Crossover formation is essential for proper segregation of homologous chromosomes during meiosis. Here, we show that *Caenorhabditis elegans* cyclin-dependent kinase 2 (CDK-2) partners with cyclin-like protein COSA-1 to promote crossover formation by promoting conversion of meiotic double-strand breaks into crossover–specific recombination intermediates. Further, we identify MutSγ component MSH-5 as a CDK-2 phosphorylation target. MSH-5 has a disordered C-terminal tail that contains 13 potential CDK phosphosites and is required to concentrate crossover–promoting proteins at recombination sites. Phosphorylation of the MSH-5 tail appears dispensable in a wild-type background, but when MutSγ activity is partially compromised, crossover formation and retention of COSA-1 at recombination sites are exquisitely sensitive to phosphosite loss. Our data support a model in which robustness of crossover designation reflects a positive feedback mechanism involving CDK-2–mediated phosphorylation and scaffold-like properties of the MSH5 C-terminal tail, features that combine to promote full recruitment and activity of crossover–promoting complexes.

**Significance**

Successful chromosome segregation during meiosis relies on crossover recombination between homologous chromosomes. Meiotic recombination initiates with the formation of numerous DNA double-strand breaks, but only a few are ultimately selected to become crossovers. How this process is regulated to ensure that each homolog pair designates at least one crossover remains poorly understood. Here, we show that *Caenorhabditis elegans* kinase CDK-2 partners with cyclin-like protein COSA-1 and promotes crossover designation through phosphorylation and activation of the MutSγ complex. Our data support a model in which scaffold-like properties of the MSH-5 C-terminal tail and its CDK-2–mediated phosphorylation combine to promote full recruitment and activity of crossover–promoting complexes, thereby generating positive feedback that contributes to the robustness of crossover designation.

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The meiosis-specific MutS homologs MSH4 and MSH5 form a heterodimeric MutSγ complex and play essential roles in crossover formation in diverse eukaryotes (24–31). MutSγ localizes to recombination intermediates as numerous foci but ultimately accumulates at sites that are destined to become crossovers (32, 33). Biochemical analyses using recombinant MSH4 and MSH5 have shown that MutSγ recognizes single-end invasion intermediates and HJs in vitro (34, 35). HJs activate the ATP hydrolysis of MutSγ and promote the exchange of bound ADP for ATP, inducing the formation of a sliding clamp that dissociates from HJs (35, 36). By iterative loading and embracing DNA duplexes within a dHJ, MutSγ is thought to stabilize crossover–specific recombination intermediates (33, 35). In addition, MutSγ recruits and activates the resolve activity of MutLγ, enabling biased processing of dHJs into crossovers during meiosis (37, 38).

A genetic screen in *C. elegans* identified a cyclin-like protein COSA-1 as a component essential for processing meiotic DSBs into crossovers (39). The mammalian ortholog CNTD1 was subsequently identified (40), and both COSA-1 and CNTD1 have been shown to localize to crossover sites (39, 41, 42). In the absence of COSA-1/CNTD1, MutSγ components persist as numerous foci in pachytene, and crossover formation is eliminated or severely compromised (33, 40), demonstrating a crucial role of COSA-1/CNTD1 in converting early recombination intermediates into crossovers. Because both COSA-1 and CNTD1 are members of the cyclin family, it is plausible that they form a complex with a cyclin-dependent kinase (CDK) and regulate the recombination process through phosphorylation.

Several lines of evidence have suggested that CDK2 might be a relevant kinase partner for CNTD1. CDK2 interacts with CNTD1 in yeast-two hybrid assays (41, 42) and localizes to interstitial chromosome sites (43, 44) in a CNTD1-dependent manner (40). Reduced CDK2 activity leads to a failure in crossover formation, while a hyperactive form of CDK2 causes an increased number of MLH1 foci (45). However, due to its requirement at telomeres in tethering chromosomes to the nuclear envelope, deletion of CDK2 leads to severe defects in SC assembly between paired homologs (synapsis) and pachytene arrest (46–49). Further, while a full-length CNTD1–specific protein of the expected size was detected (using CNTD1 antibodies) in one study (42), a short CNTD1 isoform that cannot interact with CDK2 was the predominant isoform detected in another study (using hemagglutinin antibodies in *C. elegans* Cntd1(1027/1028) mice with an epitope tag sequence inserted into the endogenous Cntd1 locus (41), raising questions regarding the extent to which CDK2 and CNTD1 might act as functional partners. Thus, it has been difficult to determine the role of CDK2 in crossover recombination. Moreover, key meiotic targets of CDK2 have not yet been identified.

We reasoned that CDK-2, the *C. elegans* homolog of CDK2, might also localize and function at crossover sites. However, global knockdown of *C. elegans* CDK-2 by RNA interference leads to cell cycle arrest of mitotically proliferating germ cells (50), thereby precluding the analysis of its requirement during meiotic prophase. To overcome this limitation and establish the meiotic function of CDK-2, we use the auxin-inducible degradation system to deplete CDK-2 from the adult germline, demonstrating that CDK-2 partners with COSA-1 to promote crossover formation during *C. elegans* meiosis. Moreover, we identify MSH-5 as a key substrate for CDK-2 and provide evidence that CDK-2 and COSA-1 partner to promote crossover designation through phosphorylation and activation of the MutSγ complex.

