arrested at the one-cell stage, consistent with previous data that PLK-1 is required for meiotic chromosome segregation (Chase et al., 2000).

Depletion of *plk-1* alone did not perturb PLK-2 localization in meiosis, SUN-1 patch formation, homolog pairing, synapsis, or bivalent formation, indicating that PLK-2 is sufficient to promote these meiotic functions (Figures 6B, 6D, S5A and S5B). Interestingly, mitotic arrest of nuclei was observed in the premeiotic zone of *plk-1(RNAi)* gonads, and was more pronounced when *plk-1* was depleted in *plk-2* mutants, indicating that PLK-2 can provide some function in the mitotic germline in the absence of PLK-1 (Figure S5C). Immunolocalization of PLK-1 revealed association with centrosomes and kinetochores in mitotic cells of the proliferating region of the germline, and PLK-2 was also faintly detected at these structures (Figures S2C and S5D). PLK-1 did not show discrete localization in meiotic prophase of wild-type animals (Figure 7A and data not shown). However, in *plk-2* mutants PLK-1 staining was detected at NE patches in early meiosis, indicating that PLK-1 can associate with PCs in the absence of PLK-2 (Figure 7A). The weaker SUN-1 patch aggregation in *plk-2* mutants suggests that PLK-1 cannot fully substitute for PLK-2, consistent with the partial defects in homolog pairing and synapsis in *plk-2* mutants.

Using SUN-1 phospho-specific antibodies (Penkner et al., 2009), we tested whether the meiotic defects in *plk-2; plk-1(RNAi)* animals were due to perturbed SUN-1 phosphorylation. In *plk-2* mutants and *plk-1(RNAi)* animals, SUN-1 was phosphorylated at both Ser8 and Ser12 upon meiotic entry, as in wild-type animals (Figure 7A and 7B). However, in *plk-2; plk-1(RNAi)* animals, Ser12 phosphorylation was not detected, while Ser8 was still phosphorylated, as described above for *ieDf2* mutants lacking PC ZnF proteins (Figures 7A and 7B). This suggests that PLKs provide the PC-associated kinase activity required for SUN-1 phosphorylation at Ser12, and for SUN-1 aggregation.

PLK-3 is divergent from PLK-1 and -2, and no defects in homolog pairing, synapsis, or meiotic chromosome segregation were observed in *plk-3(ok2812)* animals (data not shown). However, some delay in chromosome condensation at diakinesis was noted in these mutant

animals. No additive effects were observed in *plk-2; plk-3(RNAi)* animals (data not shown), indicating that PLK-3 does not play a major role in meiosis.

Discussion

Pairing centers play several separable roles to promote and monitor interhomolog interactions during meiotic prophase (Bhalla and Dernburg, 2005; MacQueen et al., 2005; Sato et al., 2009). It was unclear how the ZnF proteins associated with these sites contribute to their diverse activities, since these proteins have no obvious functional domains outside of their DNA-binding domains. Here we have demonstrated that these chromosome regions also drive large-scale reorganization of chromosomes during early meiosis by recruiting the serine/threonine kinase PLK-2. The discovery that HIM-8, and by extension the ZIM proteins, recruit PLK-2 helps to illuminate how PCs can act as a regulatory hub to coordinate the distinct mechanisms of pairing, synapsis, and the synapsis checkpoint.

We have shown that homolog pairing and synapsis require Polo-like kinase activity associated with PCs. Although PLK-2 is normally sufficient, in its absence, PLK-1 can promote incomplete homolog pairing and synapsis. When *plk-1* was depleted in *plk-2* mutants, homolog pairing was abrogated, and only limited stretches of SC were observed in late prophase, similar to observations in *chk-2* mutants. By contrast, when all PC function was eliminated by deletion of the four ZnF genes, promiscuous SC formation was observed, and our data indicate that this predominantly reflects fold-back synapsis. This is consistent with prior evidence that association of pairing centers with the nuclear envelope acts to inhibit aberrant SC formation between nonhomologous chromosome regions; this inhibition is apparently still effective in the absence of SUN-1 and ZYG-12 aggregates (*e.g.*, in *plk-2; plk-1(RNAi)* animals), but is lost when pairing centers are not associated with the NE. We speculate that in the absence of all PC ZnF proteins, PLK-2 is not appropriately sequestered in early prophase, and its precocious association with SC components promotes their assembly between nonhomologous regions, with intrachromosomal contacts being the most frequent initiation points for their loading and extension.

De novo chromosome attachment to the NE is a widely conserved feature of early meiosis. Given that several components involved in this attachment in *C. elegans*, including SUN-1, ZYG-12, and dynein, play distinct roles prior to meiotic entry, a key question has been how their meiotic activities are set in motion. The meiotic CHK-2 kinase was previously identified as an important regulator required for SUN-1 phosphorylation at Ser8 and Ser12, nuclear envelope reorganization, and homolog pairing (MacQueen and Villeneuve, 2001; Penkner et al., 2009; Sato et al., 2009). The identification of PLK-2 as a key player begs the question of how PLK-2 is conscripted at the onset of meiosis to effect this transition. PLKs are often targeted for particular functions by recruitment to interacting proteins, typically through phosphorylation of a binding partner by another kinase to create a Polo box binding motif (reviewed in Archambault and Glover, 2009). A scenario consistent with evidence presented here and in prior studies is that expression or activation of a meiosis-specific kinase creates PLK-2 binding sites by phosphorylation of the HIM-8/ZIM ZnF proteins. This concentrates PLK-2 at the inner NE, where it acts to modify the dynamic behavior of SUN-1 and to promote homolog pairing and synapsis. It is possible that CHK-2, which is

required for PLK-2 association with PCs, acts as a priming kinase. In support of this possibility, the putative PBD-binding motif in HIM-8 is part of a short sequence (PRF**STP**) that is highly conserved among HIM-8 and ZIM proteins in *C. elegans* and related species. This matches the human Chk2 consensus target motif RXXS/T (O'Neill et al., 2002). However, both human Chk2 and the yeast ortholog Rad53 have been shown to disfavor proline in the +1 position (O'Neill et al., 2002; Smolka et al., 2007), so a kinase other than CHK-2 may be required to prime PLK-2 recruitment.

