Defective Satellite DNA Clustering into Chromocenters Underlies Hybrid Incompatibility in *Drosophila*

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**Abstract**

Although rapid evolution of pericentromeric satellite DNA repeats is theorized to promote hybrid incompatibility (HI) (Yunis and Yasmineh 1971; Henikoff et al. 2001; Ferree and Barbash 2009; Sawamura 2012; Jagannathan and Yamashita 2017), how divergent repeats affect hybrid cells remains poorly understood. Recently, we demonstrated that sequence-specific DNA-binding proteins cluster satellite DNA from multiple chromosomes into “chromocenters,” thereby bundling chromosomes to maintain the entire genome in a single nucleus (Jagannathan et al. 2018, 2019). Here, we show that ineffective clustering of divergent satellite DNA in the cells of Drosophila hybrids results in chromocenter disruption, associated micronuclei formation, and tissue atrophy. We further demonstrate that previously identified HI factors trigger chromocenter disruption and micronuclei in hybrids, linking their function to a conserved cellular process. Together, we propose a unifying framework that explains how the widely observed satellite DNA divergence between closely related species can cause reproductive isolation.

**Key words:** satellite DNA, chromocenter, hybrid incompatibility, speciation.

**Drosophila** Hybrids Exhibit Chromocenter Disruption and Micronuclei Formation

The majority of repetitive satellite DNA are present at heterochromatic regions adjacent to the centromere on eukaryotic chromosomes. Our previous work showed the importance of these pericentromeric satellite DNA in encapsulating the full complement of chromosomes into a single nucleus (Jagannathan et al. 2018, 2019). Sequence-specific DNA-binding proteins cluster their cognate pericentromeric satellite DNA repeats to create physical links between heterologous chromosomes, forming a cytological structure known as chromocenter (fig. 1A). Depletion of satellite DNA-binding proteins led to the detachment of heterologous chromosomes from one another (chromocenter disruption), their subsequent loss from the primary nucleus (micronuclei) and cell death (fig. 1B). Together, we demonstrated how identical satellite DNA repeats on multiple chromosomes are important for chromocenter formation and maintenance of the entire genome in the nucleus. This prompted us to hypothesize that the highly divergent satellite DNA repeats between closely related species may fail to form chromocenters properly, ultimately resulting in hybrid incompatibility (fig. 1C). *Drosophila melanogaster* diverged ~2–3 Ma from the *Drosophila simulans* species complex (e.g., *D. simulans* and *D. mauritiana*) (Lachaise et al. 2004) but these species still maintain near complete synteny (Sturtevant and Novitski 1941; Drosophila 12 Genomes Consortium et al. 2007; Chakraborty et al. 2021). However, the satellite DNA content of *D. melanogaster* is markedly different from that of the *D. simulans* species complex (fig. 1D) (Lohe and Brutlag 1987; Jagannathan et al. 2017). This satellite DNA divergence takes the form of changes in satellite DNA abundance, changes in chromosomal location of identical satellite DNA repeats and the presence/absence of novel repeat sequences (Jagannathan et al. 2017). Most strikingly, the (AATAACATA)n, satellite DNA whose clustering into chromocenters in *D. melanogaster* is required for viability (Jagannathan et al. 2019), is completely absent in the *D. simulans* species complex (fig. 1D). Rather, these species contain an unrelated repeat (GAACAGAACATGTTC)n at the corresponding autosomal locations (fig. 1D) (Lohe and Brutlag 1987; Jagannathan et al. 2017). In addition, the (AATAT)n satellite DNA repeat, whose clustering is important for *D. melanogaster* fertility, exhibits differential abundance and chromosomal locations between these species (fig. 1D) (Lohe and Brutlag 1987; Jagannathan et al. 2017).

The cross between *D. melanogaster* females and *D. simulans/D. mauritiana* males yields lethal hybrid males and sterile hybrid females (Sturtevant 1920) (supplementary fig. S1A, Supplementary Material online). Although previous reports have indicated that adult hybrid ovaries are nearly entirely devoid of germ cells (Hollocher et al. 2000; Matute et al. 2014) (supplementary fig. S1B, Supplementary Material online), we found that larval L3 ovaries in hybrids raised at 18 °C contained a few surviving Vasa+ germ cells (supplementary fig. S1C and D, Supplementary Material online). We first examined chromocenter formation in female hybrid germ cells.
using DNA FISH probes against the (AATAT)$_n$ repeat (present in both species), the D. melanogaster-specific (AATAACATAG)$_n$ repeat, and the D. simulans species complex-specific (GAACAGAACATGGTC)$_n$ repeat.

