Introduction

Meiotic recombination occurs during prophase I when homologous chromosomes are synapsed along their entire length. Synapsis is defined as the close and stable association of homologous chromosomes through a proteinaceous structure called the synaptonemal complex (SC). In most organisms, this complex is composed of two main parts: lateral elements that attach along the axis of each homologous chromosome and transverse elements that span the central region of the SC and function to tether the homologs [1,2]. At the leptotene/zygote stages of meiotic prophase, these structural proteins begin to load onto the chromosome axes, and are completely assembled at pachyteny, when homologous chromosomes are synapsed along their entire length.

Recombination between the homologous chromosomes initiates with DNA double-strand breaks (DSBs) that are repaired as either crossovers or noncrossovers [3–5]. Crossovers establish chiasmata, which, along with sister chromatid cohesion, hold homologs together after recombination has been completed and chromosomes have dissociated their SC proteins. Chiasmata help orient the homologous chromosomes on the metaphase I spindle and ensure their proper segregation at anaphase I. The failure to establish a crossover/chiasma can result in the nondisjunction of homologs and lead to aneuploid gametes.

Crossover formation is a tightly regulated process. Mutational findings suggest a model in which the pachyteny checkpoint monitors the structure of chromosome axes and may function to promote an optimal number of crossovers.

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Author Summary

Meiosis is a specialized cell division in which diploid organisms form haploid gametes for sexual reproduction. This is accomplished by a single round of replication followed by two consecutive divisions. At the first meiotic division, the segregation of homologous chromosomes in most organisms is dependent upon genetic recombination, or crossing over. Crossing over must therefore be regulated to ensure that every pair of homologous chromosomes receives at least one reciprocal exchange. Homologous chromosomes that do not receive a crossover frequently undergo missegregation, yielding gametes that do not contain the normal chromosome number, conditions frequently associated in humans with infertility and birth defects. The pch2 gene is widely conserved and in Drosophila melanogaster is required for a meiosis-specific checkpoint that delays progression when crossover formation is defective. However, the underlying process that the checkpoint is monitoring remains unclear. Here we show that defects in axis components and homolog alignment are sufficient to induce checkpoint activity and increase crossing over across the genome. Based on these observations, we hypothesize that the checkpoint may monitor the integrity of chromosome axes and function to promote an optimal number of crossovers during meiosis.

Results

Defects in chromosome axis components cause a pch2-dependent pachytene delay

In the Drosophila gerarium, oocytes are born within cysts composed of 16 cells that are connected by ring canals. Two out of the sixteen cells, each with four ring canals, initially contain equivalent levels of SC proteins and are termed the pro-oocytes (Figure 1A). As the developing cysts travel from the anterior (region 2) toward the posterior (region 3) of the germarium, the pro-oocytes proceed through the pachytene stage of meiosis where synopsis is completed and DSB formation and recombination occurs. By region 3 of the wild-type germarium, DSB repair is completed and one of the two pro-oocytes will exit meiosis, lose its SC and become a nurse cell while the other will continue through development and form the oocyte (Figure 1A) [18].

In DSB repair and exchange-defective mutants, the transition through pachytene is delayed by pachytene checkpoint activity [14]. This results in both pro-oocytes persisting into region 3 cysts, referred to as the “two-oocyte phenotype.” Delays can be identified either by the persistence of the SC transverse filament C(3)G in both pro-oocytes [14] or by the concentration of ORB protein in the cytoplasm of two cells rather than one in region 3 cysts (Figure 1B) [19]. ORB staining, however, is less sensitive than C(3)G at detecting pachytene delays, resulting in a different frequency of the two-oocyte phenotype between the two assays [14].

Abolishing synopsis by mutation of c(3)G does not elicit the two-oocyte phenotype (Figure 1D), suggesting the pachytene checkpoint is not monitoring SC formation [14]. We further investigated the relationship between chromosome structure components and the pachytene checkpoint by determining the effects of mutations in two other genes, ord and c(2)M, which encode structural proteins.

ORD localizes to chromosome axes in oocytes independent of synopsis (i.e. in c(3)G mutants) and has roles in meiotic recombination and sister chromatid cohesion [20,21]. Although ord mutants initially display normal C(3)G and C(2)M localization, only rare structures resembling SC were observed by electron microscopy (EM), suggesting that the ultrastructure of chromosome axes was disorganized [21]. Consistent with this interpretation, C(3)G and C(2)M staining precociously deteriorates in ord mutants as the oocytes progress through pachytene [21]. We found that ord mutants displayed a high frequency of the two-oocyte phenotype (Figure 1D), indicative of a delay in meiotic progression. The two-oocyte phenotype was suppressed in ord; pch2 double mutants, indicating the delay was dependent on the pachytene checkpoint (Figure 1D) and supporting the hypothesis that the pachytene checkpoint is sensitive to defects in axis components.

C(2)M is a component of the SC lateral element and localizes adjacent to the chromosome axes even in the absence of synopsis (in c(3)G mutants), suggesting it may interact with axis components [22,23]. In c(2)M mutant oocytes, C(3)G protein fails to develop into complete strands along the lengths of each chromosome, but instead appears as small patches (Figure 1C). The most likely explanation is that SC initiates in c(2)M mutants but polymerization is defective. Similar to ord mutants, c(2)M mutants exhibited a high frequency of the two-oocyte phenotype, which was suppressed in c(2)M; pch2 double mutants (Figure 1D). The high frequency of the two-oocyte phenotype observed in c(2)M mutants was not suppressed by mutation of c(3)G (Figure 1D), demonstrating the pachytene checkpoint can signal independently of SC initiation. Together, these results suggest the pachytene checkpoint may monitor a synopsis-independent function of ORD and C(2)M, such as the formation of chromosome axes.

