Developmental Biology, Genetics and Genomics

ARID1A governs the silencing of sex-linked transcription during male meiosis in the mouse

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Abstract

We present evidence implicating the BAF (BRG1/BRM Associated Factor) chromatin remodeler in meiotic sex chromosome inactivation (MSCI). By immunofluorescence (IF), the putative BAF DNA binding subunit, ARID1A (AT-rich Interaction Domain 1a), appeared enriched on the male sex chromosomes during diplonema of meiosis I. The germ cell-specific depletion of ARID1A resulted in a pachynema arrest and failure to repress sex-linked genes indicating a defective MSCI. Consistent with this defect, mutant sex chromosomes displayed an abnormal presence of elongating RNA polymerase II coupled with an overall increase in chromatin accessibility detectable by ATAC-seq. By investigating potential mechanisms underlying these anomalies, we identified a role for ARID1A in promoting the preferential enrichment of the histone variant, H3.3, on the sex chromosomes, a known hallmark of MSCI. Without ARID1A, the sex chromosomes appeared depleted of H3.3 at levels resembling autosomes. Higher resolution analyses by CUT&RUN revealed dramatic shifts in sex-linked H3.3 associations from discrete intergenic sites and broader gene-body domains to promoters in response to the loss of ARID1A. Several sex-linked sites displayed ectopic H3.3 occupancy that does not co-localize with DMC1 (DNA Meiotic Recombinase 1). This observation suggests a requirement for ARID1A in DMC1 localization to the asynapsed sex chromatids. We conclude that ARID1A-directed H3.3 localization influences sex chromosome gene regulation and DNA repair during meiosis I.

eLife assessment

This paper presents a **useful** study regarding the meiotic functions of ARID1A, the DNA-binding component of the SWI/SNF chromatin remodeler BAF. Whereas this work suggests that ARID1A regulates chromatin composition of the sex body relative to the autosomes, the reviewers raised substantial issues with the data and interpretation. The efficiency of the conditional deletion allele seems low and the CUT&RUN experiments are **inadequate**.

Introduction

Meiosis is central to the formation of haploid gametes from diploid pluripotent progenitors. During meiosis, homologous chromosomes pair, exchange genetic material, align at the metaphase plate and then segregate during the first meiotic cell division. Unlike autosomal homologues, the X and Y chromosomes share limited homology. Unique mechanisms have evolved to ensure these chromosomes are identified, sequestered, and processed in a parallel but distinct process from autosomes.

XY chromosomes pair along the pseudo-autosomal region (PAR), leaving extensive regions of the X and Y unpaired. These regions become enriched for DNA damage response (DDR) factors, including ATR (Ataxia Telangiectasia and Rad3 related), TOPBP1 (DNA topoisomerase II-binding protein 1), and MDC1 (Mediator of DNA Damage Checkpoint 1), which amplifies γ H2A.X across XY chromatin (Alavattam et al., 2021; ElInati et al., 2017; Fernandez-Capetillo et al., 2003; Ichijima et al., 2011; Royo et al., 2013; Turner et al., 2004). XY chromatin acquires further distinguishing features when canonical histones H3.1/3.2 are exchanged for variant H3.3 in that H3K9me3 (Histone lysine9 trimethylation) becomes elevated on XY chromatin relative to autosomes (Hirota et al., 2018; van der Heijden et al., 2007; Yuen et al., 2014). As the XY chromosomes become epigenetically distinct from autosomes, they also become physically separated into a unique nuclear sub-compartment known as the XY body (Handel, 2004). Transcriptional silencing of unpaired chromatin occurs during this phase of meiosis I, known as meiotic silencing of unpaired chromatin, MSUC (Turner, 2007). MSUC of autosomal chromatin, such as in response to an abnormal karyotype, causes pachynema arrest and cell death (Baarends et al., 2005; Schimenti, 2005; Turner et al., 2005). However, silencing unpaired XY chromatin results in a different outcome known as meiotic sex chromosome inactivation (MSCI) (Alavattam et al., 2021). Sex chromosomes are required to undergo MSCI and become transcriptionally inactivated. Failure to achieve MSCI triggers arrest and cell death (Ichijima et al., 2012).

Chromatin factors that influence MSCI include the H3K9 methyltransferase, SETDB1 (SET domain, bifurcated 1), PRC1 associated SCML2 (Sex comb on midleg-like protein 2), and testis-specific reader of histone acetylation, BRDT (Bromodomain testis-specific protein) (Hasegawa et al., 2015; Hirota et al., 2018; Manterola et al., 2018). Although our previous studies demonstrated a critical requirement for SWI/SNF-directed transcriptional regulation during meiosis, evidence for its involvement in MSCI is lacking (Menon et al., 2019). This lack of evidence is also true for the PBAF (POLYBROMO1-BRG1/BRM Associated Factor) remodeler within the SWI/SNF family, which we have shown is required for meiotic cell division (Menon et al., 2021). Interestingly, the germ cell-specific depletion of the SWI/SNF catalytic subunit, BRG1 (Brahma-related gene-1), was shown to result in an early pachytene arrest (Kim et al., 2012). Whether this suggests a role in MSCI remains an open question given that BRG1 associates with both SWI/SNF remodelers, PBAF and BAF.

Here, we find that ARID1A (AT-rich Interaction Domain 1a), a BAF-specific putative DNA binding subunit, is required for the transcriptional silencing of the sex chromosomes during pachynema, thereby ensuring meiotic progression through prophase-I. Mechanistically, ARID1A is necessary to establish MSCI hallmarks, such as the eviction of elongating RNA polymerase-II (phosphorylated on Serine2 of carboxy-terminal domain; pSer2-RNAPII), limited sex-linked chromatin accessibility, and hyper-accumulation of histone H3.3 on the sex body. Additionally, ARID1A is required for the targeted localization of DMC1 to the unpaired sex chromatids, implicating BAF-A-governed sex-linked chromatin dynamics in DNA repair.

Results

Meiotic progression requires ARID1A

Meiotic progression in male mice depends on SWI/SNF-regulated gene expression (Menon et al., 2019; Menon et al., 2021). These studies raise the prospect of distinct SWI/SNF subcomplexes governing stage-specific meiotic transcription (Menon et al., 2021). To understand the meiotic functions of the biochemically distinct SWI/SNF BAF subcomplex, we examined the spermatogenic expression profiles of the BAF subunits *Arid1a* and *Arid1b* using single-cell RNA-seq data generated from testes (Ernst et al., 2019). *Arid1a* mRNA expression occurred at various spermatogenic stages with notable expression at pachynema of meiotic prophase I (Fig. S1A-C).

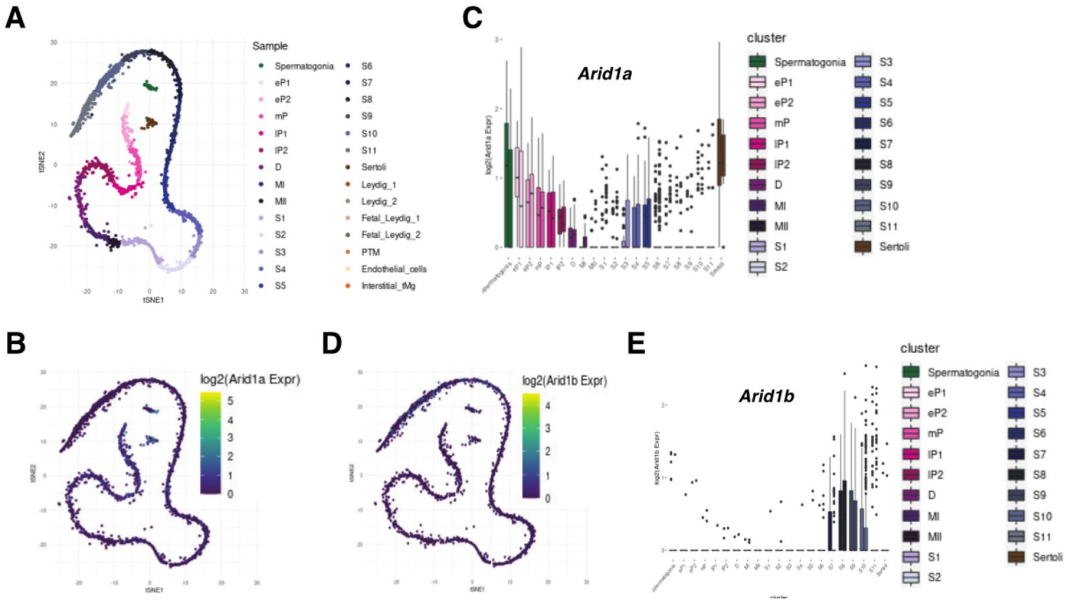


Figure S1.

ARID1A transcripts detected in pachytene spermatocytes.

scRNA-seq profiles of *Arid1a* and *Arid1b* in adult testes from B6 mice (Ernst et al., 2019). (A) tSNE plot describing the clustering of various spermatogenic stages. LOG2 normalized expression counts of (B,C) *Arid1a* and (D,E) *Arid1b* represented by tSNE plots and bar plots. (B,D) tSNE plots illustrating the first (tSNE1, x-axis) and second dimensions (tSNE2, y-axis) . (C,E) Bar plots describe the gene's mRNA abundance (y-axis) across identified germ cell clusters (x-axis). (A) eP: early pachynema, mP: mid pachynema, lP: late pachynema, MI: Metaphase-I, MII: Metaphase-II, S1-S11: Spermatid stages. Plots generated with shiny app (https://marionilab.cruk.cam.ac.uk/SpermatoShiny)

Arid1b mRNA went undetectable until late in spermiogenesis (Fig. S1A, D-E). Consistent with its mRNA expression profile, pachytene spermatocytes featured abundant ARID1A protein, which was uniformly spread (Fig. 1). Late in diplonema, ARID1A appeared preferentially enriched on the sex chromosomes as compared to autosomes (Fig. 1). These data implicate

ARID1A in meiotic sex chromosome inactivation (MSCI), a process essential for meiotic progression in males.