In the larval ovarian germ cells of pure species, both the D. melanogaster-specific (AATAACATAG)$_n$ satellite DNA and the D. simulans species complex-specific (GAACAGAACATGGTC)$_n$ satellite DNA, were clustered into approximately two to three foci (fig. 1E and F). In contrast, the larval germ cells of female D. melanogaster–D. simulans and D. melanogaster–D. mauritiana hybrids exhibited increased number of foci, indicating a striking declustering of these satellite DNA repeats (fig. 1E and F; supplementary fig. S2A, Supplementary Material online). We also observed that hybrid germ cells exhibited declustering of (AATAT)$_n$ satellite DNA in comparison to germ cells from pure species (supplementary fig. S2B, Supplementary Material online).

Male hybrids arising from the above cross (supplementary fig. S1A, Supplementary Material online) die during larval development due to the atrophy of the imaginal discs, which are tissues that are fated to form critical adult structures (Sanchez and Dübendorfer 1983; Orr et al. 1997) (supplementary table S1, Supplementary Material online). Similar to female hybrid germ cells, we observed defective chromocenter formation in hybrid male imaginal discs in comparison to the pure species controls as indicated by the increased number of satellite DNA foci (fig. 2A–C, arrows). It is suggested that karyotype and gene dosage differences between male and female hybrids (discussed in the next section) lead to female hybrids that are almost completely viable, especially at lower temperatures (Barbash et al. 2000). Interestingly, chromocenter disruption was not observed in the imaginal discs of viable female hybrids: D. melanogaster-specific (AATAACATAG)$_n$ and D. simulans-specific (GAACAGAACATGGTC)$_n$ satellite DNA were typically associated with each other, revealing intact chromocenter formation, even though these two satellite DNA repeats never coexist in the cells of pure species (fig. 2A–C, arrowheads).

Consistent with our previous reports that chromocenter disruption leads to micronuclei formation in D. melanogaster, we observed micronuclei in both the hybrid female germ cells (fig. 1G and H) as well as hybrid male imaginal discs (fig. 2D and E). In contrast, micronuclei were not observed in the intact imaginal discs of viable female hybrids (fig. 2D and E). Taken together, our data suggest that cells from atrophied tissues in hybrid animals exhibit defective chromocenter formation and micronuclei. Moreover, these cytological defects are highly correlated with cellular lethality in hybrid animals.

**Removal of Hybrid Incompatibility Genes Rescues Chromocenter Formation**

Prior work has identified D. melanogaster Hmr (Hutter and Ashburner 1987; Barbash et al. 2003), D. simulans Lhr (Watanabe 1979), and D. simulans Gfzf (Phadnis et al. 2015) as genes causing lethality in D. melanogaster–D. simulans hybrids. Lethality is primarily observed in male hybrids due to the presence of the Hmr HI gene on the D. melanogaster X chromosome; male hybrids are hemizygous for Hmr$^{mel}$ (Hmr$^{mel}$/Y) whereas female hybrids are heterozygous for Hmr (Hmr$^{mel}$/Hmr$^{sim}$). As such, the D. simulans Hmr ortholog (which is not a HI factor) or the D. simulans X chromosome more generally is thought to protect female hybrids from lethality through an uncharacterized mechanism (Barbash et al. 2000). In lethal male hybrids, the three HI genes (Hmr$^{mel}$, Lhr$^{sim}$, and Gfzf$^{sim}$) are considered to act dominantly and the removal of even one HI gene restores the viability of male hybrids (Barbash et al. 2000; Barbash 2010; Phadnis et al. 2015). Interestingly, Hmr and Lhr also localize to repetitive DNA and chromocenters in the pure species context and have been demonstrated to interact with one another (Brideau et al. 2006; Satyaki et al. 2014; Blum et al. 2017; Kochanova et al. 2020). Strikingly, we observed significant rescue of chromocenter disruption (fig. 3A and B compare with fig. 2A and B) and micronuclei formation (fig. 3C and D compare with fig. 2D and E) in the imaginal discs of hybrid males, whose viability was restored by mutating D. simulans Lhr (supplementary table S1, Supplementary Material online), suggesting that Lhr$^{sim}$ inhibits chromocenter formation in inviable hybrids. We next used a model of hybrid sterility rescue, where crossing D. melanogaster In(1)AB, Hmr$^{mel}$/FM6 females to D. simulans Lhr$^I$ males restores ovary development in female hybrids (Barbash and Ashburner 2003) (supplementary fig. S3A–C, Supplementary Material online). We found that early germ cells of rescued female hybrids (In(1)AB, Hmr$^{mel}$/++; Lhr$^I$/+) exhibited intact chromocenters (fig. 3E and F; supplementary fig. S3D and E, Supplementary Material online) and did not form micronuclei (fig. 3G and H). These results suggested that the incompatibility caused by the HI genes (Hmr and Lhr) is strongly correlated with chromocenter disruption. In contrast, germ cells of nonrescued sibling controls (FM6/++; Lhr$^I$/+) containing Hmr (supplementary fig. S3A–C, Supplementary Material online) exhibited increased declustering of the (AATAT)$_n$ satellite DNA (fig. 3E and F; supplementary fig. S3D and E, Supplementary Material online). Consistent with previous results, chromocenter disruption in the early germ cells of nonrescued hybrid ovaries was accompanied by micronuclei formation (fig. 3G and H).