Chromosomal rearrangements disrupt axis integrity and cause a pch2-dependent pachytene delay

If the pachytene checkpoint monitors the integrity of chromosome axes we reasoned that structural rearrangements would also exhibit pachytene delays. Balancers are multiply-inverted chro-
mosomes that fail to cross over with a normal homolog, presumably due to a disruption in the continuity of pairing and/or synapsis [24–26]. We characterized the integrity of SC-associated proteins in balancer heterozygotes with antibodies recognizing the SC components C(3)G and C(2)M. Single balancer heterozygotes (TM3/+) had thread-like C(3)G and C(2)M staining that was indistinguishable from wild-type (Figure 2A) [26]. Double balancer heterozygotes (CyO/++; TM3/+) also initially displayed normal C(3)G and C(2)M localization, but the staining became fragmented and sometimes undetectable in region 3 oocytes (Figure 2A). This precocious deterioration of SC proteins during pachytene is similar to what is observed in ord mutant oocytes [21], suggesting that rearrangement breakpoints might disrupt axis stability.

Using C(3)G staining to detect oocytes, we found that FM7, Bwinscy, TM2 and TM3 balancer heterozygotes each exhibited a significantly higher frequency of the two-oocyte phenotype compared to wild-type (Figure 2B), suggestive of a pachytene delay. The high frequency of the two-oocyte phenotype was suppressed to wild-type levels in FM7/++; pch2 and TM3/++; pch2

Figure 1. Pachytene delays in axis-defective mutants. (A) Schematic depiction of germline cysts and oocyte markers in a wild-type germarium. Changes in cyst morphology differentiate regions 2a (small and round cysts), 2b (flat oblong cysts), and 3 (large and round cysts). Cysts travel anterior to posterior and the age difference between each cyst is ~12–24 hrs (our unpublished data and [56]). (B) Examples of region 3 cysts with one- or two-oocytes identified with C(3)G or the cytoplasmic ORB protein. Scale bar is 5 μM. (C) Threadlike C(3)G staining is never observed in the germaria of c(2)M mutants [52], which show fragments of C(3)G staining throughout pachytene, suggestive of an assembly defect. (D) ORB staining to detect oocytes in mutants with defective axis structure. A pachytene delay phenotype is defined as a significantly greater percentage of region 3 cysts with two-oocytes when compared to wild-type (asterisks are located above each bar when P-value was <0.05). Note that the pachytene delay observed in ord and c(2)M mutants was suppressed by mutation of pch2 but not by mutation of c(3)G or mei-218. The number of cysts/germaria counted is at the bottom of each bar.

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Figure 2. SC deterioration and pachytene delays in balancer heterozygotes. (A) SC localization in region 2 and region 3 cysts from wild-type, TM3/+ and CyO/+ TM3/+ females. Oocytes in region 3 are outlined. In wild-type and TM3/+ germaria, extensive threadlike C(3)G typical of pachytene is visible in oocytes throughout the germarium. In CyO/+; TM3/+ germaria, threadlike C(3)G and C(2)M staining is detected in region 2a, but...
deteriorates in the majority of region 3 oocytes. Scale bar is 5 μM. (B) Based on C(3)G staining, a high frequency of the two-oocyte phenotype was found in heterozygotes for single translocations [T(2;3)], a single inversion [In(1)D149] and the balancer chromosomes FM7, Bwinicy, TM2, and TM3. This was suppressed by mutation of pch2, but not by mei-218. Asterisks are located above each bar when P-value was <0.05 compared to wild-type. The number of cysts counted is at the bottom of each bar. (C) Based on OR8 staining, the high frequency of the two-oocyte phenotype in In(1)D49/+ heterozygotes was not suppressed by mutation of c(3)G. (D) The average number of γ-H2AV foci as a function of relative cyst age in wild-type and balancer heterozygotes. Cyst 1 is the first to have complete SC, cyst 8 is in region 3 and cysts 9–11 are in later-stage cysts (stages 2–4), which have left the gerarium. Neither single nor double balancer heterozygotes significantly altered the appearance or disappearance of γ-H2AV foci throughout the gerarium. Error bars denote the standard error of the mean. doi:10.1371/journal.pgen.1001059.g002

females, confirming the delays were dependent on the pachytene checkpoint (Figure 2B; P<0.05 compared to either balancer heterozygote alone). pch2 had no effect on the SC morphology in single balancer heterozygotes (data not shown).

Each balancer chromosome contains several inversions. For example, the TM3 chromosome includes 10 breakpoints [27]. To investigate the effects of a more subtle disturbance in chromosome organization on the pachytene checkpoint, we tested whether a single aberration, or two breakpoints, would be enough to induce pachytene delays. Remarkably, heterozygotes of single translocations between the 2nd and 3rd chromosomes ([T(2;3)DP77/+; T(2;3)dpTP]/+, and [T(2;3)dP]/+) and a single inversion on the X chromosome [In(1)D49/+]) each exhibited a high frequency of the two-oocyte phenotype, suggesting the threshold to trigger the pachytene checkpoint is low and requires as few as two breaks in axis continuity (Figure 2B). Importantly, the delays were not dependent on C(3)G and were not significantly increased in In(1)D49 homozygotes compared to wild-type (Figure 2B and 2C), indicating the pachytene checkpoint responds to a break in alignment between homologs in a way that is independent of SC initiation.

Chromosome axis defects cause pachytene checkpoint delays independent of the MCM–related precondition genes

In addition to the delay in oocyte selection, DSB repair and exchange-defective mutants also exhibit a pch2-dependent delay in the response to DSBs [14]. To monitor DSB formation and repair in balancer heterozygotes, we stained ovaries with an antibody to γ-H2AV [28,29]. In wild-type oocytes, γ-H2AV foci are most abundant in region 2a nuclei (cyst 3 in Figure 2D) and absent by region 3 (cyst 8 in Figure 2D), indicating DSBs have been repaired. In addition, the low level of crossovers that are generated along the 2nd chromosome in mei-9 mutants are mostly suppressed in mei-9; pch2 double mutants (Figure 3A). These results suggest the residual crossovers in recombination-defective mutants depend on a mechanism facilitated by pch2.