**Results**

**CDK-2 Colocalizes with COSA-1 Both at Early Recombination Intermediates and at Crossover Sites in Pachytene.** To establish the localization and function of CDK-2 during meiosis, we used CRISPR-mediated genome editing to modify the endogenous *cdk-2* locus to express CDK-2 fused to an auxin-inducible degron (AID) and three tandem Flag epitopes (3XFlag). Self-progeny of this worm strain are fully viable (100% egg viability), indicating that the AID tag does not interfere with essential CDK-2 functions. Immunofluorescence in whole-mount adult hermaphrodite germlines (XX) revealed that CDK-2 localizes to six distinct foci per nucleus in late pachytene, which correspond to crossover–designated recombination sites as marked by COSA-1 (Fig. 1A and SI Appendix, Fig. S1A). In male germlines (XO), CDK-2 appears as five foci in pachytene nuclei (SI Appendix, Fig. S1B), consistent with its localization to crossover sites on the five autosomes.

We used nuclear spreading and three-dimensional structured illumination microscopy (3D-SIM) to examine the localization of CDK-2 in relation to an axis component, HTP-3, and other crossover factors during meiotic progression. This cytological approach shows COSA-1 localizing to numerous early recombination intermediates as faint foci prior to transition to late pachytene (32). We likewise detected 7 to 17 CDK-2 foci in early pachytene nuclei, colocalizing with COSA-1 (Fig. 1B and D). Upon transition to late pachytene, CDK-2 and COSA-1 are lost from most recombination sites and enriched together at six crossover–designated sites (Fig. 1C and D). Recent evidence has further shown that a distinct substructure emerges at the crossover site, in which MSH-5 doublets appear orthogonal to chromosome axes, flanking a central COSA-1 focus (33). CDK-2 was similarly detected as a single focus positioned between two MSH-5 foci at the crossover site (Fig. 1E). Thus, CDK-2 colocalizes with COSA-1 at early recombination intermediates as well as at crossover–designated sites in both hermaphrodite and male germlines.

**CDK-2 Is Required for Crossover Formation.** To assess CDK-2 function during meiosis, we generated a strain in which *cdk-2::AID::3×Flag* is expressed in conjunction with germline-expressed plant F-box protein TIR1, which forms an SCF (Skp1-Cul1-F-box) E3 ubiquitin ligase to target AID-tagged proteins for degradation in the presence of auxin (51) (Fig. 2A). Within 6 h of 1 mM auxin treatment, CDK-2 was no longer detected in pachytene nuclei by immunofluorescence, demonstrating its rapid and efficient degradation (Fig. 2B). The signal for COSA-1 was also completely lost in CDK-2–depleted germlines, indicating that COSA-1 localizes to recombination sites in a CDK-2–dependent manner. Likewise, CDK-2 was not detected in pachytene nuclei of animals homozygous for a null mutation of *cosa-1* (SI Appendix, Fig. S2A), demonstrating their mutual dependence.

Depletion of CDK-2 resulted in loss of crossover–based connections (chiasmata) that maintain associations between homologs in oocytes at diakinesis, the last stage of meiotic prophase. Six DAPI-stained bodies corresponding to six pairs of homologs connected by chiasmata (bivalents) are observed in wild-type diakinesis oocytes. However, following 24-h auxin treatment, CDK-2–depleted oocytes displayed 8 to 12 DAPI-stained bodies, reflecting failure to form crossovers (Fig. 2C and D). Further, the RING domain–containing protein ZHP-3, which normally becomes restricted to six crossover sites in late pachytene nuclei (12), failed to become restricted to foci in CDK-2–depleted...
gonads and instead persisted along the SC (Fig. 2E), reflecting a requirement for CDK-2 in crossover formation.

Consistent with previous studies implicating *C. elegans* CDK-2 in the mitosis-to-meiosis decision and in promoting the proliferative fate of germline stem cells (50, 52), depletion of CDK-2 by 24-h auxin treatment dramatically reduced the number of germ cells in the premeiotic region of the gonad (Fig. 3B and SI Appendix, Fig. S2B and C). However, these CDK-2–depleted gonads exhibited normal pairing of HIM-8, a protein that binds a special region on X chromosomes known as the pairing center (53), and robust synapsis (SI Appendix, Fig. S2D and E). The sole RecA recombinase in *C. elegans*, RAD-51 (54, 55), was also detected as numerous foci in both control and CDK-2–depleted gonads (SI Appendix, Fig. S2F and G). Thus, meiotic DSBs are induced in CDK-2–depleted germlines but cannot be processed into crossovers in the absence of CDK-2.