Finally, we found that female hybrids containing an extra copy of D. melanogaster Hmr raised at 29 °C, which are known to be inviable (Barbash et al. 2000) (supplementary table S2, Supplementary Material online), exhibited chromocenter disruption (fig. 3I and J) and micronuclei formation in the imaginal discs (fig. 3K and L). Furthermore, these defects were rescued in female hybrids with the D. simulans Lhr$^I$ mutation (fig. 3I–L and supplementary table S2, Supplementary Material online). Taken together, our data suggest that HI genes trigger chromocenter disruption and micronuclei formation while causing hybrid incompatibility.

**The D1 Satellite DNA-Binding Protein Is Functionally Diverged between Species**

The above data imply that the inability of chromosomes from two species to form chromocenters may cause cellular defects
such as micronuclei, leading to hybrid incompatibility. This raised the question as to whether chromocenter forming proteins such as D1 and Prod, which in D. melanogaster bind and cluster the (AATAT)_n and (AATAACATAG)_n respectively (Jagannathan et al. 2018; Jagannathan et al. 2019), might have functionally diverged. Interestingly, an evolutionary analysis on D. melanogaster chromatin-associated proteins identified that the D1 gene (but surprisingly not Prod) exhibits signs of positive selection (Parey and Crombach 2019). Strikingly, we found that the expression of D. simulans D1 (D1sim) under the control of the nos-gal4 early germ cell driver failed to rescue the germ cell depletion phenotype of the D. melanogaster D1 mutant to the same extent as D. melanogaster D1 (D1mel) (fig. 4A and B; supplementary fig. S4A and B, Supplementary Material online). Moreover, chromocenter formation was clearly impaired in the germ cells of D. melanogaster D1 mutant expressing D1sim, in comparison to germ cells expressing D1mel as determined by immunostaining for the (AATAT)_n satellite DNA-binding protein. Although we did not observe micronuclei in any of the D1sim expressing germ cells, these results reveal a functional divergence between D1sim and D1mel impacting germ cell viability, despite both proteins binding the (AATAT)_n satellite DNA (supplementary fig. S4C, Supplementary Material online). Interestingly, we observed that the Prod protein colocalized with clustered (GAACAGAACATGTTC)_n satellite DNA in D. simulans (supplementary fig. S4D, Supplementary Material online), even though this repeat is highly diverged from the (AATAACATAG)_n satellite DNA that is bound by Prod in D. melanogaster. Surprisingly, D. simulans Prod (Prodsim) was able to fully rescue the loss of viability caused by mutation of D. melanogaster Prod (supplementary fig. S4E and F, Supplementary Material online) and ectopic expression of...