PCH2 can induce interchromosomal effects on crossing over

PCH2 is required for some of the crossovers that occur in the exchange-defective mutant, hdm [14]. Consequently, hdm; pch2 double mutants exhibit an elevated frequency of nondisjunction compared to hdm single mutants. These results suggest a functional link between the pachytene checkpoint and the production of crossovers. To determine if this is a general property of mutants that exhibit pachytene delays, we tested additional double mutants with pch2. Exchange class genes Ecc1 and mei-9 encode components of an endonuclease complex of proteins that includes HDM and is required for normal levels of meiotic crossing over [30,31]. Loss of ERC1 function results in 14% X-chromosome nondisjunction, which is elevated to 30% in a pch2 mutant background, suggesting crossovers are further reduced (Table S1). In addition, the low level of crossovers that are generated along the 2nd chromosome in mei-9 mutants are mostly suppressed in mei-9; pch2 double mutants (Figure 3A). These results suggest the residual crossovers in recombination-defective mutants depend on a mechanism facilitated by pch2.

When crossing over is suppressed along a normal chromosome heterozygous to a balancer, there is an interchromosomal effect that increases crossing over on the remaining chromosome pairs [11]. Since PCH2 is responsible for the residual level of crossovers in recombination-defective mutants, we asked if it was also responsible for the increase in crossovers observed in balancer heterozygotes. Consistent with previous work on interchromosomal effects [32,33], we found that FM7/+ heterozygotes exhibit 151% of wild-type crossing over along the 2nd chromosome with an altered distribution (Table 1). Although there was little deviation from wild-type controls in the distal regions of the chromosome ([al-b], the genetic map distance was increased ~4–5 times that observed in wild-type centromere-proximal intervals (Table 1; Figure 3B). Remarkably, 2nd chromosome crossing over in FM7/+ heterozygotes was reduced to 106% of wild-type in a pch2 mutant background (p<0.00005; Table 1, Figure 3B). Similarly, introduction of the CyO chromosome increased crossing over along the X chromosome to 149% of wild-type, which was reduced to 126% in pch2 mutants (p<0.05; Table 2; Figure 3C). Interestingly, the closer the interval being tested was to the centromere, the greater the interchromosomal effect and pch2 dependence (Table 2; Figure 3C). Since pch2 single mutants exhibited normal levels of crossing over on the X and 2nd chromosome (Table 1; Table 2; Figure 3), these data show that pch2 is required for most of the interchromosomal increase in crossover levels in balancer heterozygotes.

Pachytene checkpoint activity does not lead to an increase in DSB levels

The increased crossing over observed in balancer heterozygotes could be explained by pachytene checkpoint activity increasing
Figure 3. Percentage of wild-type crossing over in balancer heterozygotes, *mei-9*, and *pch2* mutant females. (A) Percentage of wild-type crossing over in *mei-9* and *mei-9; pch2* mutant females along the 2nd chromosome. Most crossovers in a *mei-9* mutant are dependent on *pch2* (also see Table 1). (B) Percentage of wild-type crossing over along the 2nd chromosome in *pch2*, FM7/+, and FM7/+; *pch2* mutant females. *pch2* mutants...
have wild-type levels and distribution of exchange. The interchromosomal effect of the FM7 balancer was mostly suppressed in pch2 mutants (also see Table 1). (C) Percentage of wild-type crossing over along the X-chromosome in pch2, CyO/+, and CyO/+; pch2 mutant females (note the scale is reduced). The interchromosomal effect elicited by the CyO balancer was reduced in pch2 mutants, especially in the most proximal and distal intervals (also see Table 2).

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DSB levels. However, we failed to observe any significant change in the number of γ-H2AV foci in oocytes single or doubly heterozygous for FM7 and CyO compared to wild-type (Figure 2D). Since asynchrony of DSB formation can complicate measuring the total number of γ-H2AV foci, we repeated the above experiment in a γm-A (Drosophila Rad51) mutant background, in which repair of DSBs is blocked [12]. The number of γ-H2AV foci in region 3 oocytes of these mutants is expected to be close to the total number of DSBs that occurred [28,34]. γm-A mutant region 3 oocytes displayed an average of 21.0 (+/−1.5) γ-H2AV foci. Similarly, FM7/+; CyO/+; γm-A region 3 oocytes had an average of 24.0 (+/−1.4) γ-H2AV foci.

These results indicate that the ability of the pachytene checkpoint to increase crossing over in the genome is not mediated by a substantial increase in the total number of DSBs. Instead, pachytene checkpoint activity most likely increases the chance of DSBs becoming crossovers, particularly those that occur near centromeres.

PCH2 localizes to the nuclear periphery and persists when pachytene is delayed

To investigate how PCH2 affects crossover frequency, we monitored the protein localization pattern during meiosis. A transgene was constructed containing a hemagluttinin (HA) epitope at the N-terminus of the pch2 transcript under the control of an inducible UASP promoter. We expressed PCH2 using the germline specific driver P(Gal4-nos.NGT)40 [35], abbreviated as NGT, known to express in pachytene at moderate levels [36]. The NGT-driven P(HA-pch2)/TM3/+ transgenic line restored the two-oocyte phenotype in FM7/+; pch2 females to similar levels found in FM7/+ heterozygotes (Figure S1), demonstrating that the NGT-driven pch2 transgene was functional.