We found that SUN-1 phosphorylation at Ser12 depends on recruitment of PLK-2 or PLK-1 to PCs. A phosphomimetic SUN-1 mutation (S12E) is sufficient to extend the transition zone (Penkner et al., 2009), and loss of this modification could account for many of the meiotic defects observed in *plk-2; plk-1*(RNAi) animals. However, this residue may not be a direct PLK-2 target and cannot be the only relevant target, since animals expressing only SUN-1 (S12A; S16A) retain some NE patch formation and chromosome clustering (Penkner et al., 2009). We also found that introduction of a transgene expressing SUN-1^{S12E} (Penkner et al., 2009) was insufficient to restore patch formation or homolog pairing in *plk-2; plk1(RNAi)* animals (data not shown), further indicating that other targets must be required.

The extended transition zone region observed in mutants with unsynapsed chromosomes has been enigmatic. It has been hypothesized to reflect either an active surveillance mechanism that delays the zygotene stage to allow more time for pairing and synapsis, or a more passive effect in which synapsis drives the chromosomes out of their clustered configuration (Colaiacovo, 2006). Our evidence that *plk-2* mutants have short regions of clustered chromosomes despite delayed synapsis supports the idea that an active mechanism maintains the transition zone until synapsis is completed. We also found that PLK-2 is required for the synapsis checkpoint, which results in apoptosis of nuclei with unsynapsed chromosomes. Because this apoptotic response, but not extension of the transition zone, depends on the presence of an unsynapsed PC, we had regarded it as a distinct phenomenon. However, the dependence of both transition zone extension and asynapsis-dependent apoptosis on PLK-2 strongly suggests that these are two responses to the same signal. Additional components, including unsynapsed PCs and *pch-2* (Bhalla and Dernburg, 2005), are only required for apoptosis, and are therefore “downstream” of this putative signal. We speculate that unsynapsed PCs may trigger apoptosis through persistent phosphorylation of SUN-1, which has been reported to interact with the apoptosis machinery (Tzur et al., 2006).

We have found that some meiotic activities of PLK-2 can be executed, albeit less effectively, by PLK-1, and that loss of PLK-2 exacerbates the germline mitotic defects resulting from loss of PLK-1. *plk-1* and *plk-2* are highly homologous and apparently arose through a recent gene duplication event, most likely within the *Caenorhabditis* lineage. These kinases can interact with several of the same proteins in Y2H assays, including MEX-5, MEX-6, and PQN-59 (Li et al., 2004; Nishi et al., 2008). Gene duplication and divergence leading to functional specialization is thought to be a frequent event in kinase evolution. A well-studied example from budding yeast is the role of the Cdk-related kinase Ime2, which shares many substrates with Cdk1 but functions only in meiosis, differentiating the meiotic division program from mitosis (Holt et al., 2007). The partial overlap between PLK-1 and PLK-2 activities in *C. elegans* may indicate that these kinases are at an early

stage of functional divergence. Future identification of direct targets may shed light on this specialization, and will help to reveal how PLK-2 regulates diverse aspects of chromosome dynamics throughout meiotic prophase.

Experimental Procedures

C. elegans mutations and strains

Unless otherwise stated, all animals were cultured under standard conditions at 20°C. The wild-type strain was N2 Bristol. Two deletion alleles of *plk-2* were generated by the Japanese National BioResource for the Nematode (*tm1395*) and the *C. elegans* Knockout Consortium (*ok1936*). Genomic sequencing indicated that *ok1936* removes bases 12–1043 relative to the start codon, with an insertion of the 16-base sequence TGCGAAATTAGGAAAA, resulting in a frame shift and early stop codon. *tm1395* was found by the Mitani lab to be a deletion of bases 1075 to 1434 relative to the start codon, which is predicted to result in exon skipping and deletion of 145 amino acids from the middle of the protein.

Single-copy insertions of wild-type *him-8* and *him-8*(T64A) were generated by MosSCI (Frokjaer-Jensen et al., 2008). The *him-8* genomic sequence, including 943 bp upstream and 615 bp downstream of the coding region, was inserted into pCFJ151 to generate a donor template (pREG52). Quikchange™ mutagenesis was performed to generate the T64A allele, which was verified by sequencing. EG4322 animals were injected with either wild-type or mutant donor template, pJL43.1, pGH8, pCFJ90, and pCFJ104. Homozygous insertions (*ieSi13* and *ieSi14*) were confirmed by PCR, crossed into *him-8(tm611)* and assayed for rescue of the Him phenotype and expression of HIM-8 by immunofluorescence.