**Fig. 1.** Chromocenter disruption and micronuclei in the larval germ cells of D. melanogaster–D. simulans/D. mauritiana hybrids. (A–C) A model of chromocenter formation (A) and function (B) in pure species and proposed dysfunction (C) in hybrids. (D) FISH against the (AATAACATAG)_n satellite (blue), the (AATAT)_n satellite (green), and the (GAACAGAACATGTTC)_n satellite (magenta) on larval neuroblast mitotic chromosomes from the indicated species and costained with DAPI (gray). (E) FISH against the (AATAACATAG)_n satellite (green), the (GAACAGAACATGTTC)_n satellite (magenta) in the larval female germ cells from the indicated species and hybrids and costained with Vasa (blue). (F) Box-and-whisker plot of the total number of (AATAACATAG)_n and (GAACAGAACATGTTC)_n chromocenter foci per larval germ cell from D. mel y w females (n = 38), D. sim C167.4 females (n = 28), D. mel y w × C167.4 hybrid females (n = 21), and D. mel y w × mau hybrid females (n = 42). n indicates the number of germ cells analyzed, **** represents P < 0.0001 based on Tukey’s multiple comparisons test from an ordinary one-way ANOVA and crosshairs mark the mean. (G) IF against Lamin (green) and Vasa (blue) in the larval female germ cells from the indicated species and hybrids. The percentage of micronuclei-containing cells is indicated above the respective columns. n indicates the number of germ cells analyzed, **** represents P < 0.0001 from Fisher’s exact test. All scale bars are 5 μm, yellow dashed lines demarcate nuclear boundary, and white dashed lines indicate cell boundary.
Prodsim in D. melanogaster spermatocytes resulted in chromatin threads connecting heterologous chromosomes, similar to that of Prod mel (Jagannathan et al. 2019) (supplementary fig. S4G, Supplementary Material online), suggesting that Prod from D. simulans can function in the D. melanogaster background. We next tested whether D. simulans D1/Prod could complement their D. melanogaster counterparts in hybrids, where the genomes of both species are brought together in the same nucleus. To do so, we separately crossed D. melanogaster strains carrying loss-of-function alleles of Dmel\D1 (D1LL03310) and Dmel\Prod (prodk08810) to D. simulans. Consistent with previous observations that the D. melanogaster autosomes do not have any major effect hybrid lethality loci (Cuykendall et al. 2014), we did not observe a significant rescue of hybrid male lethality in comparison to the control (supplementary tables S3 and S4, Supplementary Material online). A previous study demonstrated that Prodsim was capable of complementing Prod mel function in viable female hybrids and rescued male hybrids (Itoh et al. 1999). We were able to recapitulate these findings by crossing heterozygous prod08810 females to the D. simulans C167.4 and Lhr1 strains and we observed that hybrids containing only Prodsim are at least as viable as their siblings containing both Prodmel and Prodsim (supplementary fig. S5A and B and table S3, Supplementary Material online). Similarly, we
Fig. 3. HI factors regulate chromocenter disruption and micronuclei in sterile and lethal hybrids. (A) FISH against the (AATAACATAG)_n satellite (green) and the (GAACAGAAGCATGTTC)_m satellite (magenta) on larval imaginal discs from the indicated hybrids and costained with DAPI (blue) and Hts (gray). (B) Box-and-whisker plot of total number of (AATAACATAG)_n and (GAACAGAAGCATGTTC)_m foci per cell from male \( n = 63 \) and female \( n = 63 \) D. mel y w \( \times \) Lhr\(^1\) hybrids. \( n \) indicates the number of imaginal disc cells analyzed, \( ns \) represents \( P = 0.16 \) from a Student’s t-test and crosshairs mark the mean. (C) IF against Lamin (green) in larval imaginal discs from the indicated hybrids and costained with DAPI (magenta) and phalloidin (blue). (D) Quantification of micronuclei-containing cells in the larval imaginal discs from male and female D. mel y w \( \times \) Lhr\(^1\) hybrids. The percentage of micronuclei-containing cells is indicated above the respective columns. \( n \) indicates the number of imaginal disc cells analyzed, \( ns \) represents \( P > 0.9999 \) from Fisher’s exact test. (E) FISH against the (AATAT)_n satellite (green) in the early germ cells of the indicated 0- to 2-day-old adult female hybrids. (F) Box-and-whisker plot of total number of (AATAT)_n foci per cell from Ind(1)AB, Hmr\(^{+/+}\); Lhr\(^{+/+}\) \( n = 76 \) and FM6/++; Lhr\(^{+/+}\) germ cells \( n = 73 \). \( n \) indicates the number of germ cells analyzed. ** represents \( P = 0.0074 \) from a Student’s t-test and crosshairs mark the mean. (G) IF against Lamin (green) in early germ cells from the indicated adult 0- to 2-day-old female hybrids and costained with DAPI (magenta) and phalloidin (blue). Arrows point to micronuclei. (H) Quantification of micronuclei-containing cells in the early germ cells from the indicated adult 0- to 2-day-old female hybrids. The percentage of micronuclei-containing cells is indicated above the respective columns. \( n \) indicates the number of germ cells analyzed, * represents \( P = 0.013 \) from Fisher’s exact test. (I) FISH against the (AATAACATAG)_n satellite (green) and the (GAACAGAAGCATGTTC)_m satellite (magenta) on larval imaginal discs from the indicated hybrids and costained with DAPI (blue) and Hts (gray). Arrows point to declustered satellite DNA. (J) Box-and-whisker plot of total number of (AATAACATAG)_n and (GAACAGAAGCATGTTC)_m foci per cell from Hmr-HA \( \times \) C167.4 \( n = 58 \) and Hmr-HA \( \times \) Lhr\(^1\) \( n = 62 \) female hybrids raised at 29 \(^\circ\)C. \( n \) indicates the number of imaginal disc cells analyzed, ** represents \( P = 0.0022 \) from a Student’s t-test and crosshairs mark the mean. (K) IF against Lamin (green) in larval imaginal discs from the indicated female hybrids raised at 29 \(^\circ\)C and costained with DAPI (magenta) and phalloidin (blue). Arrows point to micronuclei. (L) Quantification of micronuclei-containing cells in larval imaginal discs from the indicated female hybrids. The percentage of micronuclei-containing cells is indicated above the respective columns. \( n \) indicates the number of imaginal disc cells analyzed, ** represents \( P = 0.0017 \) from Fisher’s exact test. All scale bars are 5 \( \mu \)m, yellow dashed lines demarcate nuclear boundary, and white dashed lines indicate cell boundary.
observed that viable female hybrids and rescued male hybrids containing only D1sim are equally as viable as their siblings containing both D1mel and D1sim (supplementary fig. S5C and D and table S4, Supplementary Material online). These data suggest that D1sim and Prodsim are capable of complementing their D. melanogaster counterparts, specifically in the somatic cells of viable female hybrids and rescued male hybrids. Consistently, protein sequence alignment revealed 92.1% identity between the D1 orthologs and 97.4% identity between the Prod orthologs (supplementary fig. S6A and B, Supplementary Material online). However, our data have also shown that chromocenter formation is significantly disrupted in the atrophied imaginal discs of male hybrids and the sterile gonads of female hybrids in a HI factor-dependent manner. Therefore, in these tissues, we propose that the repeat-associated HI factors may exploit differences in the underlying sequence of species-specific D1 and Prod to impede chromocenter formation.