PCH2 staining formed foci that localized around the nucleus in syngene and early pachyten e (region 2) cells (Figure 4A). No PCH2 foci were detected in region 3 cells, suggesting the protein is rapidly degraded or no longer produced after early pachytene. Surprisingly, PCH2 foci did not overlap with the DNA stain. To determine if PCH2 foci localized within the nucleus, we counterstained with the nuclear envelope component, Lamin. We found that 73% of PCH2 foci showed a close association (i.e. touching) with the cytoplasmic side of the Lamin staining (n = 368; Figure 4B), indicating they localized adjacent to the nuclear envelope and outside the nucleus. The remaining 27% of PCH2 foci not closely associated with Lamin were found dispersed within the cytoplasm.

To determine if PCH2 localization pattern changes in mutant backgrounds that exhibit pachytene delays, we examined PCH2 expression in mutants that cause checkpoint delays: hdm, mei-9 and in TM3/+ heterozygotes. In hdm and mei-9 mutants, the number of PCH2 foci per oocyte was increased ~2-fold compared to controls (Figure 4C). In addition, the foci persisted into region 3 oocytes, which was never observed in control germaria (Figure 4A and 4C). However, PCH2 localization was not detected past stage 2 of oogenesis (data not shown), indicating the loss of PCH2 is only delayed in exchange-defective mutants. In TM3/+ heterozygotes, the levels of PCH2 foci in region 2 cells was unchanged compared to controls, but were present in region 3 (Figure 4C), revealing a correlation between the prolonged expression of PCH2 and a delay in pachytene.

The intracellular localization pattern of PCH2 did not change when pachytene was delayed since the foci remained juxtaposed to the nuclear envelope in hdm and mei-9 mutants and in TM3/+ heterozygotes at all stages (Figure 4A and data not shown). Furthermore, mutation of mei-W68, which eliminates DSB

Table 1. Effect of pch2 on crossing over on the 2nd chromosome.

| Genotypeb | ac-dp | dp-b | b-pr | cr-cn | Total ac-cn | Nc |
|-----------|------|-----|------|------|------------|---|
| Wild-type | 12.5 | 28.0 | 3.5  | 1.3  | 45.3       | 1008 |
| pch2EY    | 11.7 (94) | 26.7 (95) | 3.3 (94) | 2.1 (162) | 43.8 (97)  | 562 |
| mei-9a    | 0.67 (54) | 3.0 (10.7) | 0.5 (14.3) | 0.2 (12.3) | 4.3 (9.5)  | 993 |
| mei-9a-pch2EY | 0.0 (0.0) | 1.4 (5.0) | 0.0 (0.0) | 0.0 (0.0) | 1.4 (3.1)  | 360 |
| FM7/+     | 14.8 (118) | 30.3 (108) | 17.6 (503) | 5.6 (431) | 68.3 (151) | 568 |
| FM7/+; pch2EY | 14.6 (117) | 23.8 (85) | 7.8 (223) | 2.0 (154) | 48.2 (106) | 1176 |
| MV21 P(HA-pch2)27/+ | 12.0 (96) | 36.0 (129) | 5.3 (157) | 0.9 (69) | 54.4 (120) | 1300 |
| MV21 P(HA-pch2)27/+ | 11.5 (92) | 25.0 (89) | 7.5 (214) | 1.3 (100) | 45.3 (100) | 1404 |
| MV21 P(HA-pch2)281/+ | 9.3 (74) | 31.5 (113) | 6.5 (186) | 1.6 (123) | 48.9 (108) | 992 |
| MV21 P(HA-pch2)271 | 9.8 (78) | 41.7 (149) | 10.3 (293) | 0.8 (60) | 62.6 (138) | 1026 |

*aSecond chromosome crossing over was assayed by crossing al dp b pr cn/CyO males in the indicated backgrounds. The CyO progeny were scored for recombinants. Crossing over is expressed as cM across the intervals shown. Numbers in parentheses denote the percentage of wild-type recombinant frequency.

bN = total flies counted.

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Table 2. Effect of pch2 on crossing over on the X-chromosome.

| Genotype | pcn-cv | cv-m | m-f | fy' | Total pcn-fy' | Nc |
|-----------|-------|------|-----|-----|---------------|---|
| Wild-type | 15.4  | 18.9 | 11.9 | 7.3  | 53.5          | 657 |
| pch2EY    | 14.8 (96) | 18.5 (98) | 14.1 (118) | 6.3 (86) | 53.7 (100) | 569 |
| CyO/+     | 18.6 (121) | 27.7 (147) | 19.9 (167) | 13.6 (186) | 79.8 (149) | 1319 |
| CyO/+ pch2EY | 15.0 (97) | 26.1 (138) | 18.1 (152) | 9.1 (125) | 68.3 (128) | 1148 |

*C chromosome crossing over was assayed by crossing y pcn cv m f+y'/f females to wild-type males in the indicated backgrounds. The male progeny were scored for recombinants. Crossing over is expressed as cM across the intervals shown. Numbers in parentheses denote the percentage of wild-type recombination frequency.

N = total flies counted.

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Crossover Control in Drosophila
Figure 4. PCH2 localization during pachytene. (A) PCH2 localization in germaria when the $UASP: pch2$ transgene was driven by $P(Gal4-nos.NGT)40$ (NGT) or by $P(Gal4-VP16-nos.UTR)MVD1$ (MVD1). Single sections of region 2a oocytes are shown to the right of their corresponding germarium. Scale bars for germaria and single cells are 10 μM and 5 μM, respectively. In controls, PCH2 expression is restricted to zygotene and early pachytene cells (region 2). PCH2 expression persists into region 3 cells of $hdm$ mutants and when PCH2 is driven by MVD1. In MVD1-driven PCH2, two populations of PCH2 become present: unlocalized protein distributed evenly throughout the cytoplasm and distinct bright spots classified as foci. Due to the projection of multiple images, it is difficult to see PCH2 foci among the unlocalized staining in the MVD1+ germarium. They are visible in single sections. PCH2 expression in region 3 cells is eliminated in $sir2$ mutants. PCH2 foci localize adjacent to the DNA stain in all genotypes. (B) When the $UASP: pch2$ transgene was driven by $P(Gal4-nos.NGT)40$, PCH2 foci in region 2a oocytes localize to the cytoplasmic side of the nuclear envelope protein, Lamin. (C) Quantification of PCH2 foci. The average number of PCH2 foci per cell was increased in both region 2 and region 3 cells of $hdm$ and $mei-9$ mutants. PCH2 foci levels did not increase in $TM3$ heterozygotes or when PCH2 was overexpressed by the MVD1 driver, but did persist into region 3 cells.