The *ieDf2* deletion was constructed by MosDel (Frokjaer-Jensen et al., 2010). The *ttTi22866 Mos1* insertion strain was provided by the Ségalat lab. The presence of the expected *Mos1* element to the right of *him-8* was confirmed by PCR, and crossed to *unc-119(ed3)* to create strain CA870. A donor template was constructed by inserting a 2.6-kb genomic fragment immediately to the right of the *Mos1* insertion and a 3-kb genomic fragment immediately to the left of the *zim-1* coding region into pRL8, flanking the *C. briggsae unc-119*$^{+}$ rescuing fragment. This was coinjected into CA870 hermaphrodites with pJL43.1, pGH8, pCFJ90 and pCFJ104, as described (Frokjaer-Jensen et al., 2010). Non-Unc, mCherry-minus progeny were identified after several generations. Successful excision of the expected 20-kb genomic segment was verified by PCR, outcrossed 3 times to *unc-119(ed3)*, and maintained over *mIs11*. Loss of HIM-8 and ZIM protein expression in deletion homozygotes was verified by immunofluorescence.

plk-2(tm1395) and *(ok1936)* were outcrossed at least 6 times before analysis, and maintained as heterozygotes over the *hT2* balancer. All assays were performed on homozygous animals derived from heterozygous parents.

Feeding RNAi

RNAi targeting of *plk-1*, *plk-3* and *syp-2* was performed with clones from the Ahringer laboratory with L4440 empty RNAi vector as a negative control (Fraser et al., 2000).

Bacterial cultures were grown in LB+antibiotics overnight and concentrated 20-fold. 50 μL was spread on 60mm NGM plates with 1 mM IPTG and antibiotics. Double-stranded RNA production was induced overnight at 37°C. For *plk-1* and *plk-3* RNAi, L4 animals were placed on freshly prepared RNAi plates and transferred to new RNAi plates after several hours to minimize carryover of OP50 bacteria. Animals were dissected for cytological analysis after 45 hours on *plk-1* or *plk-3* RNAi plates. Efficacy of *plk-1* RNAi was confirmed by loss of PLK-1 immunofluorescence and one-cell embryonic arrest. For *syp-2* RNAi, *ieDf2/mIs11* adult hermaphrodites were plated on RNAi plates. After three days, F1 progeny at the L4 stage were transferred to freshly induced RNAi plates for 24 hours and dissected for cytological analysis.

Antibodies and cytological assays

PLK-2 antibodies were raised in rabbits or guinea pigs against a KLH-conjugated synthetic peptide containing the 16 N-terminal amino acids (Nishi et al., 2008, and this work), a region with minimal homology to PLK-1. Goat anti-SYP-1 antiserum was raised against a synthetic peptide containing the 22 C-terminal residues. Rabbit antibodies against REC-8, RAD-51, and COH-3 were generated by SDIX using Genomic Antibody Technology™ and are commercially available through Novus Biologicals. Polyclonal antibodies against the following antigens have been previously described: SUN-1(pSer8) and SUN-1(pSer12) (Penkner et al., 2009), HTP-3(MacQueen et al., 2005), HTP-1 (Martinez-Perez et al., 2008), HIM-8 (Phillips et al., 2005), ZIM-1, ZIM-2, and ZIM-3 (Phillips and Dernburg, 2006), PLK-1 (Budirahardja and Gonczy, 2008; Chase et al., 2000), and LAB-1 (de Carvalho et al., 2008).

Immunofluorescence was performed as previously described (Phillips et al., 2009a). Briefly, young adult hermaphrodites were dissected in egg buffer containing sodium azide and 0.1% Tween-20, fixed for 3 minutes in 1% formaldehyde in the same buffer between a Histobond slide and coverslip, and frozen on dry ice. The coverslip was removed and slides were transferred to ethanol or methanol chilled to −20°C. After 1 minute, slides were transferred to PBST (PBS containing 0.1% Tween-20), washed in 2 further changes of PBST, blocked with normal serum or Roche blocking agent, and stained with primary antibodies for at least 1 hr. Secondary antibodies labeled with Alexa 488, Cy3, or Cy5 were purchased from Invitrogen or Jackson Immunoresearch. Following immunostaining, slides were stained in 0.5 μg/ml DAPI, destained in PBST, and mounted in glycerol-based mounting medium containing n-propyl gallate.

Fluorescence in situ hybridization (FISH) procedures have also been previously described in detail (Phillips et al., 2009a). Probes used in this study included the 5S rDNA repeat (Dernburg et al., 1998), a short repeat associated with the right end of the X chromosome (Phillips et al., 2005), and a single-copy probes to the left end of chromosome V (MacQueen et al., 2005) and the left arm of the X chromosome (Phillips et al., 2005).

Apoptosis in mutant strains was quantified by introduction of the *bcIs39* ($P_{lim\text{-}7}$*::ced-1::gfp*) reporter construct (Schumacher et al., 2005). Live animals were mounted on agarose pads and evaluated using a compound objective. Only germline nuclei completely surrounded by green fluorescence were counted (Bhalla and Dernburg, 2005).

All images were acquired using a DeltaVision RT system (Applied Precision) equipped with a 100× N.A. 1.40 oil-immersion objective (Olympus), resulting in an effective XY pixel spacing of 0.067 or 0.045 μm. 3D image stacks were collected at 0.15 or 0.2-μm Z-spacing and processed by constrained, iterative deconvolution. Image scaling and analysis were performed using functions in the softWoRx software package. Projections were calculated by a maximum intensity algorithm. Composite images were assembled and some false colorizing was performed with Adobe Photoshop.