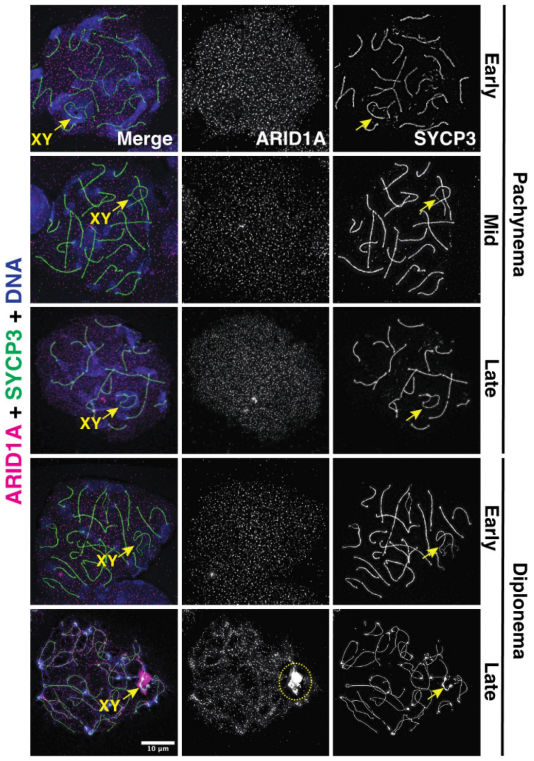


Fig 1.

ARID1A associates with the sex body late in meiotic prophase-I.

Representative pachytene and diplotene spermatocyte spreads immunolabelled for ARID1A (magenta) and SYCP3 (green) and then counterstained for DNA (blue). Scale bar:10 μm, magnification: 100x.

To determine whether meiotic spermatocytes require ARID1A, we generated a germ cell-specific knock-out of *Arid1a* using the *Stra8-Cre* transgene expressed in spermatogonia (Sadate-Ngatchou et al., 2008). Contrary to our expectations, meiosis appeared unperturbed, as evidenced by seminiferous tubules featuring round and elongated spermatids in $Arid1a^{cKO}$ testes (Fig. S2A). Furthermore, $Arid1a^{cKO}$epididymides featured mature spermatozoa, suggesting that mutants are capable of normal spermatogenesis (Fig. S2B). Although these data indicate that ARID1A is dispensable for spermatogenesis, we wanted to confirm that these results were not a technical artifact arising from inefficient *Stra8-Cre* mediated excision of the $Arid1a^{fl}$ allele.

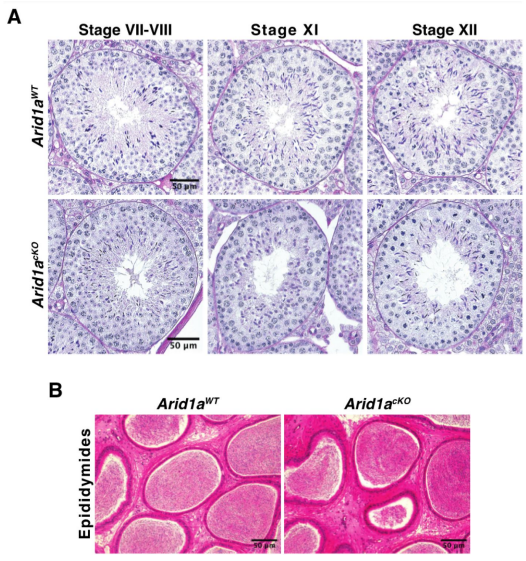


Fig S2.

Spermatogenesis appears unperturbed in $Arid1a^{cKO}$ males.

Histological examination of (A) PAS-stained seminiferous tubule sections and (B) H&E-stained cauda epididymal sections obtained from 10-week-old $Arid1a^{WT}$ *and* $Arid1a^{cKO}$ mice. The micrograph depicts seminiferous tubule staging and scale bars (50 μm). Magnification: 40x.

To address this question, we examined *Arid1a* transcripts isolated from $Arid1a^{WT}$ and $Arid1a^{cKO}$ Sta-Put purified populations of pachytene spermatocytes and round spermatids using RT-PCR (Fig. S3A). For cDNA synthesis, we used primers that amplify a 612 bp region spanning the *Arid1a* floxed exons 5 and 6. Assuming 100% CRE efficiency, we would expect to observe a 281 bp cDNA product associated with mRNA isolated from $Arid1a^{cKO}$ spermatogenic cells. Instead, we observed cDNAs representative of the floxed (*fl*) allele and, to a greater extent, the excised (*Δ*) allele from mRNA isolated from $Arid1a^{cKO}$ pachytene spermatocytes. These results indicated inefficient CRE activity (Fig. S3A). More importantly, the data suggest that a fraction of pachytene spermatocytes in $Arid1a^{cKO}$ testes fail to undergo CRE-mediated excision resulting in escapers (internal controls). An examination of ARID1A levels in testes cryosections and meiotic spreads by immunofluorescence (IF) revealed a heterogenous population of pachytene spermatocytes consisting of mutants lacking and internal controls expressing ARID1A (fig. S3B, C).

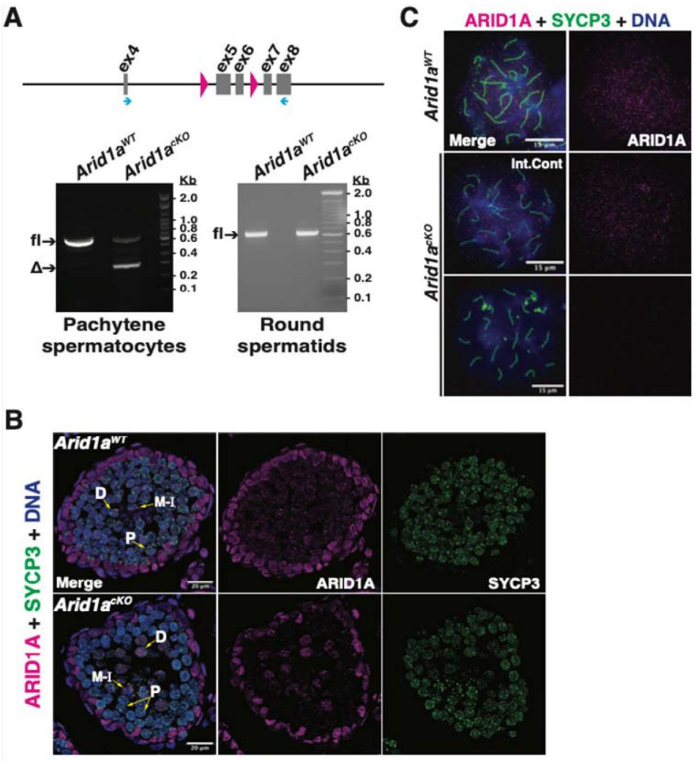


Fig S3.

Arid1a^{cKO} testes display inefficient *Stra8-Cre* activity.

(A) Schematic (top) illustrating the region of *Arid1a*^{fl} allele spanning exons (ex) 4-8 (grey boxes) along with the location of *loxP* sites (solid magenta arrowheads) and primer annealing sites (blue arrows). (A bottom): analysis of the RT-PCR of *Arid1a* transcripts from STA-PUT purified populations of *Arid1a*^{WT} *and Arid1a*^{cKO} pachytene spermatocytes (left) and round spermatids (right). DNA electrophoresis gels indicate bands corresponding to the fl: floxed and Δ: excised PCR products. *Arid1a*^{WT}and *Arid1a*^{cKO} (B) testes cryosections, and (C) spermatocyte spreads, immunolabelled for ARID1A (magenta), SYCP3 (green), and counterstained for DNA (blue). (B) Scale bar: 20 μm, magnification: 63x, P: Pachytene, D: Diplotene, and M-I: Metaphase-I spermatocytes. (C) Scale bar:15 μm, magnification: 100x.

Furthermore, examining the meiotic profile associated with $Arid1a^{cKO}$ revealed an accumulation of mutant spermatocytes (ARID1A^{-}) at mid-pachynema relative to $Arid1a^{WT}$spermatocyte spreads (Table 1). This result is indicative of a pachytene arrest. Although a few diplotene spermatocytes displaying a moderate reduction in ARID1A levels occurred in $Arid1a^{cKO}$ relative to $Arid1a^{WT}$ meiotic spreads (Table 1), mutant (ARID1A^{-}) diplotene and metaphase-I spermatocytes were undetectable in $Arid1a^{cKO}$ testes (fig. S3B). It is possible that the pachytene spermatocytes that escape CRE activity progress to subsequent meiotic stages giving rise to genotypically normal gametes. All round spermatids isolated from $Arid1a^{cKO}$ testes appeared only to express the normal transcript associated with the floxed allele (fig. S3A). Therefore, the perceived lack of a phenotype is an artifact of inefficient *Stra8-Cre* inefficiency. But more importantly, the data indicate that ARID1A is essential for progression beyond pachynema.

Table 1.

The loss of ARID1A results in a pachytene arrest.

Table outlining the distribution of P23 *Arid1a*^{WT} and *Arid1a*^{cKO}meiotic prophase-I profiles. SYCP3 staining determined meiotic staging. Co-staining of ARID1A identified mutant spermatocytes from internal controls. The total number of spermatocytes scored included 166 for *Arida*$^{fl/fl}$and 124 for *Arid1a*^{cKO}. * denotes *Arid1a*^{cKO} diplotene spermatocytes with partially reduced but not complete loss of ARID1A signal relative to controls.