**CDK-2 Is Required for Formation or Stabilization of Crossover–Specific Recombination Intermediates.** Next, we used a partial nuclear spreading protocol that maintains the temporal and spatial organization of the gonad (33, 56) to visualize the effects of CDK-2 depletion on the progression and architecture of meiotic recombination sites. To this end, we tagged MSH-5 at its C terminus with the 14-amino acid (aa)–long V5 epitope in a strain expressing CDK-2::AID::3×Flag and GFP::COSA-1. (Scale bar, 5 μm.) Inset, CDK-2 and COSA-1 colocalizing in six bright foci in nuclei following transition to late pachytene. (Scale bar, 2 μm.) (B) Full projections of SIM images of spread gonads showing the staining for HTP-3 (white), CDK-2 (red), and COSA-1 (green) from early pachytene (B) and late pachytene nuclei (C). (Scale bars, 2 μm.) (D) Quantification of CDK-2 foci in pachytene nuclei and their colocalization with COSA-1. Each diamond represents a nucleus: the gray line indicates perfect colocalization. (E) Representative SIM images of individual crossover–designated sites showing CDK-2 singlet foci localizing together with MSH-5 doublets. (Scale bar, 500 nm.)
phosphorylation of pairing center proteins (pHIM-8/ZIMs) was extended (Fig. 3 A and B and SI Appendix, Fig. S3A), reflecting delayed meiotic progression due to failure to form crossover intermediates (57–59). Further, although CDK-2–depleted germ cells did eventually lose the DSB-2 signal and transition into the late pachytene stage, multiple faint MSH-5 foci persisted along chromosome axes (Fig. 3 B), implying a failure of crossover designation in the absence of CDK-2. We note that abnormally large puncta of MSH-5 (white arrowheads) were detected in late pachytene nuclei of CDK-2–depleted germ cells (Fig. 3 C). Examination of spread nuclei using 3D-SIM further revealed a failure to establish normal crossover site architecture in CDK-2–depleted germlines. Recent work has shown that crossover–designated sites display a distinct spatial organization of recombination factors (33, 60). Specifically, cohorts of MSH-5 and the Bloom helicase HIM-6 are each detected as orthogonally localized doublets. This orientation is interpreted to reflect their association with different parts of an underlying dHJ, with COSA-1 localizing at the center of the cross formed by the HIM-6 and MSH-5 doublets (33). Whereas this organization was detected in late pachytene in controls, MSH-5 and the Bloom helicase HIM-6 were each detected as orthogonally localized doublets (33). While SC bubbles surrounding MSH-5 foci were detected in controls, such structures were not found in CDK-2–depleted germlines (Fig. 3 E). Taken together, our data

![Diagram](https://doi.org/10.1073/pnas.2117865119)
Fig. 3. CDK-2 is required for stabilizing crossover–specific recombination intermediates. (A) Animals expressing CDK-2:AID:3xFlag and TIR1::mRuby were treated with or without 1 mM auxin for 24 h after L4. Dissected gonads were spread and stained for DSB-2 (blue), MSH-5 (green), and HTP-3 (red). (Scale bar, 5 μm.) (B) Top, diagram illustrating the effect of CDK-2 depletion on meiotic progression. The DSB-2–positive nuclei (shown in blue) represent nuclei in early pachytene. Bottom, representative SIM images of nuclei from the indicated regions (1, 2, or 3) of spread gonads from control and CDK-2–depleted worms as indicated in the diagram above. White arrowheads in 3 indicate large MSH-5 aggregates. (Scale bar, 3 μm.) (C) Representative fluorescent images of recombination sites in late pachytene from control versus CDK-2–depleted germline. Stainings for MSH-5, HIM-6, and COSA-1 are shown. (Scale bar, 400 nm.) A schematic depicting the hypothesized architecture of recombination factors at the crossover–designated site is shown on the right. (D) Line scan profiles of MSH-5 (red) and COSA-1 signals (green) at recombination sites in late pachytene nuclei from control (n = 26) and CDK-2–depleted germlines (n = 26). Thin lines are individual traces, and thick lines are averages; a.u., arbitrary units. (E) Representative SIM images of spread gonads from region 2 in the diagram above and a segment of SC stretch from control and CDK-2–depleted animals. MSH-5 (green) and SYP-2 (red) stainings are shown. The yellow circle and arrowhead in the control indicate the SC bubble at the crossover site. (Scale bar, 2 μm.)
support that CDK-2 is required for maturation of early recombination sites into crossover–specific recombination intermediates.

**Phosphorylation of MSH-5 Depends on Both CDK-2 and COSA-1 In Vivo.** Given the requirement for CDK-2 in crossover designation, we hypothesized that CDK-2 might phosphorylate pro–crossover factors, such as the MutSγ complex and the ZHPI proteins, to modulate their functions. Moreover, given the colocalization and functional interdependence of CDK-2 and COSA-1, we hypothesized that COSA-1 might partner with CDK-2 to promote target phosphorylation. Experiments in which we coexpressed recombinant CDK-2–6xHis and glutathione S-transferase–COSA-1 in insect cells provided evidence consistent with CDK-2 and COSA-1 being able to form a complex in vitro (SI Appendix, Fig. S4 A and B); however, we did not pursue this in vitro approach further as both proteins were largely insoluble. Instead, we sought to identify a relevant in vivo phosphorylation target.