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formation, showed a normal staining pattern of PCH2, and hdm; mei-218 double mutants showed the same PCH2 staining pattern as hdm single mutants (Figure 4A and data not shown), consistent with our previous conclusion that the pachytene checkpoint functions independently of DSB formation [14,16]. These observations provide a connection between the nuclear envelope and pachytene checkpoint activity and suggest that PCH2’s role in nuclear events like crossing over is indirect and at a distance from the chromosomes.

Prolonged PCH2 activity leads to a pachytene delay and altered crossover distribution
To test the significance of the correlation between pachytene delays and prolonged PCH2 expression, we manipulated the timing and expression levels of PCH2 in the germline. PCH2 levels were increased by driving the UASP:pch2 transgene with P(Gal4::VP16-nos.UTR)/MV1 [37], abbreviated as MV1, known to drive high levels of expression in the germarium. MV1-driven pch2 caused the protein to persist into region 3 oocytes, which was never observed with the NGT driver in a wild-type background (Figure 4A and 4C). In addition to distinct foci, PCH2 was also distributed more evenly throughout the cytoplasm (Figure 4A). Thus, MV1-driven pch2 resulted in overproduction and prolonged expression of the protein throughout pachytene.

Pachytene delays were not observed when the pch2 transgenes were expressed using the NGT driver (Figure 5A). In contrast, MV1-driven pch2 induced a pachytene delay that resulted in a high frequency of the two-oocyte phenotype (Figure 5A). In fact, 100% (n = 10) of the germaria with PCH2 expression in region 3 cysts also contained two oocytes, as viewed by C(3)G staining. 100% (n = 10) of the germaria with PCH2 expression in region 3 cysts also contained two oocytes, as viewed by C(3)G staining, suggesting prolonged PCH2 expression is sufficient to induce a delay in pachytene progression.

Since overproducing PCH2 caused a pachytene delay, we determined if crossover frequency or distribution was also affected. We found that PCH2 expression driven by MV1 altered the distribution of exchange in all 3 transgenic lines tested (Table 1; Figure 5B). The most dramatic increase in crossover frequencies was observed in the centromere proximal interval of chromosome 2, b-pr. Although all the transgenic lines that were tested showed the same change in crossover distribution, the magnitude was different, which probably reflects different transgenic protein levels. In support of this conclusion, the presence of two transgenic copies of P(HA-pch2)71 driven by MV1 exacerbated the effect on both crossover levels and distribution (Table 1; Figure 5B). These data show that the frequency and distribution of crossing over is sensitive to the timing and level of PCH2 expression during pachytene.

sir2 is required for prolonged PCH2 expression and the pachytene checkpoint
We sought to identify factors that facilitate prolonged PCH2 expression and cause pachytene delays. The first candidate we tested was mei-218 since it is required for the pch2-dependent pachytene delays observed in DSB repair and exchange-defective mutants. mei-218 mutants, however, did not show an effect on the levels or distribution of MV1-driven PCH2 (Figure S2). Also, the two-oocyte phenotype caused by PCH2 overexpression was not suppressed in mei-218 mutants (Figure 5A), suggesting MEI-218 is not required for PCH2 localization.

The second candidate tested was Sir2, which encodes a histone deacetylase that is required for the nucleolus localization of Pch2 and the pachytene checkpoint during S. cerevisiae meiosis [15]. Five Drosophila genes belong to the Sir2 family. Of these, Sir2 is the closest homolog of the S. cerevisiae Sir2 [38]. Drosophila sir2 null alleles have no obvious effects on viability, but affect position effect variegation, heterochromatic silencing and fly life span [38–40]. sir2 mutants were fully fertile with wild-type levels of X-chromosome nondisjunction (Table S1), indicating Sir2 is dispensable for meiotic recombination.

We investigated whether Sir2 is involved in the pachytene checkpoint and found that mutation of sir2 suppressed the high frequency of the two-oocyte phenotype observed when PCH2 is overexpressed with the MV1 driver (Figure 5A). The high frequency of the two-oocyte phenotypes observed in the exchange-defective mutant hdm and DSB repair mutant spn-B were also suppressed by sir2 (Figure 6A). Likewise, Sir2 was required for the pachytene delay observed in TM3/+ heterozygotes (Figure 6A) and the delayed onset of γ-H2AV staining in spn-B mutants (cyst 2–5 in Figure 6B). Thus, like pch2, sir2 is required for the pachytene checkpoint.

Strikingly, the region 3 localization of MV1-driven PCH2 was eliminated in a sir2 mutant (Figure 4A and 4C). In contrast, loss of sir2 only slightly reduced the level of PCH2 in region 2 cells and had no effect on the peri-nuclear localization of PCH2 driven by NGT (Figure 4C and data not shown). In addition, expression of a c(2)M transgene driven by MV1 was not affected, indicating the effect of sir2 on PCH2 was not due to a reduction in the transcription of UAS-driven genes (Figure S3). These results support the hypothesis that high levels of PCH2 are dependent on Sir2 and essential for the pachytene delays.