Meiotic Profile (P23)	*Arid1a*$^{fl/fl}$ (n= 166)	*Arid1a*^{cKO} (n= 124)
Pre-Lep/Leptonema	3.6 %	13.7 %
Zygonema	-	15.3 %
Early Pachynema	13.8 %	12.9 %
Mid Pachynema	22.89 %	40.3 %
late Pachynema	7.22 %	7.2 %
Early Diplonema	38.5 %	4.8 % *
Late Diplonema	14.45 %	5.6 % *

ARID1A regulates the transcriptional silencing of the sex chromosomes at pachynema

The meiotic requirement of ARID1A and, more importantly, its association with the sex chromosomes at diplonema prompted us to examine its role in MSCI. We hypothesized that ARID1A might play a role in the transcriptional silencing of the sex-linked genes during meiosis. To address this question, we performed RNA-seq on Sta-Put purified populations of pachytene spermatocytes to profile changes in transcript abundance upon ARID1A deficiency. Differential analysis of gene expression using DeSeq2 (Love et al., 2014) revealed an equal proportion of significantly (FDR < 0.05) misexpressed genes displaying either elevated (up-regulated, n= 5824) or reduced (down-regulated, n=5821) transcript abundance in *Arid1a*^{cKO} relative to *Arid1a*^{WT} pachytene spermatocytes (Fig. 2A). Notably, we detected significant misexpression of 53 % of all the sex-linked coding genes (593/1105) in response to an ARID1A deficiency. Amongst these, 86.4% displayed elevated expression (n=512), whereas only 13.6% displayed reduced (n=81) transcript abundance. This skew was even starker when only considering sex-linked genes misexpressed by a magnitude of 2 LOG-fold or higher. Here, 97% (297/306) of the misexpressed sex-linked genes displayed increased transcript abundance in response to an ARID1A deficiency. Therefore, ARID1A predominantly affects the repression of sex-linked genes during pachynema.

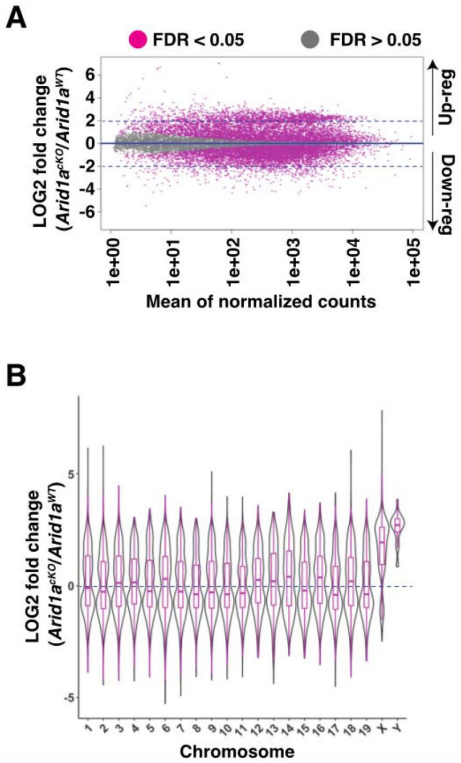


Fig 2.

Requirement of ARID1A for the repression of sex-linked genes.

(A) MA plot describing the LOG2-fold-change (LFC, y-axis) in mean expression (x-axis) of genes displaying significant (FDR≤0.05, magenta dots) changes in *Arid1a*^{cKO} relative to *Arid1a*^{WT} pachytene spermatocytes. Dashed blue lines denote 2 LFC. Gray dots (FDR≥0.05) depict non-significant changes in gene expression. (B) Violin plot describing the LOG2-fold-change (LFC, y-axis) in the median chromosome-wide gene expression (x-axis) in *Arid1a*^{cKO} relative to *Arid1a*^{WT} pachytene spermatocytes. The dashed blue line denotes no change in gene expression.

An examination of changes in the average transcript abundance on a chromosome-wide basis showed that expression from the sex chromosomes was significantly higher than that from autosomes in response to the loss of ARID1A at pachynema (Fig. 2B). Therefore, ARID1A regulates the transcriptional repression of the sex chromosomes, implicating it in MSCI. Its predominant influence on sex-linked gene regulation may result from a preferential association of ARID1A with the sex chromosomes during meiosis. This association is undoubtedly the case late in diplonema. However, earlier in pachynema, when MSCI initiates, no sex chromosome-specific pattern of ARID1A enrichment was discernible, at least by IF (Fig. 1).

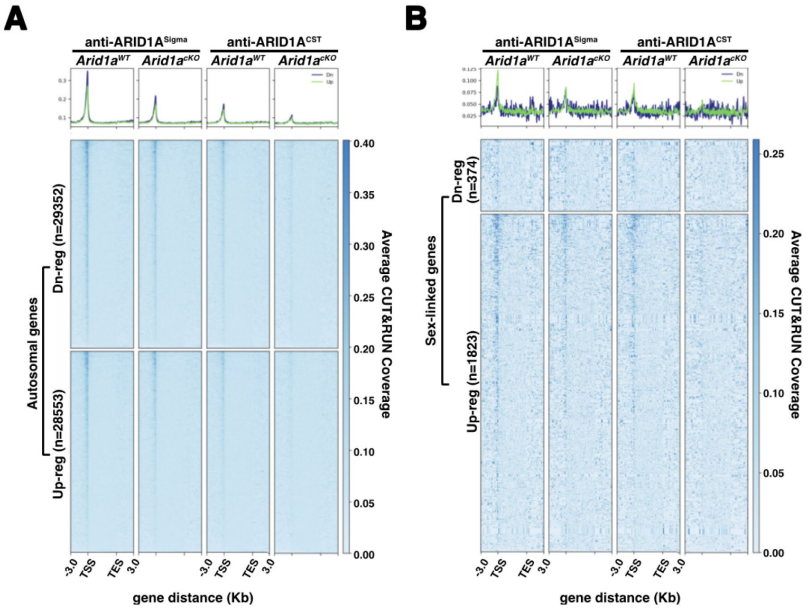


Fig S4.

Transcription start sites of differentially expressed autosomal and sex-linked genes display ARID1A occupancy.

(A-B) Heatmap (bottom) and metaplot (top) displaying the average gene-wide enrichment of ARID1A associated with differentially expressed (A) autosomal and (B) sex-linked genes in *Arid1a*^{WT} and *Arid1a*^{cKO} pachytene spermatocytes. (A-B) The number of RefSeq annotations (n) associated with differentially regulated autosomal and sex-linked genes (rows) is indicated. Up-reg: misexpressed and Dn-reg: misrepressed genes in response to the loss of ARID1A. Average ARID1A

CUT&RUN coverage was determined using two antibodies (anti-ARID1A^{Sigma}; anti-ARID1A^{CST}) plotted across RefSeq genes ± 3Kb.

We next determined ARID1A genomic localization at a higher resolution by CUT&RUN (Cleavage Under Targets & Release Using Nuclease) in pachytene spermatocytes. We detected ARID1A occupancy at promoters of differentially regulated target genes, irrespective of their chromosomal location, in $Arid1a^{WT}$ relative to $Arid1a^{cKO}$ (negative control) pachytene spermatocytes (Fig. S4). This result is consistent with the genome-wide distribution of ARID1A observed in pachytene spermatocyte spreads by IF (Fig. 1). Unnoticeable to IF, pachytene spermatocytes displayed a preferential association of ARID1A with promoters of normally repressed sex-linked genes, when detected by CUT&RUN (Fig. S4B), emphasizing its role in MSCI.

ARID1A does not influence DNA damage response signaling on the sex body

Unlike autosomal homologs that complete pairing during pachynema, the non-homologous regions of the sex chromosomes feature unrepaired DNA double-strand breaks (DSBs). These DNA DSBs recruit γH2Ax, a product of DNA damage response (DDR) signaling pathways essential for establishing and maintaining MSCI (Turner, 2007). Therefore, to determine whether ARID1A regulated MSCI by influencing DDR signaling, we first monitored the association of γH2Ax with the sex chromosomes in response to the loss of ARID1A. By IF, γH2Ax accumulation on the sex body appeared unperturbed in the mutant (ARID1A^{-}) relative to internal controls (ARID1A^{+}) and $Arid1a^{WT}$ pachytene spermatocytes (Fig. S5A). Consistent with this, the recruitment of ATR (Ataxia Telangiectasia and Rad3 related), a kinase known to phosphorylate γH2Ax and initiate MSCI (Turner et al., 2004; Royo et al., 2013), appeared normal in the absence of ARID1A. These data indicate that sex-linked DDR signaling occurred independently of ARID1A (Fig. S5B). Furthermore, MDC1 (Mediator of DNA damage Checkpoint 1), a known γH2Ax reader that is essential for sex body formation (Ichijima et al., 2011), remains robustly associated with the asynapsed sex chromosomes in the absence of ARID1A during pachynema (Fig. S5C). These data show that the mechanisms underlying ARID1A-mediated repression of the sex-linked transcription are mutually exclusive to DDR pathways regulating sex body formation.

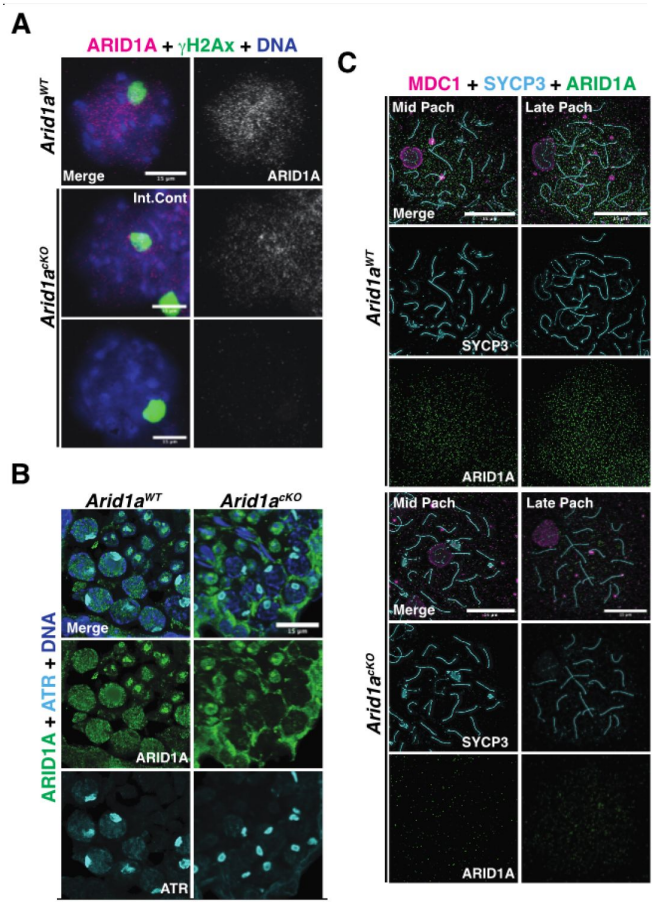


Fig S5.