Chromocenter Disruption Is a Common Phenotype among Drosophila Hybrids

Due to their recent divergence ~250,000 years ago, the satellite DNA content of D. simulans and D. mauritiana are more similar to each other in comparison to D. melanogaster (Jagannathan et al. 2017). However, a few satellite DNA repeats remain distinct between these species (e.g., the D. simulans contains Y-specific (AATAAAC)n, and (AAGAGAG)n repeats, which are lacking in D. mauritiana), whereas other satellite DNA show different abundances and locations on the chromosomes. For example, the (GAACAGAACATGTTC)n, satellite DNA is restricted to chromosomes 2 and 3 of D. simulans while being present on chromosomes X, Y, 2, and 3 of D. mauritiana (Jagannathan et al. 2017). Crosses between D. simulans females and D. mauritiana males result in fertile female progeny but sterile male progeny (supplementary fig. S7A, Supplementary Material online), with the testes of sterile males reported to exhibit loss of premeiotic germ cells (Lachaise et al. 1986; Kulathinal and Singh 1998). We confirmed that the testes of male hybrids exhibited dramatic early germ cell loss with age (supplementary fig. S7B, Supplementary Material online). Moreover, we observed chromocenter disruption of the (GAACAGAACATGTTC)n, satellite DNA (fig. 4F and G) and micronuclei formation (fig. 4H and I), occurring specifically in the male germ cells of hybrid testes. In contrast, germ cell content and tissue morphology were intact in the fertile female hybrids between D. simulans and D. mauritiana (supplementary fig. S7C, Supplementary Material online). Therefore, chromocenter disruption due to differences in satellite DNA composition, even between very recently diverged species, may drive micronuclei formation and cell death and promote hybrid incompatibility.