Yeast 2-hybrid analysis

Yeast 2-hybrid screening was performed using the ProQuest™ Two-Hybrid System (Invitrogen). A poly-T-primed cDNA library was constructed from young adult hermaphrodites and cloned into pDEST22. The full-length PLK-2 coding sequence was amplified by RT-PCR, sequence-verified, and cloned into pDEST32. A C-terminal fragment containing amino acids 280–632, which spans the Polo box domain but omits the kinase domain, was cloned into the same bait vector. Both baits retrieved numerous cDNA clones (Supplementary Information, Tables S2A and S2B), which were then verified by pairwise interaction tests using *MEL1, HIS3, and LacZ* reporters.

Full-length coding sequences of HIM-8 and ZIM-3 were amplified from a cDNA library, sequence-verified, and cloned into pDEST22. The resulting HIM-8 prey vector was used as a template for site-directed mutagenesis to generate the T64A and S85F mutations. Expression of the mutant proteins was verified by Western blotting. Various fragments of HIM-8 coding sequence (Figure 4B) were also cloned into the same prey vector.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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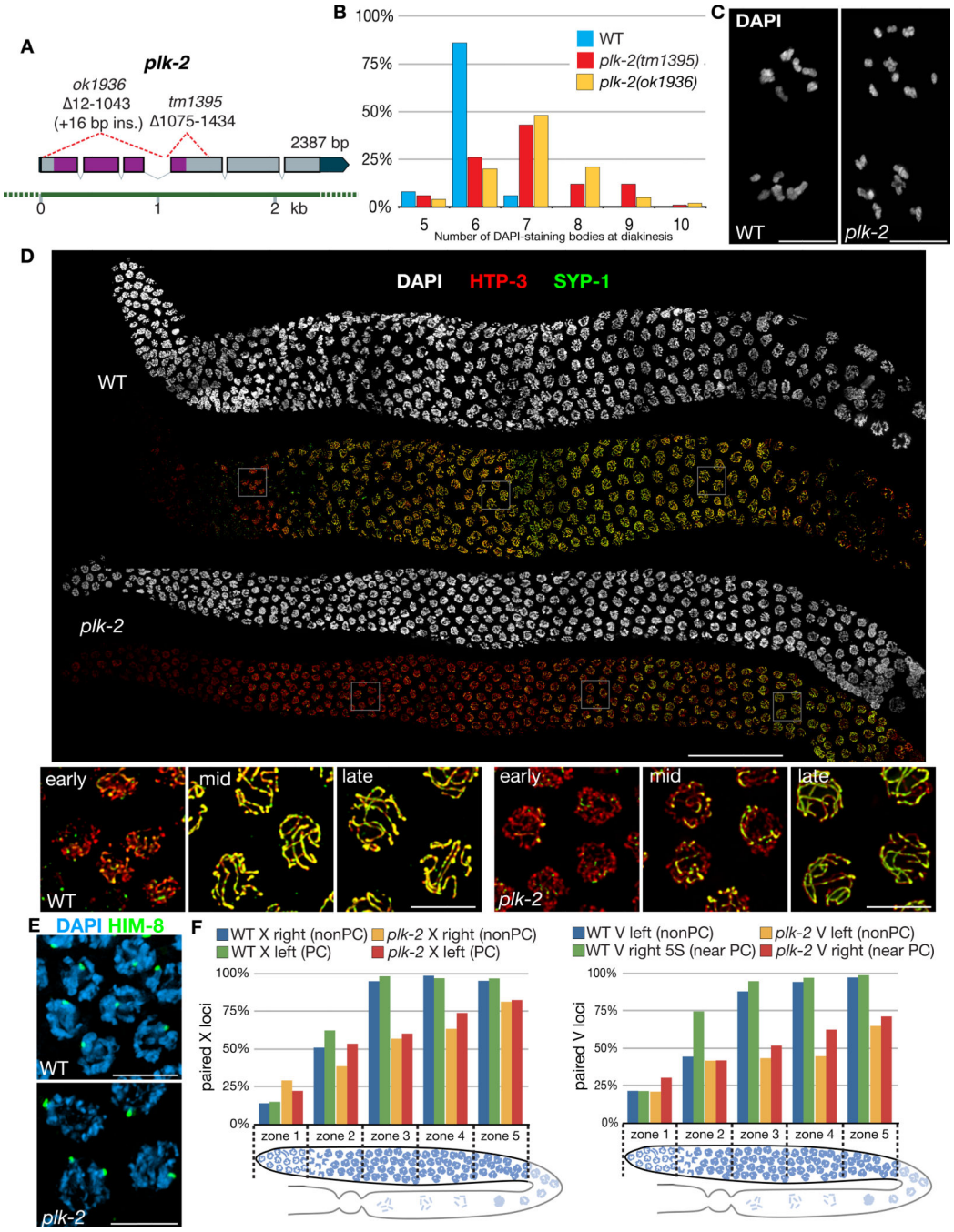