Discussion
The pachytene checkpoint is sensitive to defects in chromosome axes
We have previously shown that removing the SC central element component C(3)G does not cause pch2-dependent delays in Drosophila meiotic prophase [14]. Therefore, the pachytene checkpoint is not monitoring the process of synopsis per se. Instead, two lines of evidence suggest the pachytene checkpoint is sensitive to defects in chromosome axes. First, mutations in genes that encode structural axis components, C(2)M and ORD, cause pch2-dependent pachytene delays. Second, heterozygous chromosomal rearrangements also cause a pch2-dependent delay. Homozygous rearrangements do not cause delays; therefore, the pachytene checkpoint is sensitive to any discontinuity in the alignment between homologous chromosomes. Since the delays do not depend on C(3)G, the defect must be prior to or independent of synopsis initiation. The misalignment of homologous sequences could destabilize the integrity of chromosome axes, such as the assembly of ORD or C(2)M, and expose substrates that trigger the pachytene checkpoint. Indeed, females doubly heterozygous for balancer chromosomes show deterioration of C(2)M staining in pachytene oocytes similar to ord mutants [21], suggesting the axial elements are compromised by the heterozygous inversion breakpoints.

Two genetically distinct pathways can trigger the pachytene checkpoint
The delays observed in c(2)M mutants and balancer heterozygotes do not depend on the MCM-related precondition genes such as mei-218, which are required for the pachytene delays in DSB repair and exchange-defective mutants [14]. Balancer heterozygotes also do not cause a delayed response to DSBs or increase in the number of PCH2 foci as observed in DSB repair and exchange-defective mutants. Therefore, two pathways probably lead into a pch2-dependent checkpoint: a mei-218-dependent
Figure 5. PCH2 overexpression leads to pachytene delays and altered crossover distribution. (A) Three different PCH2 transgenic lines driven by MVD1 exhibit a high frequency of the two-oocyte phenotype whereas PCH2 driven by NGT does not. The pachytene delay in MVD1-driven PCH2 was suppressed by mutation of sir2, but not by mei-218 (also see Figure S1). Asterisks are located above each bar when P-value was <0.05 compared to wild-type. The number of cysts counted is at the bottom of each bar. (B) Percentage of wild-type crossing over along the 2nd chromosome in three different transgenic lines where PCH2 is overexpressed by the MVD1 driver. All lines display a similar altered distribution pattern with elevated exchange in the b-pr interval, yet each exhibits a different level of effect on total crossover levels. Two copies of the transgene driven by MVD1 have the greatest effect on both crossover distribution and levels.
doi:10.1371/journal.pgen.1001059.g005
pathway involving the early function of DSB repair proteins and a mei-218-independent pathway involving the structure of chromosome axes.

Of the two pathways in Drosophila, the pachytene checkpoint in other organisms has similarities to the mei-218-independent pathway involving chromosome structure. Heterozygous inversions and translocations induce a pachytene delay, suggesting a model in which the pachytene checkpoint can respond to breaks in axis continuity between paired homologs. The C. elegans pachytene checkpoint also monitors meiotic chromosome structure since a defect in a SC-nucleating “pairing center” triggers a Pch2-dependent response [16]. Similarly, the budding yeast pachytene

**Figure 6. sir2 is required for the pachytene delays.** (A) Mutation of sir2 suppressed the high frequency of the two-oocyte phenotype observed in the exchange-defective mutant hdm, DSB repair mutant spn-B and in TM3 heterozygotes. Asterisks are located above each bar when P-value was <0.05 compared to wild-type. The number of cysts counted is at the bottom of each bar. (B) The average number of γ-H2AV foci is plotted as a function of relative cyst age in wild-type, spn-B and sir2; spn-B mutants. Mutation of sir2 suppressed the delayed onset of γ-H2AV (see cyst 2–5) in spn-B mutants. spn-B mutants also have a block in DSB repair that cause γ-H2AV to accumulate into late stages of oogenesis, which is not suppressed by sir2. Error bars denote the standard error of the mean. doi:10.1371/journal.pgen.1001059.g006
checkpoint has been proposed to monitor SC-dependent events that may involve the relationship between recombination complexes and chromosome axes [41–43]. Therefore, a common feature of the pachytene checkpoints in these organisms is their sensitivity to the axis continuity between paired homologs with the main difference being SC-dependent defects (yeast and nematodes) versus SC-independent axis defects (Drosophila). Interestingly, both yeast Pch2 and mouse Trip13/Pch2 have been proposed to have a checkpoint-independent role in the organization of chromosome axis proteins [43,44]. We do not know as of yet, however, if this is related to the sensitivity of paired axes at the Drosophila pachytene checkpoint, although it is tempting to suggest such a model.

Pachytene checkpoint activity in budding yeast is associated with prolonged Pch2 expression that requires Sir2 [15]. As in budding yeast, Drosophila sir2 mutants are defective in the pachytene checkpoint and our overexpression studies suggest Sir2 is also required for the prolonged expression of PCH2. These results provide evidence for an evolutionarily conserved role of Pch2 and Sir2 in monitoring changes in chromosome structure during meiotic prophase from yeast to a metazoan (Figure 7). Drosophila may have evolved an additional mei-218-dependent pachytene checkpoint, not shared with yeast and nematodes, which is sensitive to DSB repair complexes.

Chromosomal rearrangements induce pch2-dependent interchromosomal effects on crossing over

The effect of inversion heterozygosity on the frequency of crossing over has been known since the work of Sturtevant in 1919. Most often these intrachromosomal rearrangements cause an interchromosomal increase in recombination levels. Exhaustive work has been carried out investigating the interchromosomal effect and several models have been proposed in order to account for the increase in crossing over [11]. The most recent and generally accepted model was last described by Lucchesi and Suzuki in 1968 who proposed a timing model where pairing and crossover formation are coupled during the pachytene stage of prophase [11]. They suggested that when the pairing process between one set of homologs is perturbed or delayed by chromosome rearrangements, pachytene was lengthened and the opportunity to make crossovers was prolonged. We propose a modified version of the timing model where breaks in homology cause disruptions in the axis structure, resulting in a checkpoint-mediated delay (Figure 7).