The divergence of satellite DNA repeats between species has been long postulated to mediate hybrid incompatibility (HI) and reproductive isolation. However, poor conservation of these noncoding repeats across species is also one of the main reasons why satellite DNA is typically considered to be “junk DNA,” making it difficult to speculate on the “incompatibility of (useless) junk.” Our recent work identified a conceptual and mechanistic framework to understand how satellite DNA functions within species: pericentromeric satellite DNA association between heterologous chromosomes (chromocenters) bundles the entire set of chromosomes within the nucleus (Jagannathan et al. 2018; Jagannathan et al. 2019). In this study, we demonstrate that this framework can explain how divergent satellite DNA repeats impede the viability and fertility of hybrids. Indeed, we provide the first proof-of-concept evidence that cells from hybrid animals containing distinct satellite DNA repeats exhibit phenotypes unique to chromocenter disruption. Our study lays the foundation for understanding hybrid incompatibility at a cellular level in Drosophila as well as other eukaryotes.

What is the potential mechanism behind chromocenter disruption in affected hybrid tissues? We have previously shown that binding of satellite DNA repeats by sequence-specific binding proteins forms DNA-protein modules, which interact with each other to form chromocenters. Our data in this study indicate that HI factors (Hmrsim, Lhrtsim, and potentially Gfzfsim) are upstream of this process and directly impede chromocenter formation in hybrids. Previous work from other groups have shown that Hmr and Lhr can localize to chromocenters (Brudeau et al. 2006; Satyaki et al. 2014; Kochanova et al. 2020) and exhibit increased expression levels in lethal male hybrids (Thomae et al. 2013). Moreover, Hmr and Lhr colocalize/form complexes with Gfzf in both pure species and hybrids (Cooper et al. 2019; Lukacs et al. 2021). We therefore speculate that a complex of HI factors in hybrids could inhibit either the protein–DNA or protein–protein interactions that contribute to chromocenter formation (fig. 4J). Interestingly, recent work has demonstrated that Hmr and Lhr facilitate the detachment of pericentromeric heterochromatin from sister chromatids during anaphase in D. melanogaster (Blum et al. 2017). A tempting possibility is that Hmr/Lhr may perform this function aberrantly in hybrids and detach pericentromeric heterochromatin from heterologous chromosomes during interphase, thus triggering chromocenter disruption.

Finally, we postulate that it is the rapid divergence of satellite DNA repeats that necessitates coevolution of proteins involved in the formation and regulation of chromocenters, with each species continually fine-tuning a “chromocenter strategy” for its own unique repeat content. Encapsulating the genomes of two organisms in a single cell (hybrids) could trigger a clash of strategies that results in chromocenter disruption and loss of cellular and organismal viability. We are therefore struck by the potential for satellite DNA turnover within species to alter evolutionary trajectories of chromocenter-associated proteins, thereby planting the seed for subsequent reproductive isolation and speciation.