We focused on MSH-5, as it contains numerous CDK consensus motifs ([S/T]P) in an extended and highly disordered C-terminal tail (Fig. 4A) and was previously demonstrated to be phosphorylated by human CDK1 in vitro (39). Using mass spectrometry, we mapped three sites on the MSH-5 C-terminal tail (T1009, T1109, and S1278) that are phosphorylated by human CDK1 in vitro (SI Appendix). We successfully generated phopho-specific antibodies against only one of these sites (MSH-5 pT1009), and we used these antibodies to demonstrate that MSH-5 is indeed phosphorylated in vivo in a CDK-2–and COSA-1–dependent manner. Immunofluorescence on spread nuclei revealed that the MSH-5 pT1009 signal was found at numerous recombination sites in early pachytene and became enriched at crossover–designated sites in late pachytene, colocalizing with MSH-5 and COSA-1 throughout meiotic progression (SI Appendix, Fig. S4C and Fig. 4B). Further, this phospho–MSH-5 signal is lost in worms expressing a mutant version of MSH-5 that includes a T1009A substitution (SI Appendix, Fig. S4D). Importantly, the MSH-5 pT1009 signal was abolished from recombination sites in both CDK-2–depleted and cosa-1–mutant gonads (Fig. 4B and C), indicating that the MSH-5 C-terminal tail is phosphorylated in vivo in a manner dependent on both CDK-2 and COSA-1.

**The C-Terminal Tail of MSH-5 Is Essential for Accumulation of Pro–Crossover Factors at Recombination Sites.** Whereas the N-terminal 60% of the MSH-5 protein shows a high level of conservation with its orthologs throughout the eukaryotic kingdoms, the long C-terminal tail is unique to its Caenorhabditis orthologs (SI Appendix, Fig. S5A). Moreover, primary sequence conservation within this tail domain is very low among the Caenorhabditis MSH-5 orthologs, which are similar primarily in that they contain multiple (8–23) CDK consensus motifs embedded within a protein segment predicted to be highly disordered (SI Appendix, Fig. S5B). These features of the MSH-5 C-terminal tail suggest that the presence of the disordered tail and/or its ability to serve as a substrate for CDK-2 might contribute to the essential functions of MSH-5 in meiosis.

To address this, we used CRISPR to generate worm strains expressing a series of MSH-5 C-terminal truncations by inserting V5 coding sequences followed by premature stop codons (Fig. 5A). Western blotting analysis showed that all four truncated proteins (∆178 aa, ∆270 aa, ∆339 aa, and ∆414 aa) are expressed at their expected sizes, albeit at modestly reduced (50

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**Fig. 4.** CDK-2 is responsible for MSH-5 phosphorylation within its C-terminal tail. (A) Schematic showing the domain structure and putative CDK phosphorylation sites in MSH-5 (adapted from the SMART database). Low-complexity regions are shown in magenta, and in vitro phosphorylation sites mapped by mass spectrometry analysis are indicated in red. An asterisk indicates the MSH-5 pT1009 epitope. The IUPred disorder score profile for MSH-5 is shown below the schematic. (B and C) Immunofluorescence images of spread pachytene nuclei from control (no auxin), CDK-2–depleted (1 mM auxin treatment for 24 h), and cosa-1(tm3298) germlines. (C) Nonspecific signals from the MSH-5 pT1009 antibody that do not colocalize with MSH-5 were occasionally detected at the nuclear periphery. Staining for HTP-3 (white), MSH-5 (green), and MSH-5 pT1009 (red) is shown. (Scale bars, 5 μm.)
to 70%) levels (SI Appendix, Fig. S6A and B). Strikingly, truncations of MSH-5 led to a marked reduction in egg viability, and the percentage of males among surviving progeny also increased, reflecting meiotic impairment (SI Appendix, Fig. S6C). In particular, animals harboring two large truncations (msh-5::V5539AA and msh-5::V5344AA) displayed an average of ~11 DAPI bodies in diakinesis oocytes (Fig. 5B and C and SI Appendix, Fig. S6D) and only zero to one bright COSA-1 foci in late pachytene nuclei (Fig. 5D and SI Appendix, Fig. S6E). As msh-5 is not haploinsufficient (28), these data indicate that the C-terminal tail of MSH-5 is crucial for its function in crossover formation. Immunofluorescence of spread gonads from msh-5::V5339AA mutants revealed that COSA-1 was diffusely present in the nucleoplasm (Fig. 5E and SI Appendix, Fig. S6F). Additionally, after spreading, dim COSA-1 foci were detected at up to six sites along chromosome axes, which colocalized with ZHP-3 and the truncated MSH-5 (Fig. 5E and F and SI Appendix, Fig. S6F). However, ZHP-3 signal persisted along the SC in msh-5::V5339AA mutants (Fig. 5F), and most recombination intermediates failed to mature into crossovers. Thus, the C-terminal tail of MSH-5 is essential for accumulation of pro–crossover factors at COSA-1–marked recombination sites.