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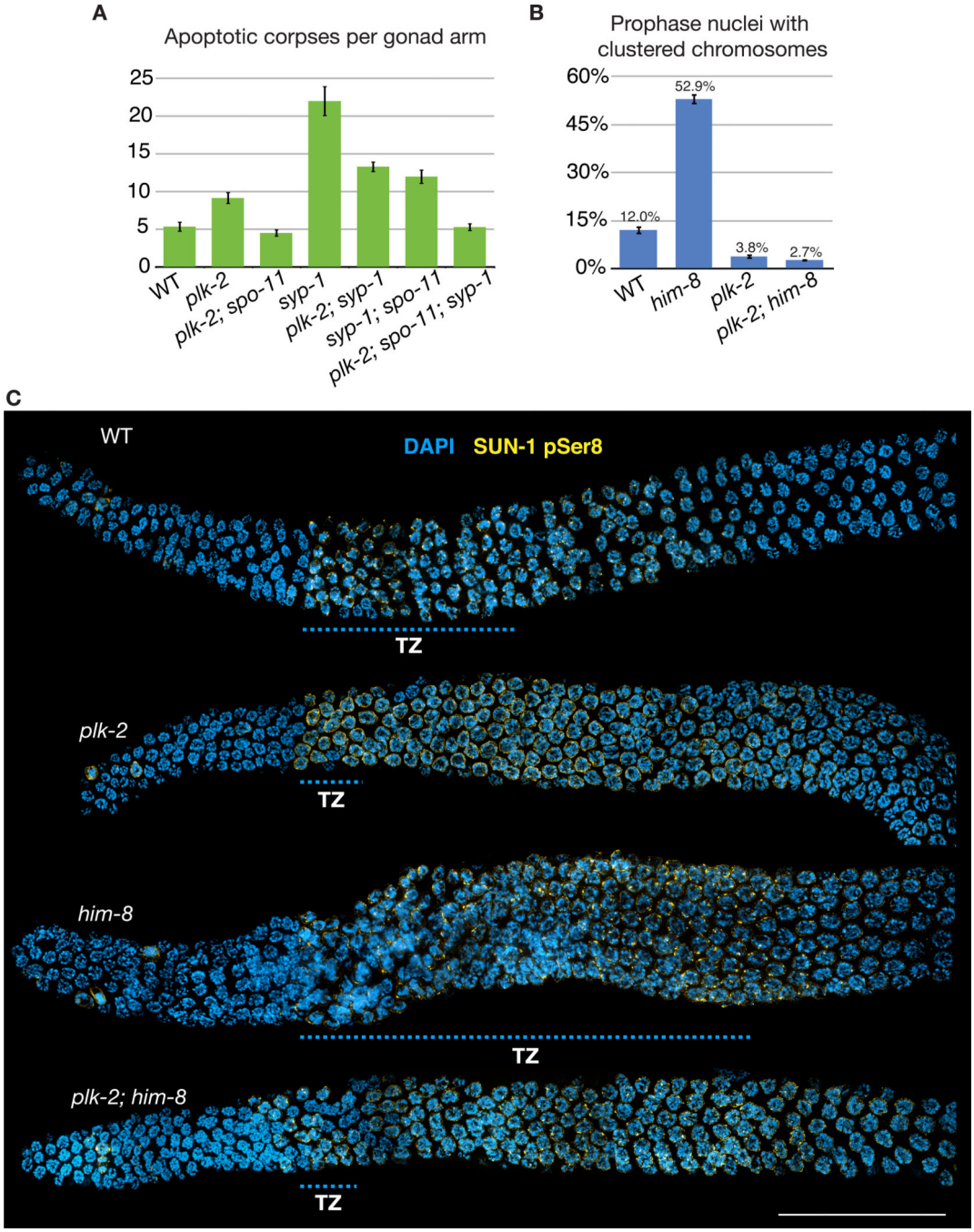
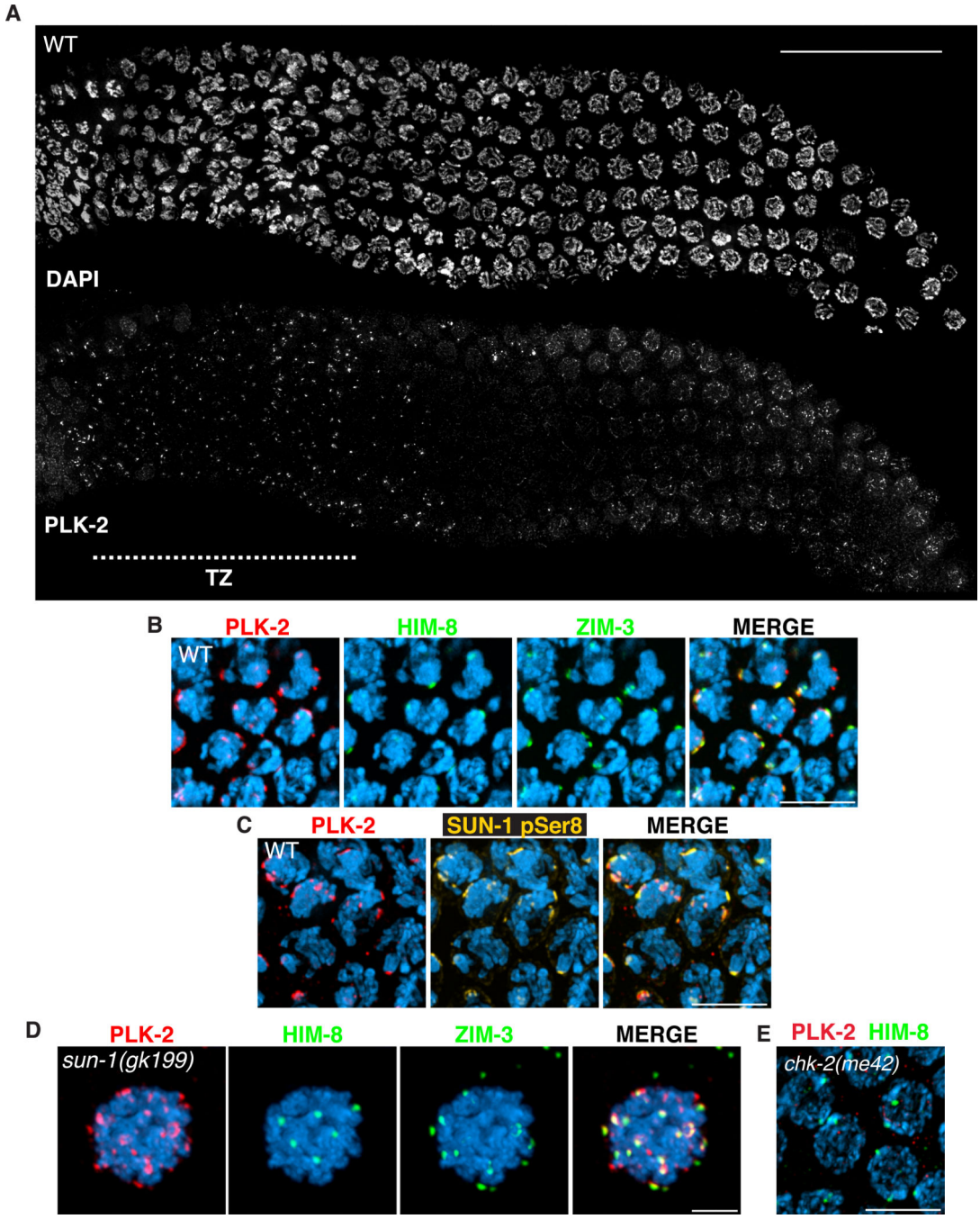