Materials and Methods

Fly Husbandry and Strains
All fly stocks were raised on standard Bloomington medium at 25 °C unless otherwise indicated. D. melanogaster y w was...
FIG. 4. Functional divergence of the D1 chromocenter forming protein from D. melanogaster and D. simulans. (A) D1 mutant (D1<sup>1L03170</sup>) testes expressing HA-tagged UAST-D1<sup>mel</sup> and UAST-D1<sup>sim</sup> under the control of nos-gal4 were stained for Vasa (gray). Scale bars are 25 μm. (B) Quantification of HA<sup>+</sup> germ cells in testes from 0- to 4-day-old and 12- to 16-day-old males of the following genotypes, nos>D1<sup>mel</sup>; D1<sup>LL03310</sup> (n = 29, 0–4 days) and (n = 31, 12–16 days) and nos>D1<sup>sim</sup>; D1<sup>LL0310</sup> (n = 31, 0–4 days) and (n = 32, 12–16 days). n indicates the number of testes analyzed, ** represents P = 0.006 (0–4 days) and P = 0.001 (12–16 days) from a Student’s t-test. (C) IF against Prod (magenta), HA (green), and Lamin (blue) in spermatogonial cells of the indicated genotypes. (D) Box-and-whisker plot of HA foci/cell in D1 mutant (D1<sup>1L03170</sup>) spermatogonia expressing D1<sup>mel</sup>-HA (n = 57) or D1<sup>sim</sup>-HA (n = 60). n indicates the number of germ cells analyzed, **** represents P < 0.0001 from Student’s t-test and crosshairs mark the mean. (E) Box-and-whisker plot of Prod foci/cell in D1 mutant (D1<sup>1L03170</sup>) spermatogonia expressing D1<sup>mel</sup>-HA (n = 57) or D1<sup>sim</sup>-HA (n = 60). n indicates the number of germ cells analyzed, **** represents P < 0.0001 from Student’s t-test and crosshairs mark the mean. (F) FISH against the (GAACAGAACATGTTC)<sub>n</sub> satellite (magenta) in spermatogonial cells from the indicated 0- to 3-day-old males and costained with DAPI (blue) and Lamin (green). (G) Box-and-whisker plot of large autosomal (GAACAGAACATGTTC)<sub>n</sub> foci per spermatogonial cell from D. sim C167.4 (n = 39) and D. sim C167.4 × D. mau <sup>+</sup> (n = 31), n indicates the number of germ cells analyzed, ** represents P = 0.0067 from a Student’s t-test and crosshairs mark the mean. (H) IF against Lamin (green) in spermatogonial cells from the indicated 0- to 3-day-old males and costained with DAPI (magenta) and phalloidin (blue). Arrows point to micronuclei. (I) Quantification of testes containing at least one cell with a micronucleus from 0- to 4-day-old D. sim C167.4 pure species males (n = 18) and 0- to 4-day-old D. sim C167.4 × D. mau hybrid males (n = 26). The percentage of micronuclei-containing testes is indicated above the respective columns. n indicates the number of testes analyzed, ** represents P = 0.0027 from Fisher’s exact test. All scale bars (except panel A) are 5 μm and yellow dashed lines demarcate nuclear boundary. (J) A speculative model of how hybrid incompatibility factors may inhibit chromocenter formation by species-specific satellite DNA-binding proteins in cells of affected hybrid tissues.
used as a wild type stock. The following fly stocks were obtained from the Drosophila species stock center: D. simulans w$^+$ (DSSC#14021-0251.195), D. mauritiana w$^+$ (DSSC#14021-0241.60), D. mauritiana w$^+$ wild type (DSSC#14021-0241.150), D. simulans Lhr$^+$ (DSSC# 14021-0251.023), D1$^{L03310}$ (DGRC140754), FRT42D prod$^{508810}$ (DGRC111248), and D. simulans C167.4 (DGRC107850) were obtained from the Kyoto stock center. prod$^+$ (BDSC42686) was obtained from the Bloomington Drosophila stock center. Hmr-HA (Satyaki et al. 2014) and In(1)AB, Hmr$^+$/F86 (Barbash and Ashburner 2003) were gifts from Daniel Barbash, whereas the D. simulans Tsimbazaza strain (Hollocher et al. 2000) was a gift from Patricia Wittkopp. nos-gal4 (Van Doren et al. 1998) and bam-gal4 (Chen and McKearin 2003) and pUASt-GFP-Prodmel (Jagannathan et al. 2019) have been previously described.

Transgene Construction
For construction of pUASt-GFP-Prodmel, a codon optimized Prodmel ORF was subcloned into the NotI and KpnI sites of pUASt-EGFP-attB (Salzmann et al. 2013) resulting in pUASt-GFP-Prodmel. For construction of p400-GFP-Prodmel, a 400 bp promoter upstream of the Prod start site from D. melanogaster was PCR amplified using the following primer pair, GATCGAATTCCCGGGTATCCTTGCTC and subcloned into the HindIII and EcoRI sites on pUASt-GFP-Prodmel, replacing the UAS sequence. Transgenic flies were generated for both plasmids using PhiC31 integrase-mediated transgenesis into the attP0 site (BestGene). The following plasmids, pUASt-D1mel-HA and pUASt-D1sim-HA were obtained from Dan Barbash (Ferree and Barbash 2009) and transgenic flies were generated using PhiC31 integrase-mediated transgenesis into the attP2 site (BestGene).

Immunofluorescence Staining and Microscopy
For Drosophila tissues, immunofluorescence staining was performed as described previously (Cheng et al. 2008). Briefly, tissues were dissected in PBS, transferred to 4% formaldehyde in PBS, and fixed for 30 min. Tissues were then washed in PBS-T (PBS containing 0.1% Triton-X) for at least 60 min, followed by incubation with primary antibody in 3% bovine serum albumin (BSA) in PBS-T at 4 °C overnight. Samples were washed for 60 min (three 20-min washes) in PBS-T, incubated with secondary antibody in 3% BSA in PBS-T at 4 °C overnight, washed as above, and mounted in VECTASHIELD with DAPI (Vector Labs). The following primary antibodies were used: rabbit anti-Vasa (1:200; d-26; Santa Cruz Biotechnology), rat anti-Prod (gift from Tibor Torok, 1:5000), and guinea pig anti-Mec (generated using the synthetic peptide DGENDANDGYSDNYNDSSEVAA [Covance]). Fluorescent images were taken using a Leica TCS SP8 confocal microscope with 63× oil-immersion objectives (NA = 1.4). Brightfield images were acquired using a Keyence microscope. Images were processed using Adobe Photoshop software.