The timing model proposed by Lucchesi and Suzuki predicts that a factor exists which controls the timing of meiotic prophase and can affect the level of exchange [11]. The pachytene checkpoint may regulate this timing mechanism. Although pch2 is dispensable for crossing over in a wild-type background, it is required for most of the residual crossovers that occur in recombination-defective mutants. pch2 is also required for most of the interchromosomal effect and pachytene delays observed in inversion heterozygotes. To our knowledge, pch2 is the first example of a gene in Drosophila required for the interchromosomal effect that is not required for crossing over in general. Prolonged PCH2 expression may facilitate the formation of more crossovers by simply delaying a pachytene transition and extending the crossover determination phase, thereby allowing more crossover sites to be selected. An alternative explanation is that pch2, while not required for crossover formation in wild-type,
is required for a crossover mechanism active only in axis-defective situations. Since the interchromosomal effect is not mediated by an increase in DSBs, PCH2 most likely increases the chance of DSBs becoming crossovers at the expense of noncrossovers.

**PCH2 function, localization, and mechanism of checkpoint activity**

Drosophila PCH2 localizes to peri-nuclear foci in zygote and early pachytene cells and is rapidly degraded or no longer made at mid-pachytene. In mutants in which pachytene delays are observed, PCH2 expression persists longer than in wild-type. The observation that overexpressing PCH2 induces effects on both timing and crossover levels indicates prolonged PCH2 expression is necessary and sufficient for the pachytene checkpoint’s downstream effects. Since the duration of early pachytene correlates with the domain of PCH2 expression, we suggest that degradation of PCH2 turns off checkpoint activity and allows progression through pachytene, which ends the crossover determination phase (Figure 7).

We observed PCH2 localization to the outside of the nuclear envelope. These results were surprising considering the effect a pch2 mutation has on nuclear events like crossing over. While we cannot rule out the possibility that a small undetectable fraction of PCH2 protein enters the nucleus and interacts with the chromosomes, PCH2 may indirectly affect nuclear events by facilitating interactions between the chromosomes and the nuclear envelope. In budding yeast, the pachytene checkpoint requires the localization of Pch2 to the nucleolus [15]. Therefore, like budding yeast, PCH2 in Drosophila may mediate the pachytene checkpoint at a distance from the recombination sites. Intriguingly, the nuclear envelope has been linked to several cellular processes at a distance from the recombination sites. Intriguingly, the nuclear envelope has been linked to several cellular processes. These results were surprising considering the effect a pch2 mutation has on nuclear events like crossing over. While we cannot rule out the possibility that a small undetectable fraction of PCH2 protein enters the nucleus and interacts with the chromosomes, PCH2 may indirectly affect nuclear events by facilitating interactions between the chromosomes and the nuclear envelope. In budding yeast, the pachytene checkpoint requires the localization of Pch2 to the nucleolus [15]. Therefore, like budding yeast, PCH2 in Drosophila may mediate the pachytene checkpoint at a distance from the recombination sites. Intriguingly, the nuclear envelope has been linked to several cellular processes at a distance from the recombination sites.

**Materials and Methods**

**Drosophila strains**

Drosophila stocks and crosses were maintained on a standard medium at 25°C. The following mutant alleles were used unless otherwise noted:- ord<sup>10</sup> [20], c(2)M<sup>44</sup>, pch2<sup>B101778f</sup> (pch2<sup>B17</sup>), c(3)G<sup>66</sup> [18], hid<sup>R</sup>, mei-218<sup>B</sup>, rec<sup>1</sup> and rec<sup>2</sup> [19], Exc1<sup>Y</sup> [30], mei-9<sup>-</sup>, spm<sup>A</sup>, spm<sup>B</sup>, sir2<sup>17</sup> [38], and mei-w68<sup>G52</sup>. The deficiency Df(2L)BSC245 deletes cytological bands 33F3-34A9, which includes the sir2 locus. T(2;2)<sup>DPF</sup> and T(2;3)<sup>dpf</sup> translocations were obtained from the Bloomington Stock Center. T(2;2)<sup>DPF</sup> breakpoints are at 26E-27A on the 2nd and 85C on the 3rd, T(2;3)<sup>dpf</sup> breakpoints are at 25A on the 2nd and 93B-D on the 3rd. The T(2;3)<sup>dpf</sup> translocation has breakpoints at 40 (heterochromatin) on the 2nd and 95A3 on the 3rd and was obtained from B. Wakimoto [50].

**Genetic techniques**

X-chromosome nondisjunction was assayed by crossing females to y/a<sup>18</sup> males. The frequency of X-chromosome nondisjunction is calculated as 2[Bar females + Bar<sup>-</sup> males]/[2[Bar females + Bar<sup>-</sup> males] + Bar<sup>-</sup> females + Bar males]. To estimate wild-type X chromosome crossing over frequency, y/y <i>paw</i> cm f y<sup>*</sup> female flies were crossed to C(1;1)1, v<sup>;</sup> f: (+); C(4)RM, cI cI males, and X chromosome recombinants were scored in males. Second chromosome crossing over was assayed by crossing al dp b pr cn/cn females to al dp b pr cn/CY0 males and scoring for recombinants among the CY<sup>+</sup> progeny. P-values were calculated using the Fisher’s exact test.