**Phosphosites within the MSH-5 C-Terminal Tail Contribute to the Pro–Crossover Activity of the MutSγ Complex.** To determine the significance of phosphorylation of the MSH-5 tail, we sequentially mutated the codons corresponding to the 13 predicted and/or mapped CDK phosphorylation sites in its C terminus using CRISPR (Fig. 6A and SI Appendix, Fig. S7A). Contrary to our initial expectations based on the truncation mutants, animals homozygous for msh-5 phosphomutations (4A, 11A, and 13A) did not show obvious phenotypes in meiosis, exhibiting nearly normal progeny viability (SI Appendix, Fig. S7B). Even the msh-5::V513A mutant, in which all 13 CDK consensus sites were mutated to alanines, was able to designate crossovers and form bivalents as in wild type (SI Appendix, Fig. S7C–E and Fig. 6B).

As the conserved presence of multiple CDK phosphorylation sites suggests a functional importance, we hypothesized that preventing phosphorylation of the MSH-5 C terminus did not cause overt phenotypes because phosphorylation is only one of multiple parallel pathways that converge to ensure a robust outcome of meiosis. Thus, we used a temperature-sensitive allele of him-14(it44); msh-5::V5WT in the MutSγ complex, as a sensitized genetic background to weaken the signal of a functional deficit for msh-5 phosphomutants. him-14(it44) harbors a missense mutation (D406N) within its conserved DNA-binding domain and is characterized by a temperature-sensitive reduction in crossover formation (24, 61). Analysis of phosphosite mutations in this sensitized context provided clear evidence that phosphorylation of the MSH-5 tail functions to augment the pro–crossover activity of MutSγ in vivo.

First, we found that the msh-5::V513A mutation showed strong synthetic phenotypes with him-14(it44) at 15 °C, a temperature that is normally permissive for him-14(it44) (Fig. 6C and D). Whereas DAPI staining of diakinesis oocytes did not reveal meiotic defects in him-14(it44); msh-5::V5WT control animals at 15 °C, 12 univalents were consistently observed in him-14(it44); msh-5::V513A oocytes, indicating a failure of crossover formation. Further, nuclear spreading and immunofluorescence revealed that while wild-type MSH-5 pared down to six bright COSA-1 foci at crossover–designated sites in him-14(it44) at 15 °C, the MSH-513A protein persisted as numerous foci throughout pachytene in the him-14(it44); msh-5::V513A double mutant (Fig. 6B), similar to the phenotypes observed in CDK-2–depleted germlines (Fig. 3). Moreover, in him-14(it44); msh-5::V513A animals, one to three COSA-1 foci initially associated with MSH-5, but this colocalization was lost in late pachytene (only 33% of COSA-1 signals colocalized with MSH-5 in him-14(it44); msh-5::V513A versus 98% in him-14(it44); msh-5::V5WT; Fig. 6E and SI Appendix, Fig. S8A). Interestingly, ZHP-3 was detected together with COSA-1 in late pachytene nuclei of him-14(it44); msh-5::V513A (SI Appendix, Fig. S8B), suggesting that phosphorylation of the MSH-5 C terminus is required for retaining the association of MutSγ with other pro–crossover factors in him-14(it44) animals.

Second, analysis of him-14(it44)–double mutant animals carrying phospho-null mutations at 1, 7, or 11 phosphosites (1A, 7A, and 11A) further revealed that multiple phosphorylation sites in the MSH-5 tail can contribute to promoting MutSγ activity (Fig. 6A and D and SI Appendix, Fig. S8C). The him-14(it44); msh-5::V511A mutant was particularly informative, as analysis of diakinesis oocytes revealed only mild meiotic impairment at 15 °C, in striking contrast to the complete failure of crossover formation observed in him-14(it44); msh-5::V513A oocytes under the same conditions. This suggests that phosphorylation of as few as two sites within the MSH-5 tail can sustain sufficient pro–crossover activity of the partially compromised MutSγ complexes. However, severe impairment of crossover formation is observed in both him-14(it44); msh-5::V511A and him-14(it44); msh-5::V513A when MutSγ function is further compromised at the semipermissive temperature of 20 °C (Fig. 6D, Right). This suggests that phosphorylation at additional sites can contribute to augmentation of MutSγ activity.

