Supplementary Information for

Title: Ensuring meiotic DNA break formation in the mouse pseudoautosomal region

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SUPPLEMENTARY DISCUSSION

PAR axis dimensions and DSB formation

Because the PAR is estimated to be less than one Mb long[3,4] and the average DSB frequency in mouse spermatocytes is estimated at fewer than one DSB per ten megabase pairs (Mb)[2], this would predict fewer than one DSB for every ten meioses if the PAR behaved like average, i.e., a >90% failure rate. The autosomal genome average DNA content per μm of axis is approximately 15 Mb/μm, based on direct measurement and comparison of genome size to total SC length at pachynema[2,63]. Therefore, one DSB per ten Mb leads to an expectation of ~1.5 DSBs per μm of axis on average. The PAR is about 1 Mb/μm and undergoes at least 1–2 DSBs per meiosis (based on frequency of recombination protein foci), consistent with axis length being a critical contributor to DSB frequency[2]. However, the observation that a long PAR axis is not sufficient to ensure DSB formation in oocytes indicates that axis length is not the sole contributor to PAR DSB heat.

PAR ultrastructure vs. thickening of chromosome ends late in prophase I

There are superficial similarities between the PAR ultrastructure we document here and previously described thickening of chromosome ends at late pachynema/diplonema[64]. However, we note that there are substantial temporal and functional differences that establish that these are distinct axis behaviors: i) Axis splitting at chromosome ends occurs much later than PAR axis splitting. ii) End splitting occurs on synapsed chromosomes, whereas splitting of PAR axes is inhibited by synapsis. iii) Late axis splitting occurs on all chromosome ends and is more pronounced on centromeric ends, and thus must be mo-2-independent. iv) End structure is different from the PAR structure in that it has a more triangular shape and is enriched for SYCP3 in the center. v) End splitting is independent of RMMAI proteins because RMMAI proteins do not localize to all chromosome ends, and because ANKRD31 is dispensable for this phenomenon (data not shown).

Determinants of the collapse of elongated PAR axes

In wild-type spermatocytes, shortening of PAR axes is coincident with the initiation of X-Y synapsis at early pachynema. Because homologous synapsis requires a DSB on at least one of the two homologs and because RPA2 foci are always detectable in the PAR at early pachynema, we can conclude that collapse of the PAR loop–axis structure in early pachynema occurs after DSB formation. Furthermore, because X PARs that have non-homologously synapsed in *Spo11*$^{-/-}$ spermatocytes have the short-axis configuration typical of normal pachynema, we further infer that synapsis in the absence of recombination is sufficient to trigger collapse of the extended PAR axis structure.

We have not yet been able to similarly test the reciprocal possibility that DSB formation without synapsis is sufficient as well, in part because of the premature spermatocyte death in mutant mice that would be relevant to address this issue. However, several lines of evidence suggest it could be the case: i) MEI4 foci are not colocalized with RPA or DMC1 foci[16]. ii) MEI4 foci persist in *Spo11*$^{-/-}$ spermatocytes[22]. iii) MEI4 foci are undetectable at early-to-mid pachynema on the centromere-proximal portion of the X chromosome axis, where DSB

formation occurs but X–Y synapsis does not. Taken together, these findings suggest that DSB formation (followed by assembly of DSB repair proteins) may be sufficient to promote the local disassembly of RMMAI proteins[16,22]. Hence, a similar process could apply to the PAR and autosomal mo-2 RMMAI blobs as well.

DSB formation at mo-2 regions in oocytes

Because delaying or blocking PAR synapsis is accompanied by an increase in local RPA2 foci in oocytes, our findings are consistent with the possibility that there is little intrinsic difference between oocytes and spermatocytes with respect to the DSB-forming behavior of mo-2 regions. In other words, given enough time without synapsis, perhaps oocytes can realize the DSB potential of mo-2 regions as fully as spermatocytes can. However, autosomal mo-2 regions (into which synapsis can spread from interstitial initiation sites in both sexes) appear to experience a greater level of DSB formation in spermatocytes than oocytes (**Extended Data Fig. 8b**). This might indicate that spermatocytes have a greater intrinsic DSB potential for mo-2 regions. Oocytes may lack or have lower levels of protein factors or post-translational modifications that foster full DSB potential of mo-2 regions. Other differences between spermatocytes and oocytes that could contribute to different DSB potential include differences in duration of the stages of prophase I (spermatocytes spend considerably longer in pachynema, for example), different dynamics or timing of telomere-led chromosome movements, and different temperature.

Oocytes also appear to be less efficient than spermatocytes at pairing X and Y chromosomes. This could reflect a lesser ability to ensure that a DSB is formed, but even the cells with unsynapsed X and Y had usually succeeded in acquiring at least one PAR-associated RPA2 focus (**Extended Data Fig. 9h**), suggesting that DSB failure was not the cause of pairing and synapsis failure. One possibility is that the lack of REC8 enrichment on PARs in oocytes results in less efficient homologous recombination once a DSB has formed.

SPO11 and formation of PAR DSBs

Previous studies showed that expressing only one of the two major splicing isoforms of *Spo11* (*Spo11β*) or tagging SPO11 with the DNA binding domain of yeast Gal4 confers a specific defect in PAR DSB formation[2,65]. Our findings raise the possibility that the specialized properties of the PAR uniquely sensitize it to otherwise subtle defects in SPO11 activity.

Separation of PAR sister chromatid axes

Sister chromatids develop individualized axes late in prophase I (diplonema or diakinesis) in many species[10], and expansion microscopy of *Drosophila melanogaster* SCs suggests that sister chromatids have structurally distinct axes during pachynema[66]. Moreover, splitting of sister chromatid axes occurs in cohesion-defective mutants of mouse (*Rec8*$^{-/-}$) and *Sordaria macrospora* (*spo76*)[67,68]. Thus, there is ample precedent for individualization of sister chromatids into separate axes, but to our knowledge, splitting of the PAR in late zygonema represents the earliest stage yet documented for this phenomenon in wild-type meiosis in any species.

The purpose of PAR axis splitting in spermatocytes remains unclear. One possibility is that it helps inhibit sister chromatid recombination by physically separating the loops of the sister chromatids from one another. We do not favor this interpretation because it requires explanation as to why the mechanism(s) that controls recombination partner choice within the context of conjoined sister axes elsewhere in the genome is not sufficient in the PAR. Another possibility is that sister axis splitting is a consequence of a specialized cohesin assembly whose purpose is to stabilize the extremely distal chiasma formed by crossing over in the PAR. This hypothesis is motivated in part by the spermatocyte-specific enrichment of REC8 (not seen on PARs in oocytes) and the requirement for REC8 to maintain cohesion specifically at the distal tip of the PAR. Because REC8 is depleted across the mo-2-containing region in spermatocytes, and because REC8 is required to maintain conjoined sister axes, one possibility is that the separation of sister axes is a secondary consequence of how REC8 is localized, and that the splitting serves no specific purpose per se.

Supplementary References

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