**Cytology and immunofluorescence**

For immuno localization experiments, females were aged at room temperature for about 16 hours and ovaries were dissected and fixed using the “Buffer A” protocol [31]. The antibody to γ-H2A.V was described by Melrho et al. [20] and used at a 1:500 dilution. Additional primary antibodies included mouse anti-C:3/G antibody used at 1:500 [18], rabbit anti-C:2/M antibody used at 1:400 [32], a combination of two mouse anti-Orb antibodies (4H6 and 6H4) used at 1:100 [53], mouse anti-Lamin antibody developed by Fisher, P.A. used at 1:800, and a rat anti-HA antibody (Roche) used at 1:15.

The secondary antibodies were Cy3 labeled goat anti-rabbit (Jackson Labs) used at 1:250, Cy3 labeled goat anti-mouse (Jackson Labs) used at 1:100 and Alexa fluor 488 goat anti-mouse (Invitrogen) used at 1:100. Chromosomes were stained with Hoechst 33342 at 1:50,000 (10 mg/ml solution) for seven minutes at room temperature. Images were collected using a Leica TCS SP2 confocal microscope with a 63X, N.A. 1.3 lens. In most cases, whole germaria were imaged by collecting optical sections through the entire tissue. These data sets are shown as maximum projections. The analysis of the images, however, was performed by examining one section at a time.

**Counting the frequency of the two-oocyte phenotype and calculating P-values**

The oocytes were observed using an anti-C:3/G antibody. In some cases, such as when C:3/G staining was not visible, anti-ORB staining was used to identify the oocyte(s). A cell was scored as an oocyte if complete SC filaments were clear and distinct or by a concentration of ORB staining in the cytoplasm of a cell [54]. P-values were calculated using the Fisher’s exact test. The P-value from the test compares the ratio of one-oocyte to two-oocyte cysts that were observed in two genotypes.

**Counting γ-H2AV foci in repair-proficient and repair-defective backgrounds**

The γ-H2AV foci were counted from germaria where the foci were clear and distinct. Foci numbers in wild-type were at a maximum in region 2a (early pachytene) and few foci were visible by region 2b (mid pachytene). Therefore, to compare foci numbers in different genotypes, we used a method that calculates all cysts with γ-H2AV foci, averaging the amount in each pair of pro-oocytes. We compared the average foci in all the pro-oocytes or oocytes of each germarium, starting with the youngest cysts at the anterior end, by examining a full series of optical sections.

For counting γ-H2AV foci in repair-defective backgrounds, ORB staining was used to identify oocytes in region 3. The foci were counted from germaria where the foci were clear and distinct. The foci were counted manually by examining each section in a full series of optical sections containing complete pro-oocyte nuclei.

**Plotting γ-H2AV foci as a function of relative cyst age**

Since the position of a cyst in the germarium is only a rough estimate of its meiotic stage, the foci were first counted in all the pro-oocytes/oocytes (identified by C:3/G staining) in the germarium. The meiotic stage of each pro-oocyte was then normalized according to the relative position of the entire cyst within the
germarium since the relative position is more important than absolute position. The pro-oocytes from 13 wild-type germaria, 4 FM7/+; 4 CyO/+; 5 FM7/++; CyO/+; 5 spn-BBu, and 4 sir2/pgc9/Df; spn-BBu were arranged according to their relative age. The average number of γH2AV foci per pro-oocyte at each stage was then calculated and plotted as a function of relative cyst age.

**Construction of PCH2 transgenes**

The annotated coding region of pch2 was obtained from Flybase and amplified off the pch2 cDNA clone LD24646 [55] by PCR. The coding region of pch2 was then cloned into the Gateway® pENTR™4 vector (Invitrogen). An LR ‘clonase’ reaction was then performed to recombine pch2 into the pHW destination vector (Invitrogen) that contains 3 copies of an N-terminus HA-tag under the control of an inducible UASP promoter. The construct was injected into fly embryos by Model System Genomics at Duke University. To express the transgenic lines, they were crossed to flies expressing Gal4 using either the NGT (P(Gal4-nos.NGT)4) [35] or MVD1 (P(Gal4:VP16-p.LTR:UASP) drivers [37]).

**Counting PCH2 foci**

The HA-PCH2 foci were counted from germaria where the foci were clear and distinct. We counted the average foci surrounding nuclei in all the cysts at region 2 and region 3 of each germarium by examining a full series of optical sections.

**Supporting Information**

**Figure S1** Transgenic rescue of pch2-dependent delay. Transgenic pch2 expressed by the NGT driver restored the high frequency of the two-oocyte phenotype found in FM7/+ heterozygotes. The two oocyte phenotype was assayed by C5/G staining and an asterisk is located above each bar when P-value was <0.05 compared to wild-type. The number of cysts counted is at the bottom of each bar.

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**Figure S2** PCH2 localization in mei-218 mutants. MVD1-driven PCH2 expression persists into region 2b and region 3 in a mei-218 mutant. To the right is shown a single section of an early pachytene oocyte with PCH2 foci adjacent to the DNA stain, indicating that mei-218 has no effect on the localization pattern of PCH2 within a cell.

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**Figure S3** C(2)M Expression by the P(UAS:c(2)MHA) transgene in wild-type and sir2 mutants. Germinaria are stained with anti-HA under the control of an inducible UASP promoter. The construct was injected into fly embryos by Model System Genomics at Duke University. To express the transgenic lines, they were crossed to flies expressing Gal4 using either the NGT (P(Gal4-nos.NGT)4) [35] or MVD1 (P(Gal4:VP16-p.LTR:UASP) drivers [37]).

**Table S1** X-Chromosome nondisjunction in ExcI, pch2 and sir2 mutants.

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**Author Contributions**

Conceived and designed the experiments: EFJ KSM. Performed the experiments: EFJ. Analyzed the data: EFJ KSM. Wrote the paper: EFJ KSM.
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