Figure 2.



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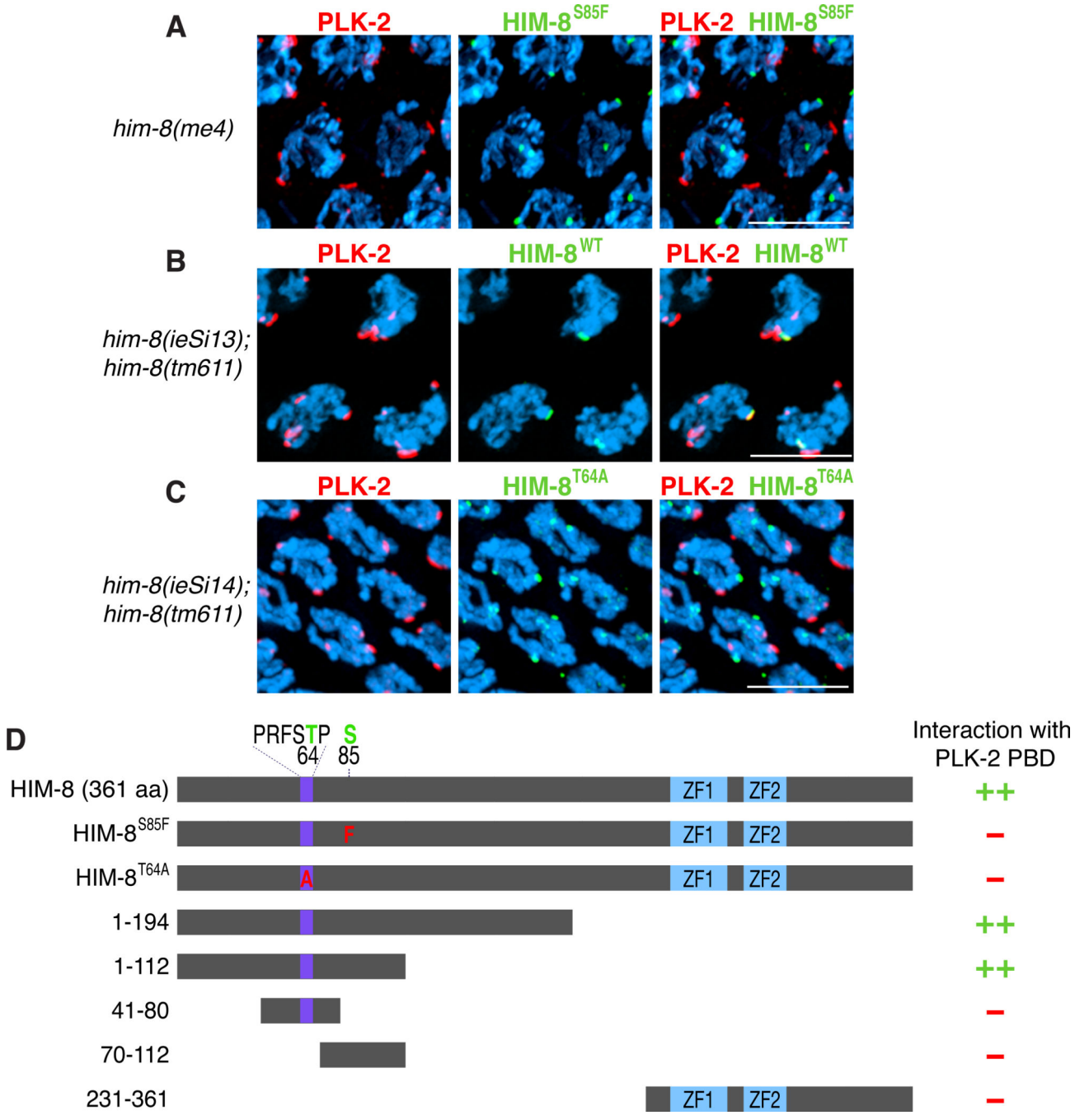