**MSH-5 Phosphosites Are Required for Suppression of the him-14(it44) Crossover Deficit by Elevated COSA-1 Levels.** The mels8 transgene expressing green fluorescent protein (GFP): COSA-1 can partially alleviate the crossover deficit observed at semipermissive temperatures in the him-14(it44) mutant (62). Using qRT-PCR, we determined that cosα-1 mRNA levels are up-regulated by twofold in mels8 worms compared to wild-type controls (SI Appendix, Fig. S8D). Thus, we hypothesized that this suppression might reflect elevated activity of a putative CDK-2/COSA-1 complex, which compensates for the impaired MutSγ activity in him-14(it44) through hyperphosphorylation of downstream targets, including MSH-5. To test whether suppression of him-14(it44) by mels8 was dependent on phosphosites in the MSH-5 C-terminal tail, we compared the effect of mels8 in him-14(it44); msh-5::V511A and him-14(it44); msh-5::V513A genetic backgrounds, reasoning that reducing the number of available phosphosites might abrogate suppression. Experiments were conducted at 22 °C, a semipermissive temperature at which the suppression of him-14(it44) by mels8 is evident. At 22 °C, him-14(it44); msh-5::V5WT diakinesis oocytes displayed an average of 9.8 DAPI bodies, reflecting a mixture of bivalents and univalents resulting from a partial impairment of crossover formation, but the number of DAPI bodies was reduced to 8.5 in the presence of the mels8 transgene (Fig. 6F and SI Appendix, Fig. S8E), indicating an increase in bivalent formation reflecting increased success in crossover formation. However, there was no significant difference in the number of DAPI bodies, with or without the mels8 transgene, in the him-14(it44); msh-5::V513A background (Fig. 6F and SI Appendix, Fig. S8E).
This finding strongly suggests that phosphorylation of the MSH-5 C-terminal tail is required for suppression of him-14(it44) phenotypes by elevated CDK-2 activity, supporting the conclusion that the MSH-5 tail is the major CDK-2 target whose phosphorylation is responsible for augmenting the residual MutSγ activity in him-14(it44) animals.

Discussion

C. elegans CDK-2 Partners with COSA-1 to Promote Crossover Formation. Here, we demonstrate that C. elegans CDK-2 localizes to recombination intermediates and partners with COSA-1 to promote crossover formation. Using superresolution...
microscopy, we show that CDK-2 and COSA-1 colocalize together within the same subcompartment at crossover sites, with a spatial positioning distinct from those exhibited by other recombination factors, such as MutSγ and Bloom helicase. Further, we show that CDK-2 and COSA-1 are interdependent for localization, are both required for in vivo phosphorylation of MutSγ, and their absence/depletion has identical consequences for the progression of recombination. Based on these collective findings and conservation of the predicted cyclin/CDK interface (39), the most parsimonious explanation is that CDK-2 and COSA-1 combine to form a dedicated meiotic CDK/cyclin complex that functions to convert a subset of meiotic DSBs into interhomolog crossovers by stabilizing crossover–specific recombination intermediates.

Fig. 6. Phosphosites within the C-terminal tail of MSH-5 contributes to the pro-crossover activity of the MutSγ complex. (A) Diagram of msh-5–mutant series harboring phosphorylation-defective mutations within its C-terminal tail, indicating with white boxes the positions of S/T residues that were replaced by A residues. (B) Oocyte nuclei at diakinesis from msh-5::V5WT and msh-5::V513A. Adult hermaphrodites grown at 20°C (48 h after L4) were stained with DAPI. (Scale bar, 4 μm.) (C) Oocyte nuclei at diakinesis from him-14(it44) unc-4; msh-5::V5WT and him-14(it44) unc-4; msh-5::V513A. L4 hermaphrodites were grown at 15°C for 48 h and were dissected for DAPI staining. (Scale bar, 4 μm.) (D) Graph showing the number of DAPI bodies in diakinesis oocytes from indicated genotypes grown at 15°C (Left) and 20°C (Right). The numbers of nuclei scored are shown below. The mean ± SD is shown; ****P < 0.0001; ***P = 0.0004 to 0.0009; *P = 0.0158; ns, not significant by Mann–Whitney U test. (E) Immunofluorescence images of spread pachytene nuclei from indicated genotypes grown at 15°C showing staining for HIM-3 (white), MSH-5::V5 (red), and GFP::COSA-1 (green). Cyan arrowheads indicate COSA-1 foci that colocalize with MSH-5, while orange arrowheads indicate the ones that do not overlap with MSH-5. (Scale bar, 3 μm.) (F) Graph showing the number of DAPI bodies in diakinesis oocytes from indicated genotypes grown at 22°C. Numbers of nuclei scored are shown below. The mean ± SD is shown; ****P < 0.0001; ns, not significant by Mann–Whitney U test.
While our data strongly support *C. elegans* CDK-2 and COSA-1 working together as a functional unit in promoting meiotic crossover formation, it remains unresolved whether this functional partnership is conserved in mammalian spermatocyte meiosis. On the one hand, mouse CNTD1 and CDK2 colocalize at late crossover sites (41, 42), and loss of *Cnd1* function (40, 42), a *Cnd1*Δ allele that disrupts a Y2H interaction between CNTD1 and CDK2 (42), and a partial loss-of-function *Cdka2*Δ allele (45) all cause very similar phenotypes, including elevated/persistent MSH4 foci, loss of late crossover markers, and a deficit of chiasmata. While these findings support a functional partnership, however, no immunoprecipitation studies to date have detected in vivo association between CNTD1 and CDK2. Further, detection of a short isoform of CNTD1 lacking a domain essential for cyclin/CDK interaction has led to the proposal that CNTD1 might function through alternative binding partners independently of CDK (41).