ARID1A does not influence sex body formation.

(A) *Arid1a*^{WT}and *Arid1a*^{cKO} spermatocyte spreads immunolabelled for ARID1A (magenta) and γH2Ax (green). Scale bar:15 μm, magnification: 100x. (B) Cross section of adult (3-month-old) *Arid1a*^{WT}and *Arid1a*^{cKO} seminiferous tubules immunolabelled for ARID1A (green) and ATR (cyan). Scale bar:15 μm, magnification: 63x. (C) *Arid1a*^{WT}and *Arid1a*^{cKO} pachytene spermatocytes immunolabelled for MDC1 (magenta), SYCP3 (cyan), and ARID1A (green). Scale bar:15 μm, magnification: 100x. (A-B) DNA counterstained with DAPI (blue).

ARID1A limits RNAPII localization to the sex chromosomes during pachynema

Apart from γH2Ax, the association of the sex chromosomes with RNA polymerase II (RNAPII) distinguishes the sex body from the autosomes during pachynema. Normally, pachytene spermatocytes display reduced levels of RNAPII on the sex body relative to autosomes (Khalil et al., 2004). This sub-nuclear localization of RNAPII coincides with increased transcriptional output, which peaks at diplonema (Ernst et al., 2019), underscoring the importance of targeted mechanisms regulating sex chromosome repression. Therefore, we were curious to test whether ARID1A influenced the nuclear localization of RNAPII during pachynema. We performed IF to monitor the localization of the actively transcribing (elongating) form of RNAPII marked by Serine2 phosphorylation (pSer2) on its carboxy-terminal domain (Noe Gonzalez et al., 2021). We monitored elongating RNAPII (pSer2) because its pausing regulates meiotic transcription (Alexander et al., 2022). By co-staining for RNAPII (pSer2) and SYCP3, we could stage pachynema and identify the sex chromosomes. While RNAPII (pSer2) localization to the autosomes appeared similar, we noticed increased levels of RNAPII (pSer2) association with the sex chromosomes in *Arid1a*^{cKO} relative to *Arid1a*^{WT} mid and late pachytene spermatocytes (Fig. 3A). By quantifying the RNAPII (pSer2) signal generated by IF, we detected a 1.5-fold increase (p=0.0003) in the average RNAPII (pSer2) fluorescence associated with the sex chromosomes in *Arid1a*^{cKO}relative to *Arid1a*^{WT} pachytene spermatocytes (Fig. 3B).

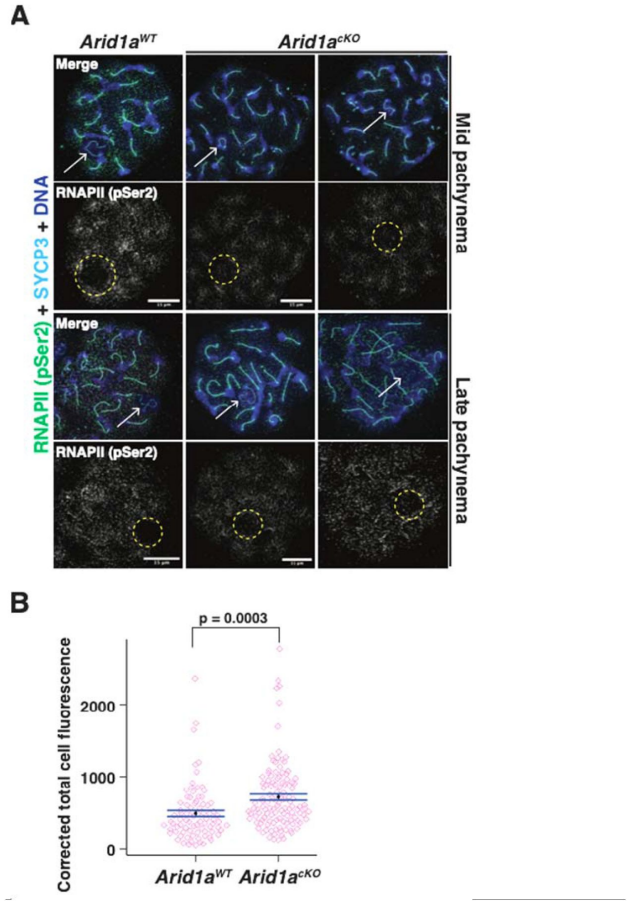


Fig 3.

ARID1A limits RNA polymerase II (RNAPII) localization to the sex body.

(A) *Arid1a*^{WT}and *Arid1a*^{cKO} pachytene spermatocytes immunolabelled for pSer2-RNAPII (green), SYCP3 (cyan), and counterstained with DAPI (blue). Scale bar:15 μm, magnification: 100x. The sex chromosomes (white arrow) and sex body (yellow dashed circle) are labeled. (B) Dot plot describing the corrected total pSer2-RNAPII fluorescence (y-axis) measured from *Arid1a*^{WT} (n = 82) and *Arid1a*^{cKO} (n= 119) pachytene spermatocytes (3 replicates per genotype). Empty diamonds (magenta) represent independent data points. Significance determined by a two-tailed unpaired Student's t-test p values. Data expressed as mean (black dot) ± SEM.

Due to technical difficulties, we could not simultaneously stain for ARID1A alongside SYCP3 and RNAPII (pSer2), making it impossible to distinguish internal controls from mutants in the *Arid1a*^{cKO} meiotic spreads. This result suggests that the 1.5-fold increase in RNAPII association with the sex body in *Arid1a*^{cKO}relative to *Arid1a*^{WT} pachytene spermatocytes is likely underestimated. Therefore, our results indicate that ARID1A facilitates sex-linked gene repression by limiting the association of elongating RNAPII with the sex chromosomes during pachynema.

ARID1A regulates promoter accessibility during pachynema

Next, we hypothesized that ARID1A-governed chromatin remodeling might underlie its role in limiting RNAPII accessibility on pachytene sex chromosomes. Therefore, we profiled changes in chromatin accessibility in response to the loss of ARID1A using ATAC-seq. Normally, ATAC-seq peaks (accessible chromatin) are mapped comparably across promoters (34%), intergenic (22.8%), and intronic (21.5%) regions. In contrast, the loss of ARID1A resulted in a dramatic shift towards predominantly promoter-associated ATAC-seq peaks with only a minority mapping to intergenic and intronic regions (Fig. 4A).

Consistent with an increase in the proportion of promoter-associated peaks, we detected an increase in accessibility across transcription start sites (TSS) associated with both autosomal and sex-linked DEG's in *Arid1a*^{cKO} relative to *Arid1a*^{WT} pachytene spermatocytes (Fig. 4B,C). Furthermore, in the case of autosomal targets, chromatin accessibility at their TSSs was enhanced irrespective of their transcriptional status, appearing indistinguishable between

down-regulated and up-regulated genes upon the loss of ARID1A (Fig. 4B). In contrast, on the sex chromosomes, the loss of ARID1A seemed to have a prominent effect on TSS's associated with up-regulated genes, which displayed greater chromatin accessibility relative to their down-regulated counterparts (Fig. 4C). Therefore, the increased promoter accessibility of normally repressed sex-linked genes may underlie their persistent transcription by RNAPII upon the loss of ARID1A. Overall, these data highlight a role for BAF complexes in limiting promoter accessibility, especially on the sex chromosomes during pachynema.

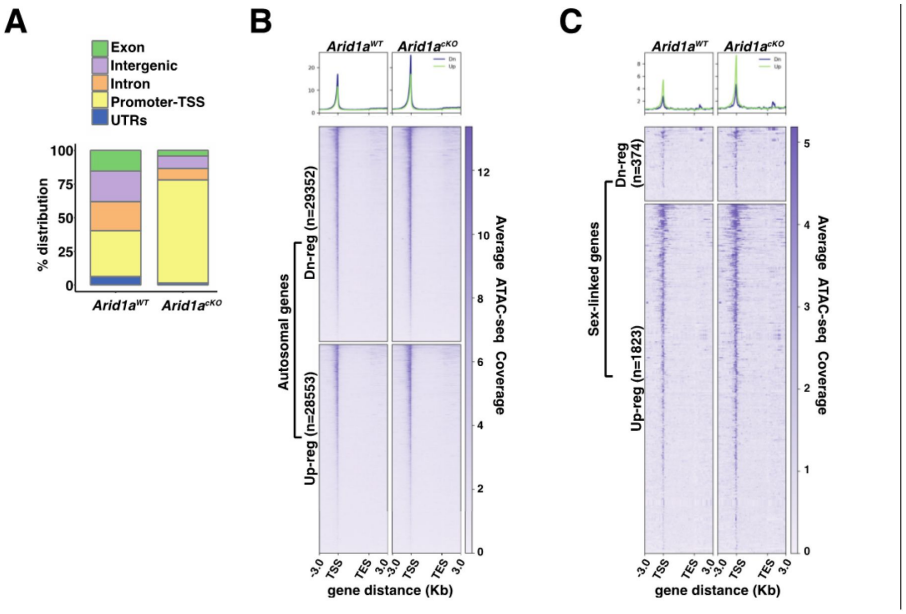


Fig 4.