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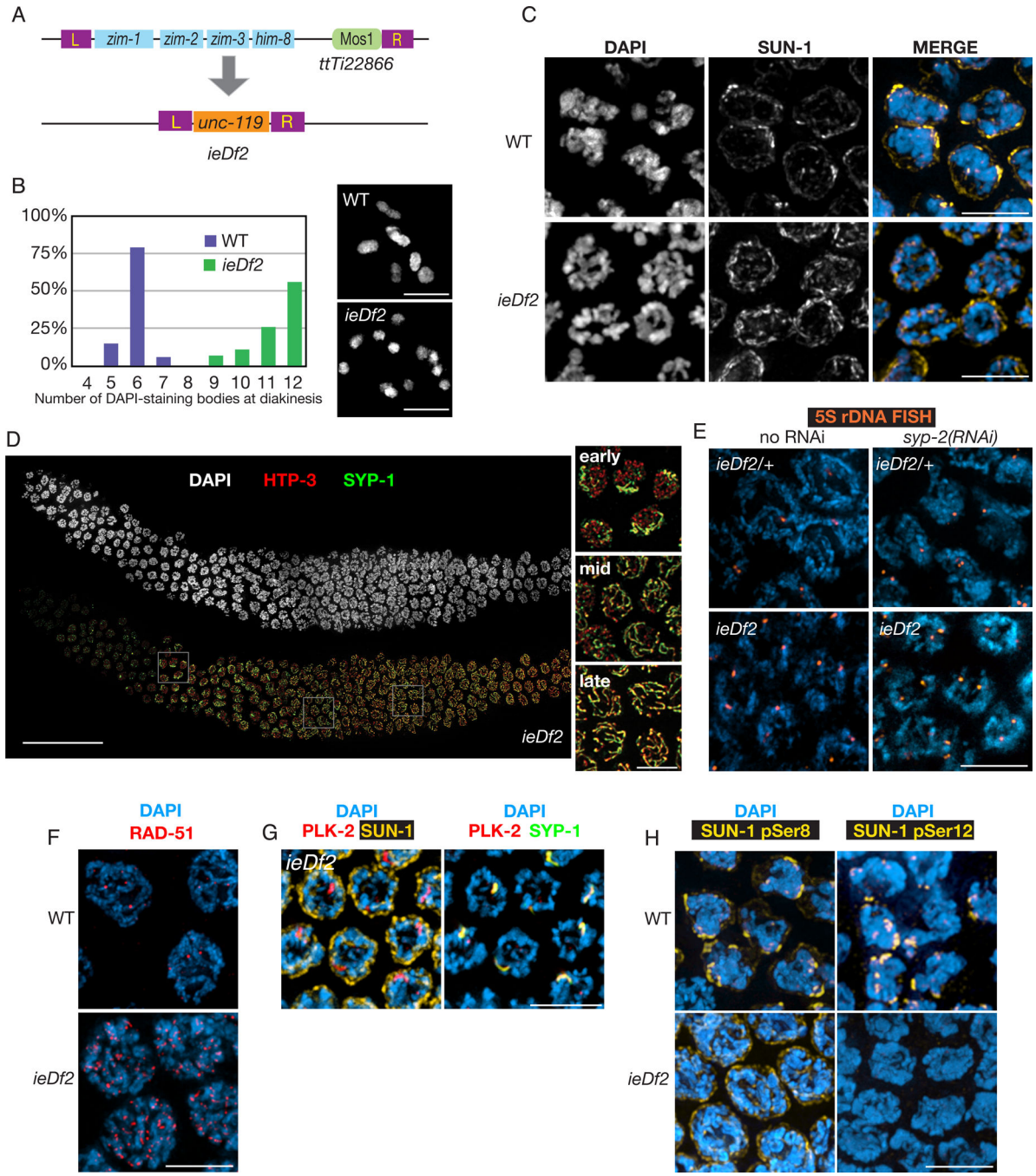

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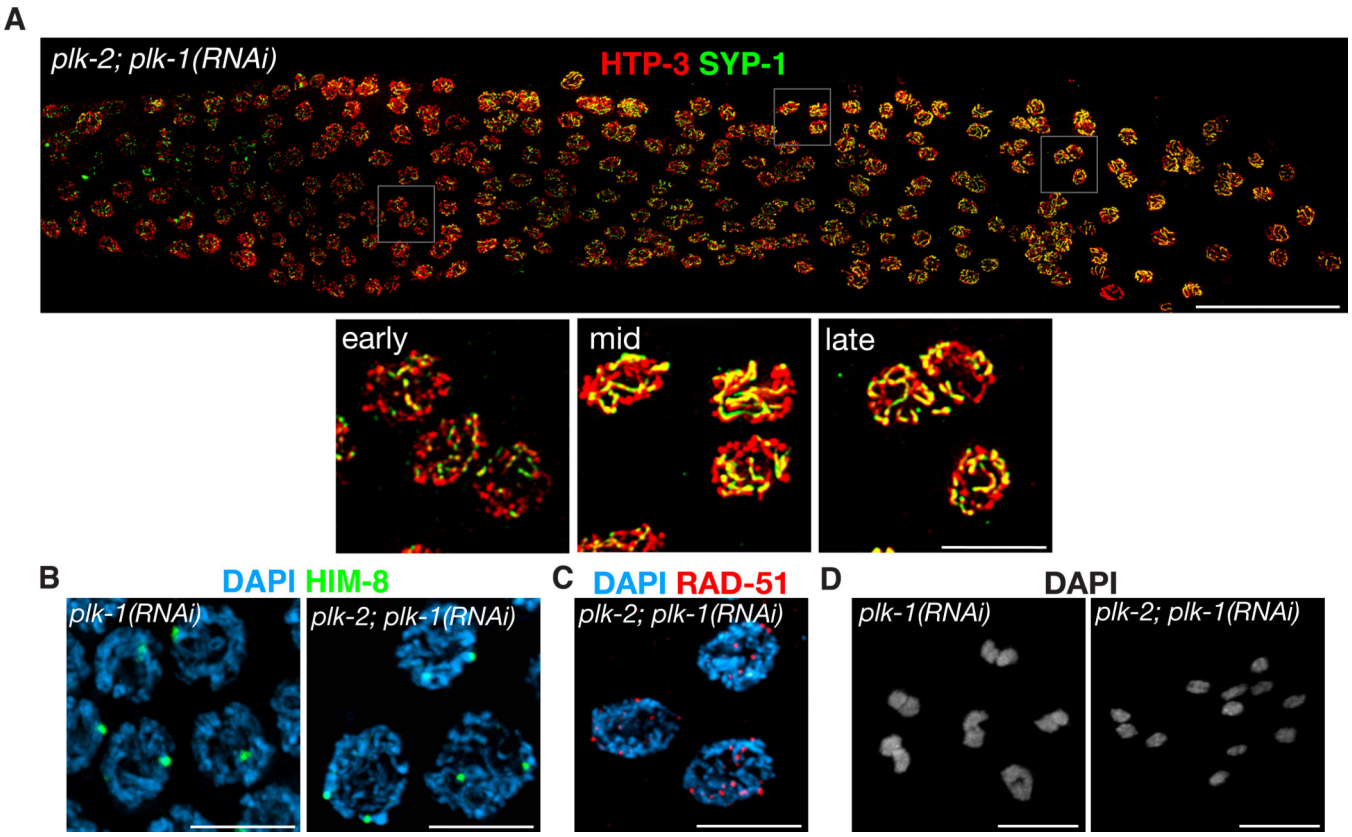