**Evidence That CDK-2 Promotes Crossover Designation through Phosphorylation of MSH-5.** Here, we identify MutSy as a key meiotic target of CDK-2 and demonstrate that MSH-5 is phosphorylated in a COSA-1– and CDK-2–dependent manner within its disordered C-terminal tail. As mutating all 13 C-terminal CDK motifs in MSH-5 does not alone result in meiotic defects, CDK-2 likely has additional substrates essential for crossover formation. However, severe consequences of phosphosite loss are evident when the activity of MutSy is compromised, indicating the importance of phosphorylation within the MSH-5 tail for enabling success of meiosis under suboptimal conditions. The significance of this kinase–substrate relationship is further supported by dosage repression, in which extra copies of the *cosa-1* gene enable him-14(it44) animals to form higher levels of crossovers at a semipermissive temperature (62). We have shown that suppression of him-14(it44) phenotypes by COSA-1 overexpression requires MSH-5 phosphorylation within its C-terminal region. Thus, we propose that MSH-5 is a key substrate of CDK-2/COSA-1 and that phosphorylation within the MSH-5 C-terminal tail potentiates the overall activity of MutSy in stabilizing crossover–specific recombination intermediates (Fig. 7).

**The C-Terminal Tail of MSH-5 as a Scaffold to Accumulate Other Pro–Crossover Factors.** CDK phosphorylation sites in MSH-5 are clustered within its disordered C-terminal domain, which we have shown to be essential for crossover formation. Because the MSH-5 C-terminal tail is outside of its enzymatic core or the dimerization interface mapped for human MSH4 and MSH5 (36), it is unlikely that deleting the C-terminal tail affects the ATP hydrolysis rate or the formation of the HIM-14/MSH-5 heterodimer. In worms expressing a truncated MSH-5 (msb-5Δ339Δ33), MSH-5 localizes to no more than six COSA-1–marked recombination sites, suggesting that crossover site designation may have occurred. However, pro–crossover factors do not accumulate to wild-type levels at these sites, depletion of ZHP-3 from along the length of the SC does not occur, and lack of chiasmata connecting homologs at diakinesis indicates a failure to process these recombination intermediates into crossovers (Fig. 5). We propose that the C-terminal tail of MSH-5 serves as a scaffold to accumulate proteins required for crossover formation. Indeed, intrinsically disordered proteins frequently contain short linear motifs that mediate interactions with diverse targets and have emerged as major hubs in cellular signaling (63). Recent work in mice has identified a novel proline-rich protein, PRR19, that functions with CNTD1 to promote crossover formation (42). Intriguingly, the MSH-5 tail is also enriched in prolines (CDK is a proline-directed kinase) and thus may act as a functional substitute for PRR19 to stably recruit CDK-2/COSA-1 in *C. elegans*. Determining how the MSH-5 tail mediates higher-order assemblies of pro–crossover factors will be an important topic for future research.

The phenotype observed in *msb-5Δ339Δ33* animals is in sharp contrast to that in *msb-5Δ*:V5Δ33; him-14(it44), where one to three bright foci of COSA-1 initially localize to a subset of MutSy-positive recombination intermediates but lose their association in late pachytene (Fig. 6B). Thus, although the MSH-5 C-terminal tail itself is required for concentrating pro–crossover factors at recombination sites, it appears to have deleterious effects on their retention in its unphosphorylated form. We speculate that the C-terminal tail of MSH-5 may also act as a scaffold for recruiting antirecombinases, which is counteracted by CDK-2–dependent phosphorylation. Recent evidence in *Saccharomyces cerevisiae* has demonstrated that phosphorylation of the N-terminal degron within Msh4 protects it from proteolysis at recombination sites, thereby activating its pro–crossover activity (64). However, phosphorylation of the MSH-5 C-terminal tail does not seem to have similar stabilizing effects, as we did not observe an increased level of MSH-5 in our C-terminal truncation mutants. As ZHP-3 is depleted from the SC in *msb-5Δ*:V5Δ33; him-14(it44) mutants while colocalizing with COSA-1, CDK-2/COSA-1 may also phosphorylate ZHP-3/4 and trigger their relocation from the SC to crossover–designated sites (Fig. 7).

**Multisite Phosphorylation, Positive Feedback, and Propensity for Aggregation Provide a Robust Mechanism for Crossover Designation.** CDK phosphorylation sites are often clustered in disordered regions (65), and multisite phosphorylation by CDK can set a threshold to elicit an ultrasensitive response (66, 67). Our analysis of *msb-5* phosphosite mutants revealed that crossover formation becomes highly sensitive to the number of phosphorylation sites available in the MSH-5 C-terminal tail when the activity of MutSy is compromised by a temperature-sensitive mutation in him-14. At more permissive temperatures where HIM-14 is largely functional, fewer phosphosites are needed to achieve a threshold level of MutSy activity required to ensure crossover formation. Conversely, under more restrictive conditions where HIM-14 is less functional, more phosphosites are required to achieve a threshold level of MutSy activity. Although all MSH-5 orthologs in the *Caenorhabditis* species possess numerous CDK consensus motifs in the disordered C-terminal region, these sites are poorly conserved. Thus, we speculate that the number of phosphorylation sites, rather than their exact position, influences the pro–crossover activity of MutSy, in line with several precedents controlled by multisite phosphorylation (68, 69). Further, as phosphorylation within the MSH-5 tail promotes the stable association of CDK-2/COSA-1 to recombination sites, it can also generate positive feedback that further enhances MSH-5 phosphorylation, thereby conferring a switch-like behavior that contributes to the robustness of crossover designation.