ARID1A limits promoter accessibility.

(A) Genomic associations of MACS2 derived ATAC-seq peak calls from *Arid1a*^{WT} and *Arid1a*^{cKO} pachytene spermatocytes. Percent (%) distribution of genomic annotations is indicated. (B-C) Heatmap (bottom) and metaplot (top) displaying the average ATAC-seq signal associated with differentially expressed (B) autosomal and (C) sex-linked genes in *Arid1a*^{WT}and *Arid1a*^{cKO} pachytene spermatocytes. (B-C) The number of RefSeq annotations (n) associated with differentially regulated genes (rows) is indicated. Up-reg: misexpressed, and Dn-reg: misrepressed genes in response to the loss of ARID1A. Average ATAC-seq coverage was plotted across RefSeq genes ± 3Kb.

ARID1A regulates the chromatin composition of the sex body

Chromatin remodelers regulate DNA accessibility by altering nucleosome positioning or composition (Clapier et al., 2017). The latter outcome is interesting in the context of MSCI, given that the sex body typically displays a striking enrichment of the variant histone H3.3 while concomitantly appearing depleted of the canonical histones H3.1/3.2 (van der Heijden et al., 2007; Yuen et al., 2014). Given that human ARID1A is known to regulate H3.3 genomic associations (Reske et al., 2022), we tested whether a similar mechanism governed H3.3 localization to the sex chromosomes. To address this, we monitored H3.3 localization in pachytene spermatocytes by IF, simultaneously staining for ARID1A and HORMAD1. The former aided in mutant identification, while the latter labeled the pachytene sex chromosomes. Consistent with previous reports, H3.3 can be seen preferentially coating the sex chromosomes distinguishing it from autosomal chromatin during normal pachynema (Fig. 5A). Interestingly, this sex body association of H3.3 was detected less frequently early in pachynema (17%), becoming more pervasive during the mid (70 %) and late (95%) stages of pachynema. In contrast, in the absence of ARID1A, H3.3 staining on the sex chromosomes was indistinguishable from their autosomal counterparts throughout pachynema (Fig. 5A). Notably, the majority of mutant mid (89%) and late (77%) pachytene spermatocytes lacked

the typical sex body enrichment of H3.3 (Fig. 5A), indicating that ARID1A dictates the preferential accumulation of H3.3 on the sex body.

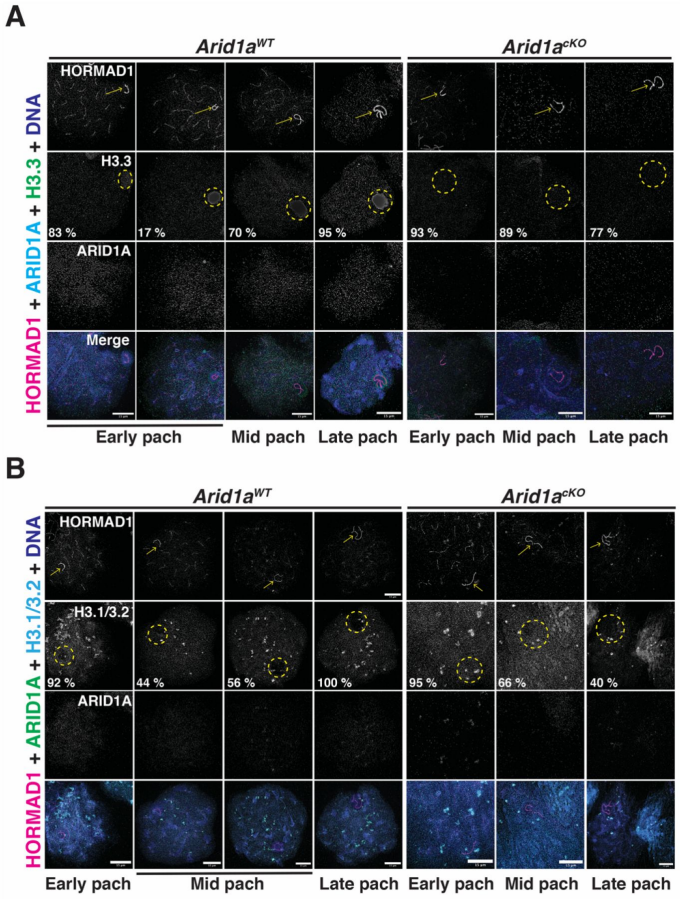


Fig 5.

ARID1A influences the chromatin composition of the sex body.

(A-B) *Arid1a*^{WT} and *Arid1a*^{cKO} pachytene spermatocytes immunolabelled for HORMAD1 (magenta), (A) ARID1A (cyan) and H3.3 (green), (B) ARID1A (green) and H3.1/3.2 (cyan). (A-B) Proportion (%) of *Arid1a*^{WT} and *Arid1a*^{cKO} early, mid, and late pachytene spermatocytes displaying distinct (A) H3.3, (B) H3.1/3.2 localization patterns with the sex body. For H3.3 immunostaining, the total number of *Arid1a*^{WT} pachytene spermatocytes: early = 144, mid= 274, late= 95; *Arid1a*^{cKO} pachytene spermatocytes: early = 43, mid= 86, late= 55, were scored, from 3 replicates each. For H3.1/3.2 immunostaining, the total number of *Arid1a*^{WT} pachytene spermatocytes: early = 91, mid= 124, late= 45; *Arid1a*^{cKO} pachytene spermatocytes: early = 22, mid= 116, late= 58, were scored, from 3 replicates each. DNA counterstained with DAPI (blue). The sex chromosomes (yellow arrow) and sex body (yellow dashed circle) are labeled. Scale bar:15 μm, magnification: 100x.

Next, we were curious to know the consequence of reduced H3.3 association on H3.1/3.2 occupancy on the sex body in response to the loss of ARID1A. We performed IF to monitor the association of H3.1/3.2 with the sex chromosomes marked by HORMAD1 in response to the loss of ARID1A. Like H3.3, canonical H3.1/3.2 appeared uniformly distributed genome-wide in most early pachytene spermatocytes (92%). At the onset of pachynema, sex chromosomes mostly displayed comparable H3.3 and H3.1/3.2 levels (Fig. 5A, B). However, this balance changed during the subsequent sub-stages of pachynema. Although robust H3.3 levels remained, H3.1/3.2 appeared excluded from the sex chromosomes in most mid (56%) and all late pachytene spermatocytes (Fig. 5A, B). In contrast, the loss of ARID1A increased the proportion of mid (66%) and late (40%) pachytene spermatocytes retaining H3.1/3.2 on sex-linked chromatin (Fig. 5B). The abnormal retention of canonical H3.1/3.2 coincides with a lack of H3.3 enrichment on sex-linked chromatin in response to the loss of ARID1A.

To determine whether these abnormal kinetics result from a genome-wide deficiency in H3.3, we compared its levels by western blotting to find no difference in H3.3 abundance in *Arid1a*^{cKO} relative to *Arid1a*^{WT} spermatocyte enriched populations (Fig. S6A). We also considered an alternative possibility that ARID1A might promote the sex-linked enrichment of H3.3 by influencing the expression of cognate chaperones like DAXX and HIRA during murine pachynema (Rogers et al., 2004; van der Heijden et al., 2007). We addressed this by monitoring DAXX and HIRA levels in response to the loss of ARID1A using IF. In the case of DAXX, we examined meiotic spreads to find no changes in either the overall levels or sex chromosome localization of DAXX in response to the loss of ARID1A (Fig. S6B). The same was

also true of HIRA, whose levels and nuclear localization in mutants appeared comparable to that seen in internal (from *Arid1a*^{cKO}) and normal (*Arid1a*^{WT}) spermatocytes (Fig. S6C). Therefore, ARID1A impacts H3.3 accumulation on the sex chromosomes without affecting its expression or incorporation during pachynema. Overall, our data suggest that ARID1A influences MSCI by regulating the composition of sex-linked chromatin.

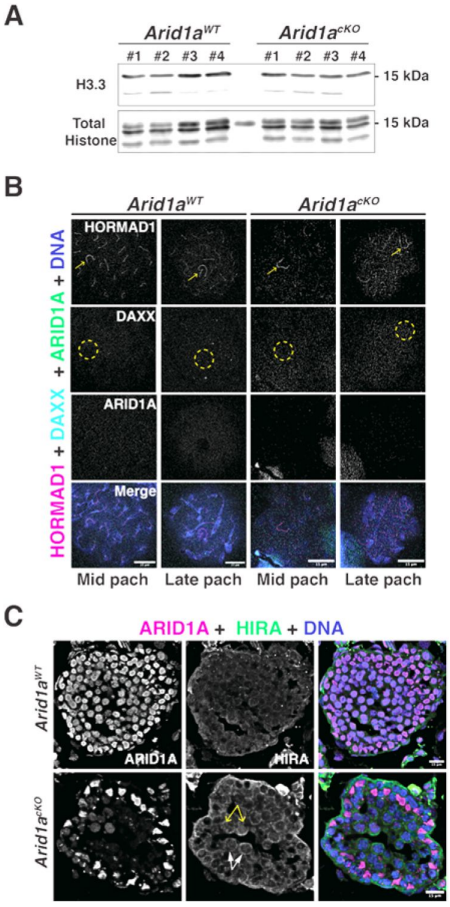


Fig S6.

ARID1A does not influence the expression or incorporation of H3.3.

(A) Western blots on acid-extracted histones obtained from four independent replicates of P19 *Arid1a*^{WT} and *Arid1a*^{cKO} spermatogenic cells, displaying H3.3 abundance. Total histone levels from each sample are displayed. (B) *Arid1a*^{WT} and *Arid1a*^{cKO} pachytene spermatocytes immunolabelled for HORMAD1 (magenta), DAXX (cyan), and ARID1A (green). DNA counterstained with DAPI (blue). The sex chromosomes (yellow arrow) and sex body (yellow dashed circle) are labeled. Scale bar:15 μm, magnification: 100x. (C) *Arid1a*^{WT}and *Arid1a*^{cKO} testis cryosections immunolabelled for ARID1A (magenta) and HIRA (green). DNA counterstained with DAPI (blue). Representative mutant (white arrows) and internal control (yellow arrows) pachytene spermatocytes labeled in *Arid1a*^{cKO} testis cryosection. Scale bar: 15 μm, magnification: 63x.