DNA Fluorescence In Situ Hybridization
Whole mount Drosophila tissues were prepared as described above, and optional immunofluorescence staining protocol was carried out first. Subsequently, samples were postfixed with 4% formaldehyde for 10 min and washed in PBS-T for 30 min. Fixed samples were incubated with 2 mg/ml RNase A solution at 37 °C for 10 min, then washed with PBS-T + 1 mM EDTA. FISH using heat denaturation was carried out as follows: samples were washed in 2xSSC-T (2xSSC containing 0.1% Tween-20) with increasing formamide concentrations (20%, 40%, and 50%) for 15 min each followed by a final 30-min wash in 50% formamide. Hybridization buffer (50% formamide, 10% dextran sulfate, 2× SSC, 1 mM EDTA, 1 μM probe) was added to washed samples. Samples were denatured at 91 °C for 2 min, then incubated overnight at RT. For hybrid tissues, FISH using acid denaturation was used instead of heat denaturation and carried out as follows: samples were washed in 2xSSC-T, DNA was denatured using a 30-min incubation with 2 N HCl at RT followed by three rinses in ice-cold PBS-T. Hybridization buffer (60% formamide, 10% dextran sulfate, 2× SSC, 1 mM EDTA, 0.5–1 μM probe) was added to washed samples and incubated overnight at RT. For mitotic chromosome spreads, larval 3rd instar brains were squashed according to previously described methods (Larracuent and Ferree 2015). Briefly, tissue was dissected into 0.5% sodium citrate for 5–10 min and fixed in 45% acetic acid/2.2% formaldehyde for 4–5 min. Fixed tissues were firmly squashed with a cover slip and slides were submerged in liquid nitrogen until bubbling ceased. Coverslips were then removed with a razor blade and slides were dehydrated in 100% ethanol for at least 5 min. After drying, hybridization mix (50% formamide, 2× SSC, 10% dextran sulfate, 200 ng of each probe) was applied directly to the slide, samples were heat denatured at 95 °C for 5 min and allowed to hybridize overnight at room temperature. Following hybridization, slides were washed three times for 15 min in 0.2× SSC and mounted with VECTASHIELD with DAPI (Vector Labs). The following probes were used for Drosophila in situ hybridization: (AATAT)$_6$ (AATAACATAG)$_3$ and (GAACAGAAACATGTCGCAAGAACAATGTCGGAACA) and have been previously described (Jagannathan et al. 2017).

Rescue Experiments
The pUASt-D1mel-HA and pUASt-D1sim-HA transgenes were each recombined with the D1$^{L03310}$ mutant allele to generate two rescue strains (pUASt-D1mel-HA, D1$^{L03310}$, and pUASt-D1sim-HA, D1$^{L03310}$). These rescue strains were crossed to flies containing the nos-gal4 germ cell driver and the D1 mutant allele (nos-gal4;VP16; D1$^{L03310}$) at room temperature as follows,

$D1^{mel}$ rescue: nos-gal4;VP16/CyO; D1$^{L03310}$/TM2×pUASt-D1mel-HA, D1$^{L03310}$/TM6B
D1sim rescue: nos-gal4::VP16/Cyo; D1<sup>LEO3310</sup>/TM2×pUASt-D1sim-HA, D1<sup>LEO3310</sup>/TM6B

We collected 0- to 4-day-old and 12- to 16-day-old D1 mutant flies expressing D1<sup>med</sup> and D1sim (nos>UASt-D1<sup>med</sup>; D1<sup>LEO3310</sup>/D1<sup>LEO3310</sup> and nos>UASt-D1sim; D1<sup>LEO3310</sup>/D1<sup>LEO3310</sup>) and dissected testes to score the number of HA+ germ cells and assess the extent of rescue. The Prod<sup>sim</sup> rescue allele was generated by recombining the p400-GFP-Prodsim transgene with prod<sup>408810</sup>. The Prod<sup>sim</sup> rescue allele and the FRT42D prod<sup>408810</sup> allele as a control were each crossed to prod<sup>4</sup> in vials at 25 °C. The percent of transheterozygous prod mutant flies was quantified in each replicate.

Supplementary Material

Supplementary data are available at Molecular Biology and Evolution online.

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