We further speculate that the propensity for MSH-5 to form aggregates in late pachytene germ cells, revealed upon CDK-2 depletion, may also be a feature that promotes robustness of the crossover designation mechanism. Given the intrinsically disordered protein sequence in which phosphosites are embedded, we hypothesize that the MSH-5 C-terminal tail may have a capacity to undergo phase separation that can be modulated
by phosphorylation, which could potentially contribute to the formation of ellipsoidal protein structures that have long been recognized as “recombination nodules” (70). The idea that formation of biomolecular condensates may contribute to crossover designation is supported by recent modeling of cytological data from Arabidopsis, in which HEI10 is proposed to accumulate in a few sites through diffusion-mediated coarsening at the expense of smaller foci (71). By enriching pro-crossover factors at crossover-designated intermediates while depleting them from other recombination sites, the formation of biomolecular condensates can serve as a general mechanism for controlling both crossover designation and positioning.

Materials and Methods

C. elegans Genetics, Genome Engineering, and Auxin-Mediated Depletion of CDK-2. All strains used in this study were maintained on NGM (nematode growth medium) plates seeded with OP50-1 bacteria under standard conditions as described in ref. 72. All experiments were performed at 20 °C except where noted. All C. elegans strains were derived from a Bristol N2 background. SI Appendix, Tables S1 and S3 summarize all mutations and strains used in this study. The strains expressing CDK-2::AID:3×Flag and variants of MSH-5::V5 were generated by Cas9/CRISPR-mediated homologous recombination (73). Details of procedures, CRISPR RNAs, repair templates, and genotyping primers (SI Appendix, Table S2) are provided in the SI Appendix.

Auxin-mediated degradation of CDK-2 from the C. elegans germline was performed as previously described (51). Briefly, auxin plates were prepared by diluting a 400 mM auxin solution (indole-3-acetic acid in ethanol) into NGM (cooled after autoclaving) to a final concentration of 1 mM. Plates were dried at room temperature and stored at 4 °C protected from light for up to 1 wk prior to use. Plates were spread with concentrated OP50-1 bacterial cultures and incubated overnight at 37 °C. Age-matched young-adult hermaphrodites were picked and left for various hours at 20 °C prior to immunofluorescence.

Scoring DAPI-Staining Bodies in Diakinesis Oocytes of him-14(its44) Animals. All strains carrying him-14(its44) unc-4(e120) were maintained at 20 °C with the mnc1 balance: unc-4(e120), closely linked to him-14 on chromosome II, was used as a marker for easy genotyping and does not elicit meiotic phenotypes. Homozygote fourth larval (L4) stage worms (Unc, nongreen) were picked and transferred to experimental temperatures (15 °C, 20 °C, or 22 °C) for 44 h. To score DAPI-staining bodies in diakinesis oocytes, worms were picked on to a slide with a minimal volume of M9. Excess liquid was wicked away, and animals were fixed in 15 μL of 95% ethanol. Once dry, ethanol was reapplied, and this process was repeated a total of three times. The slides were mounted using VECTASHIELD containing DAPI (Vector Laboratories, H-1200-10) and sealed. Slides were stored at 4 °C for no longer than 4 d before imaging using a standard fluorescent microscope. DAPI bodies in the nuclei of diakinesis oocytes in the −1 to −3 positions were counted.

Phosphopeptide Antibody Production and Affinity Purification. A synthetic phosphopeptide (TAHIPP[T]PIQMGEC) corresponding to the C. elegans MSH-5 sequence flanking threonine 1009 was generated using standard methods. The phosphopeptide was conjugated to keyhole limpet hemocyanin and injected into rabbits (Covance). To affinity purify polyclonal MSH-5 pT1009 antibodies, immune serum was first passed through SulfoLink coupling resin (Thermo Fisher, 20401) coupled to a nonphosphopeptide (TAHIPP[T]PIQMGEC). Flow-through was then bound and eluted from phosphopeptide-coupled resin. The specificity of the antibodies was verified by dot blot and immunofluorescence of worm strains carrying phosphorylation-defective mutations in msh-5.

Immunofluorescence. Immunofluorescence experiments involving whole-mount gonads and spread nuclei were conducted as in refs. 33 and 56 with modifications. The antibodies used, details of procedures, and imaging acquisition and processing are provided in the SI Appendix. Additional methods are described in the SI Appendix.

Data Availability. All study data are included in the article and/or SI Appendix. Materials used in this research are available on request from A.M.V. and Y.K.

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