ARID1A prevents the promoter accumulation of H3.3

To define the changes in the sex-linked associations of H3.3 at a higher resolution, we performed CUT&RUN on pachytene spermatocytes isolated from *Arid1a*^{WT} and *Arid1a*^{cKO}testes. Despite the appearance of a robust H3.3 IF signal from the sex body during normal pachynema (Fig. 5A), the Macs2 peak caller (Zhang et al., 2008) identified very few sex-linked peaks (n =224). These peaks were overwhelmingly associated with intergenic regions (Fig. S7A). Comparatively, a dramatically higher number of peaks (n=12183) primarily associated with genic (promoter, intron, and exon) regions occurred in *Arid1a*^{cKO}pachytene spermatocytes (Fig. S7A). More interestingly, there appeared to be a shift from few but overwhelmingly sex-linked H3.3 peaks (n=224; 98%) to autosomal H3.3 peaks (n=11512; 94.5 %) in response to the loss of ARID1A (Fig. S7A). Additionally, while the proportion of H3.3 sex-linked peaks in *Arid1a*^{cKO} pachytene spermatocytes were few (n=671; 5.5%), they outnumbered their *Arid1a*^{WT} counterparts by 3-fold. These data suggest that the loss of ARID1A strongly influences H3.3 genomic associations.

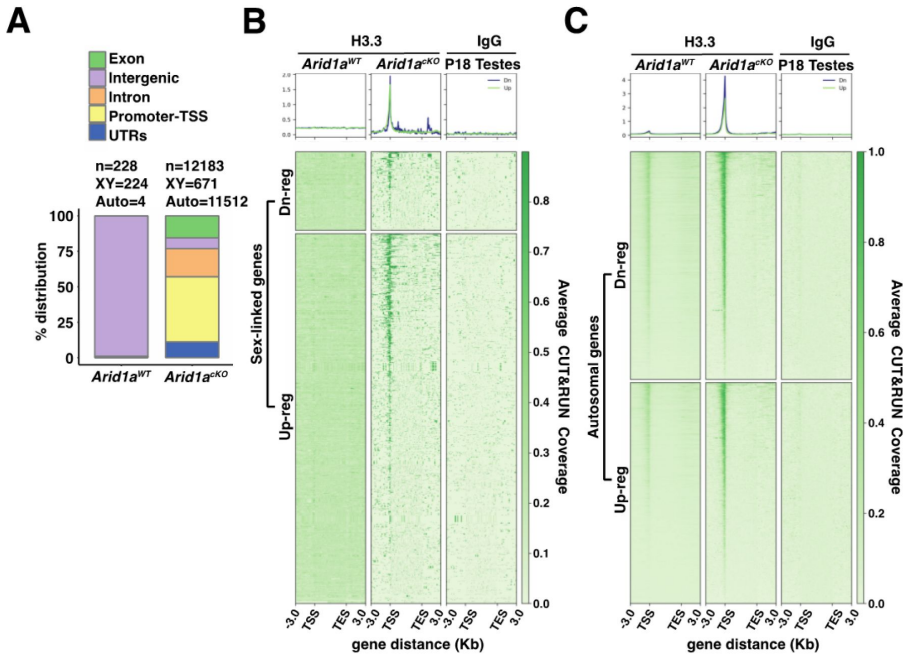


Fig S7.

ARID1A limits H3.3 occupancy at promoters

(A) Genomic associations of MACS2 H3.3 peak calls from *Arid1a*^{WT} and *Arid1a*^{cKO} pachytene spermatocytes. Percent (%) distribution of genomic annotations is indicated. (B-C) Heatmap (bottom) and metaplot (top) displaying the average H3.3 signal associated with differentially expressed (B) sex-linked and (C) autosomal genes in *Arid1a*^{WT} and *Arid1a*^{cKO} pachytene spermatocytes. (B-C) The number of RefSeq annotations (n) associated with differentially regulated genes (rows) are indicated. Up-reg: misexpressed, and Dn-reg: misrepressed genes in response to the loss of ARID1A. Average H3.3 coverage plotted across RefSeq genes ± 3Kb.

Due to the increased representation of promoter-associated peaks in response to the loss of ARID1A, we monitored H3.3 association with TSSs of genes differentially regulated by ARID1A. Consistent with the genomic annotation of H3.3 peaks, we could not detect H3.3 enrichment at TSSs associated with ARID1A-regulated sex-linked genes relative to IgG control. H3.3 signal spread gene-wide in *Arid1a*^{WT} pachytene spermatocytes (Fig. S7B, Fig. S8A-top panel). In contrast, we observed a striking accumulation of H3.3 at the TSSs of sex-linked genes misexpressed without ARID1A (Fig. S7B, Fig. S8A). There is a transition from the gene-wide spreading of H3.3 to promoter-proximal enrichment in response to the loss of ARID1A. Concomitantly, X and Y-linked intergenic regions undergo a significant decrease in H3.3 occupancy upon ARID1A loss (Fig. S8A). A similar loss of H3.3 occupancy was also observed at a handful of autosomal intergenic peaks (Fig. S7A, Fig. S8B – top).

Unlike the sex-linked targets, TSSs of ARID1A regulated autosomal genes displayed H3.3 enrichment relative to the IgG control (Fig. S7C, Fig. S8B – bottom). However, this TSS occupancy appeared enhanced in *Arid1a*^{cKO} relative to *Arid1a*^{WT} pachytene spermatocytes (Fig. S7C, Fig. S8B). Therefore, TSSs of both sex-linked and autosomal gene targets display concordant changes in H3.3 occupancy. Overall, ARID1A occupancy at TSSs (Fig.S4) of differentially regulated genes prevents the accumulation of H3.3 at target promoters.

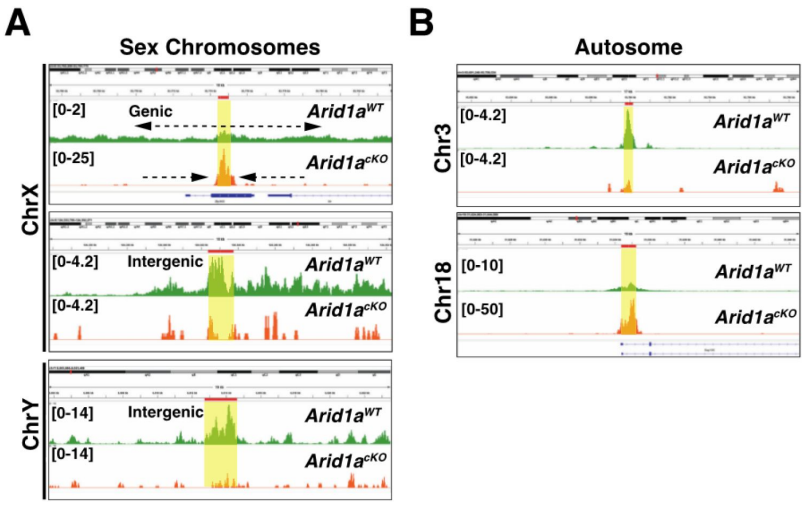


Fig S8.

Genome browser views of ARID1A governed H3.3 genomic associations.

H3.3 CUT&RUN coverage from *Arid1a*^{WT} (green tracks) and *Arid1a*^{cKO}(Orange tracks) pachytene spermatocytes. Solid red bars and yellow highlights denote H3.3 peak calls (MACS2) from *Arid1a*^{WT} and *Arid1a*$^{cK-O}$pachytene spermatocytes. Vertical viewing limits within parentheses.

ARID1A restricts H3.3 occupancy to intergenic regions on the sex chromosomes

Our analysis of the genome-wide localization of H3.3 in response to the loss of ARID1A would argue that the abnormal increase in promoter-associated H3.3 results from its redistribution from high-affinity sites (peaks) that are predominantly sex-linked and intergenic during normal pachynema (Fig. S7A, Fig. S8). We focused our attention on these H3.3-occupied intergenic sex-linked sites. Although the Macs2 algorithm identified only 224 sex-linked intergenic peaks, we speculated that this approach filters out regions enriched for H3.3 that fail to meet the peak calling threshold compounded by the pervasive spreading of H3.3 on the sex chromosomes (Fig. 5A, Fig. S7B, S8A). We adopted an alternative strategy that involved identifying potential H3.3 bound sites by monitoring their occupancy at ARID1A-governed accessible chromatin (Fig. 4A). We compared *Arid1a*^{WT} and *Arid1a*^{cKO} pachytene spermatocyte associated ATAC-seq peaks to identify mutually exclusive and overlapping peaks using BEDTools (Quinlan and Hall, 2010). The resulting peaks could be categorized into lost (n=9), common (n=7194), or gained (n=2246) based on their absence, persistence, or appearance, respectively, in *Arid1a*^{cKO} relative to *Arid1a*^{WT} pachytene spermatocytes (Fig. 6A). Next, we examined the ATAC-seq signal at these regions to find an increase in accessibility not only at gained peaks as expected but also at common peaks in *Arid1a*^{cKO} relative to *Arid1a*^{WT}pachytene spermatocytes.