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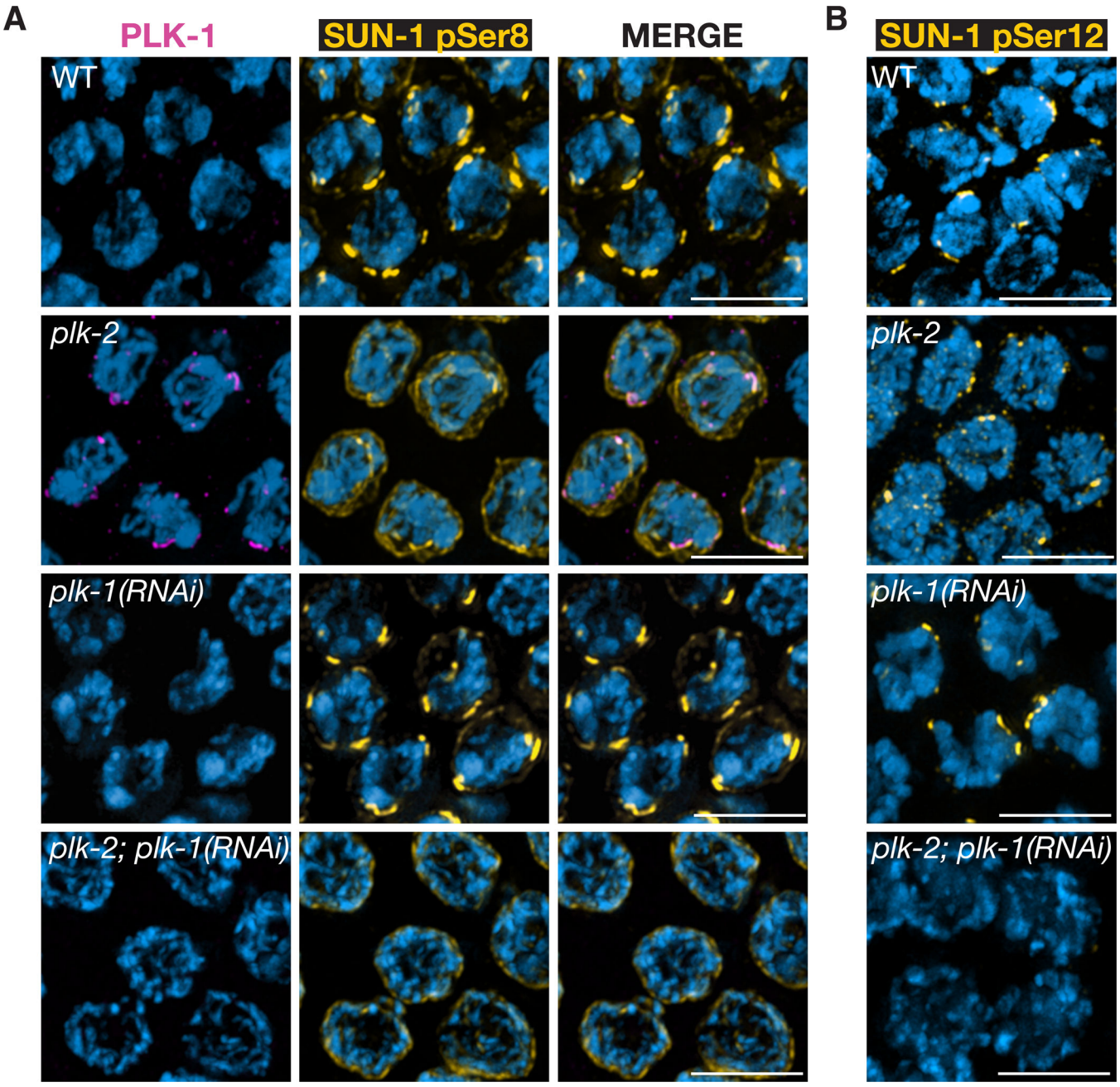

Figure 7.