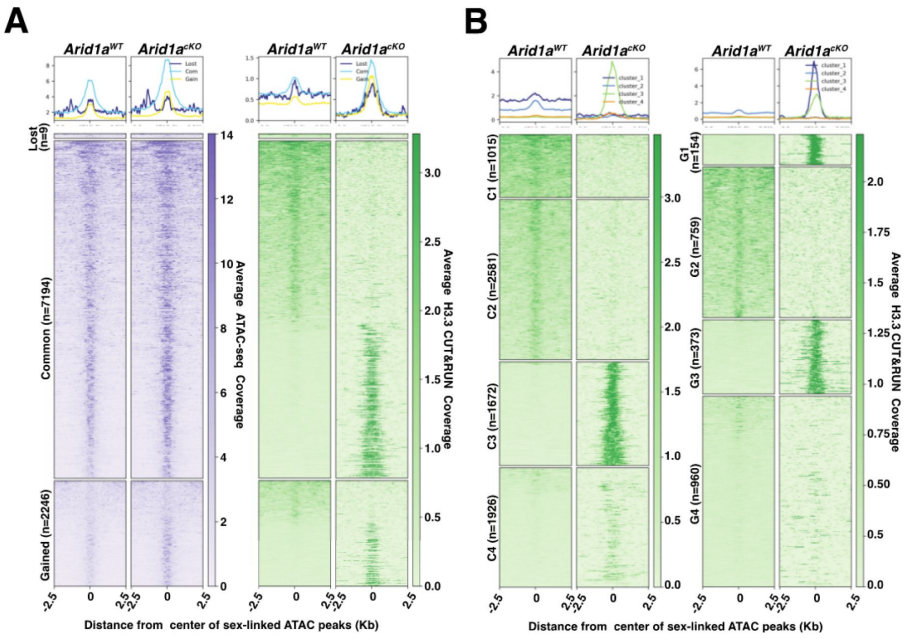


Fig 6.

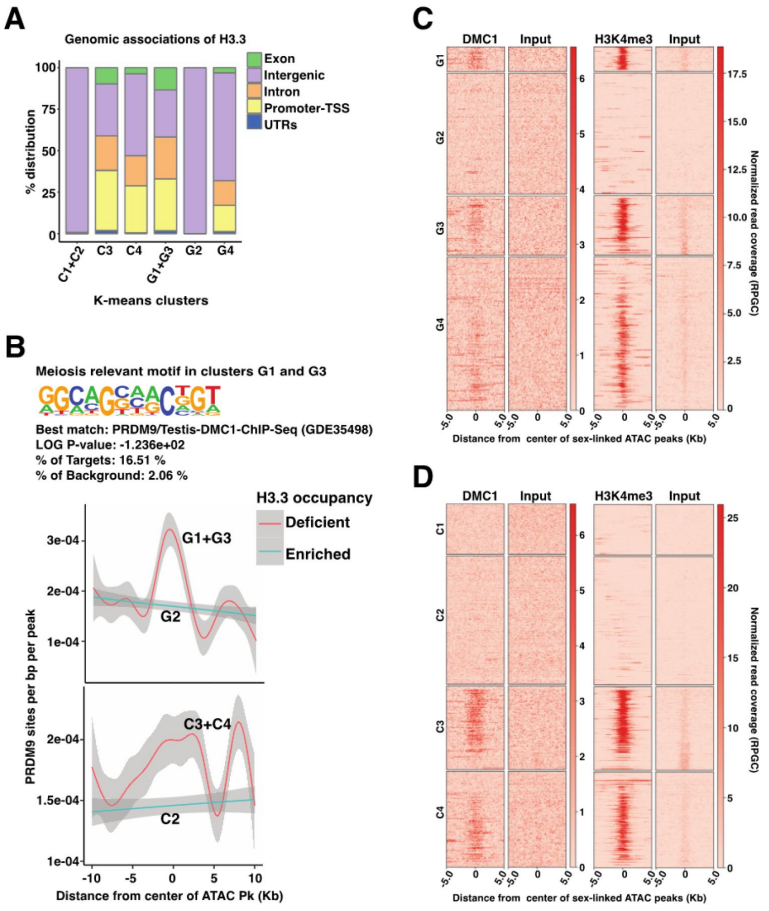
H3.3 displays differential occupancy across ARID1A governed sex-linked open chromatin.

(A-B) Heatmaps (bottom) and meta-plots (top) displaying average (A) chromatin accessibility (purple heatmap) and H3.3 enrichment (green heatmap) associated with lost, common, and gained ATAC-seq peak calls (MACS2), (B) enrichment of H3.3 at k-means clusters associated with common (C1-C4, left) and gained (G1-G4, right) ATAC-seq peaks, in *Arid1a*^{WT}and *Arid1a*^{cKO} pachytene spermatocytes. (A-B) ATAC-seq and H3.3 coverage plotted over a 5 Kb window centered at ATAC-seq peaks (MACS2). Number of ATAC-seq peak calls (n) associated with each category is indicated.

Despite being labeled as lost, these regions did not display the expected loss in ATAC-seq signal in *Arid1a*^{cKO} relative to *Arid1a*^{WT} pachytene spermatocytes (Fig. 6A, left panel). The ATAC signal appears unchanged, highlighting the possibility that lost regions were labeled as such because they failed to satisfy Macs2 peak thresholds in *Arid1a*^{cKO}pachytene spermatocytes, especially given the comparatively higher magnitude of ATAC signal surrounding the common and gained peaks (Fig. 6A, left panel). We restricted our analysis to common and gained sites constituting 99 % of sex-linked regions subject to ARID1A-regulated chromatin accessibility. By plotting the average H3.3 coverage at sites associated with common and gained peaks, we detected striking differences in H3.3 occupancy. Briefly, both common and gained open chromatin regions consisted of loci differentiated by either a loss or robust increase in H3.3 occupancy in *Arid1a*^{cKO} relative to *Arid1a*^{WT} pachytene spermatocytes (Fig. 6A, left panel). To gain further insight, we performed k-means clustering, based on H3.3 occupancy, to identify 4 clusters associated with either common (C1-C4) or gained (G1-G4) peaks (Fig. 6B). The clusters associated with common ATAC-seq peaks displayed contrasting changes in H3.3 occupancy in response to the loss of ARID1A. C1 and C2 represented regions deficient for H3.3, while C3 and C4 represent regions that gained H3.3 binding in *Arid1a*^{cKO} relative to *Arid1a*^{WT} pachytene spermatocytes (Fig. 6B, left panel). Similarly, clusters associated with gained ATAC-seq peaks, namely, G1 and G3, represented sites that gained H3.3 occupancy, while G2 identified sites that lost H3.3 occupancy. We also noticed a gain in H3.3 binding at regions associated with G4 in response to the loss of ARID1A, albeit at significantly reduced coverage relative to G1 and G2 (Fig. 6B, right panel). Genomic annotations of common and gained k-means clusters revealed that the sites that displayed a loss of H3.3 binding (represented by C1 + C2, G2) in response to the loss of ARID1A were intergenic (Fig. S9A).

In contrast, those that gained H3.3 localization (represented by C3, C4, G1+G3, and G4) distributed differentially between intergenic and genic regions (Fig. S9A). Therefore, these

data support our idea that ARID1A restricts H3.3 occupancy primarily to intergenic sites on the sex chromosomes. Furthermore, the loss of ARID1A triggers an abnormal redistribution of H3.3 to genic regions. Therefore, the increased promoter occupancy of H3.3 is an indirect consequence of ARID1A loss.



ATAC-seq peaks (MACS2).

Fig S9.

H3.3 occupancy is antagonistic to DMC1 associations in non-homologous sex-linked regions.

(A) Genomic associations of common (C1-C4) and gained (G1-G4) k-means clusters in pachytene spermatocytes. Percent (%) distribution of genomic annotations is indicated. (B) Results of PRDM9 motif enrichment analyses at gained (G1 and G3), k-means clusters (top), and plots describing the frequency of PRDM9 motifs (y-axis) spanning a 5 Kb window centered at ATAC-seq peaks (x-axis) associated with gained (middle) and common (bottom) k-means clusters that are either deficient (orange trend line) or enriched (cyan trend line) for H3.3 occupancy. Trend lines were generated using generalized additive mode smoothing (gam). 95% confidence intervals (gray shading) are indicated. (C-D) Heatmaps displaying testes DMC1 (left) and pachytene spermatocyte associated H3K4me3 (right) enrichment relative to input at k-means clusters associated with (C) gained (G1-G4), and (D) common (C1-C4) ATAC-seq peaks. (C-D) DMC1 and H3K4me3 coverage plotted over a 10 Kb window centered at

ARID1A-governed H3.3 localization influences the sex-linked association of DMC1

To gain further insight into the mechanisms regulating H3.3 associations on the sex chromosomes, we examined the regions associated with various k-means clusters to investigate the enrichment of relevant motifs. To our surprise, we detected an enrichment of the motif associated with the DSB hotspot specifier, PRDM9 (PR domain-containing protein 9), in clusters related to gained peaks, G1 and G3 (Fig . S9B, top). Additionally, we observed an increased frequency of PRDM9 motif centered at gained ATAC-seq peaks associated with G1 and G3 (Fig . S9B, middle).

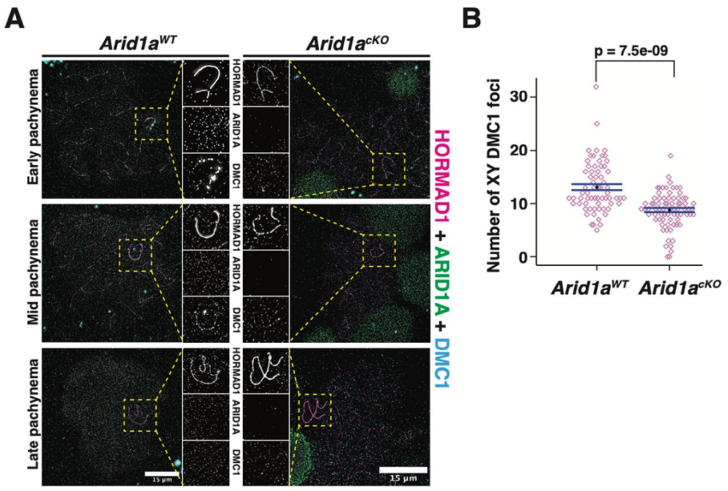


Fig 7.

ARID1A influences the axial association of DMC1 with the XY during pachynema.

(A) *Arid1a*^{WT}and *Arid1a*^{cKO} pachytene spermatocytes immunolabelled for HORMAD1 (magenta), ARID1A (green), DMC1 (cyan), and counterstained with DAPI (blue). Scale bar:15 μm, magnification: 100x. A magnified view of the sex chromosomes is indicated (yellow dashed square). (B) Dot plot displaying the number of sex-linked DMC1 foci (y-axis) quantified from *Arid1a*^{WT} (n = 66) and *Arid1a*^{cKO}(ARID1A^{-}, n= 75) pachytene spermatocytes, obtained from 3 replicates each. Empty diamonds (magenta) represent independent data points. Significance determined using a two-tailed unpaired Student's t-test p values. Data expressed as mean (black dot) ± SEM.

Interestingly, regions associated with G1 and G3 usually are devoid of H3.3 (Fig. 6B), implying a potential antagonism between H3.3 occupancy and the occurrence of meiotic DNA DSBs. Gained ATAC-seq peaks associated with G2, which usually displays H3.3 occupancy, appeared devoid of PRDM9 motif occurrences relative to G1 and G3 (Fig. S9B, middle). Next, we determined the frequency of the PRDM9 motif at k-means clusters associated with common ATAC-seq peaks. These regions also displayed a dichotomous association with H3.3 (Fig. 6B). C3 and C4, usually deficient in H3.3, showed an increased frequency of PRDM9 motifs relative to C2, which typically displays H3.3 binding (Fig. S9B, bottom). These data confirm an antagonistic relationship between H3.3 and meiotic DNA DSBs.

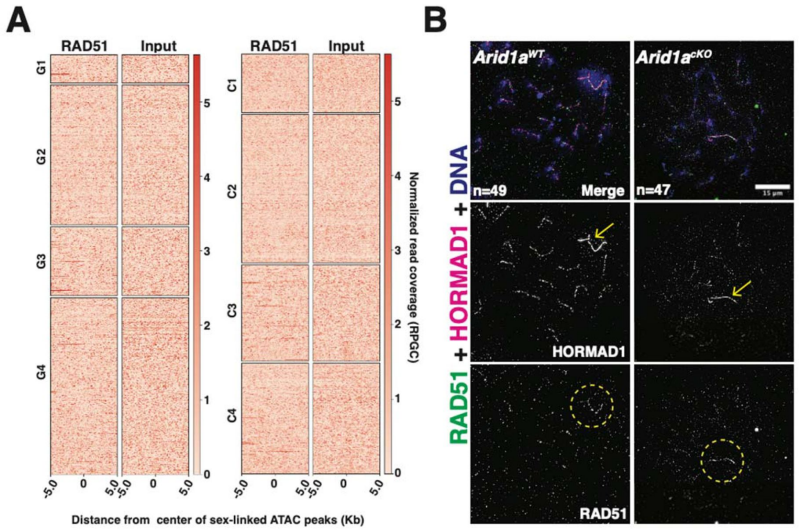


Fig S10.

ARID1A does not affect the axial association of RAD51 with the XY during pachynema.

(A) Heatmaps displaying testes RAD51 enrichment relative to input at k-means clusters associated with gained (G1-G4, left) and common (C1-C4, right) ATAC-seq peaks. RAD51 coverage plotted over a 10 Kb window centered at ATAC-seq peaks (MACS2). (B) *Arid1a*^{WT} and *Arid1a*^{cKO} pachytene spermatocytes immunolabelled for HORMAD1 (magenta), RAD51 (green) and counterstained with DAPI (blue). Scale bar:15 μm, magnification: 100x. Yellow arrows and dashed circles label the sex chromosomes.

Since the PRDM9 motifs identified from SSDS (Single-Stranded DNA Seq) data were generated to map the genomic associations of DNA repair factor DMC1(Brick et al., 2012), we monitored its enrichment at the various k-means clusters. We also monitored the association of markers of meiotic DNA DSBs, such as the PRDM9 catalyzed H3K4me3 at common and gained k-means clusters using published ChIP-seq data from pachytene spermatocytes (Maezawa et al., 2018a). Consistent with the motif analyses, DMC1 occupancy and H3K4me3 enrichment occurred at gained and common ATAC-seq peaks associated with k-means clusters, normally devoid of H3.3, namely, G1, G3, G4, C3, and C4 (Fig. S9C, D).

These data suggest that ARID1A might indirectly influence DNA DSB repair on the sex chromosomes by regulating the localization of H3.3. To test this conclusion, we monitored the association of DMC1 on the sex chromosomes, in response to the loss of ARID1A, by IF. Normally, DMC1 foci appear distributed along the non-homologous arms of the X and Y chromosomes from early to mid-pachynema, only disappearing by late pachynema (Fig. 7A, left, panel insets) (Moens et al., 2002). In contrast, in the absence of ARID1A, we observed a marked reduction in the number of DMC1 foci (Fig. 7A, right, panel insets). Quantification of DMC1 foci in Arid1a^{cKO} relative to Arid1a^{WT} pachytene spermatocytes revealed a significant decrease in the sex-linked association of DMC1 in the absence of ARID1A (Fig. 7B). Along with DMC1, the mitotic DNA recombinase, RAD51, is also known to localize to the non-homologous arms of the sex chromosomes (Moens et al., 1997; Moens et al., 2002). Therefore, we determined whether ARID1A also influenced the sex-linked association of RAD51. First, we analyzed previously generated RAD51-SSDS data (Hinch et al., 2020) to monitor its enrichment across the various k-means clusters associated with common and gained ATAC-seq peaks. Unlike DMC1, we found no detectable levels of RAD51 at any k-means clusters (Fig. S10A). Furthermore, the formation of RAD51 foci along the asynapsed axes of the sex chromosomes appeared unchanged in *Arid1a*^{cKO} relative to *Arid1a*^{WT}pachytene spermatocytes (Fig. S10B). Therefore, ARID1A is necessary for the normal sex-linked association of DMC1 but not RAD51. More importantly, our data highlight a dual role for ARID1A in regulating sex chromosome repression and DNA repair (Fig. 8).

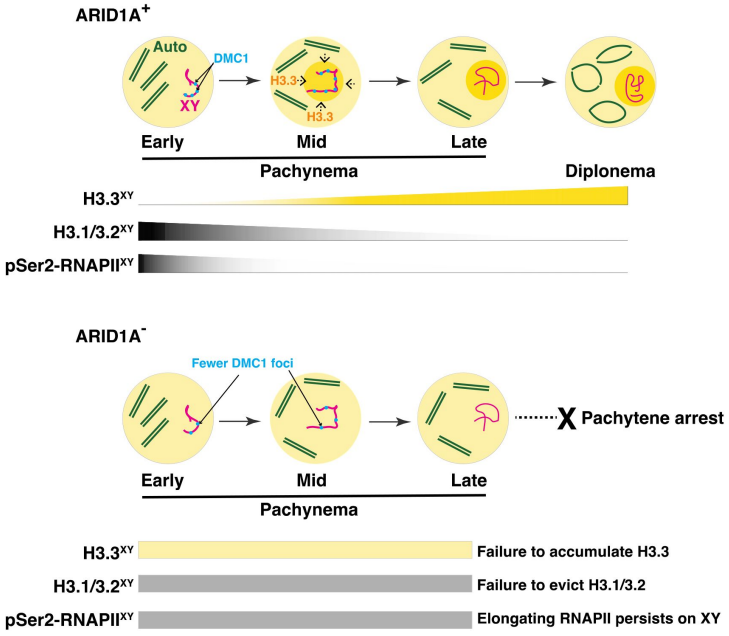


Fig 8.

A model describing the role of ARID1A sex-linked chromatin regulation.

During meiosis, the sex chromosomes undergo transcriptional repression at the onset of pachynema. A dramatic change in the composition of sex-linked chromatin accompanies this chromosome-wide repression. From mid to late pachynema, spermatocytes display a hyper-accumulation of the variant histone H3.3 (Ochre shading and gradient) on the sex chromosomes (magenta) relative to autosomes (green). Concomitantly, the levels of the canonical histones H3.1/3.2 and elongating pSer2-RNAPII complex (grey gradients) appear depleted from the sex body by late pachynema. The loss of ARID1A dramatically alters the chromatin composition of the sex body, which features low H3.3 (yellow bar) association at levels indistinguishable from autosomes throughout pachynema. Concomitantly, canonical H3.1/3.2 and pSer2-RNAPII levels (grey bar) on the sex body remain abnormally stable throughout pachynema. These sex-linked chromatin

aberrations, along with persistent transcription owing to the association of pSer2-RNAPII with mutant sex body, fail meiotic sex chromosome inactivation (MSCI) and, consequently, pachytene arrest. This defect also coincides with an abnormal loss of DMC1 (blue foci) localization to the unpaired sex chromatids in response to the loss of ARID1A. Therefore, along with transcriptional repression, ARID1A-governed chromatin dynamics appear to influence DNA repair on the sex chromosomes.

Discussion

We previously demonstrated a spermatogenic requirement for the mammalian SWI/SNF complex based on its role in coordinating germline transcription (Menon et al., 2019). Furthermore, we showed that the SWI/SNF PBAF subcomplex activates genes essential for reductional meiosis and gamete formation (Menon et al., 2021). These data highlight that distinct SWI/SNF subcomplexes govern specific meiotic transitions.

Our current study demonstrates a requirement for the ARID1A-associated BAF (BAF-A) subcomplex for meiotic sex chromosome inactivation (MSCI). It does so by promoting the accumulation of the variant histone H3.3 on the sex chromosomes at the onset of mid-pachynema, coinciding with the eviction of canonical histones H3.1/3.2 and RNA polymerase II from the sex body (Model, Fig. 8). In contrast, when depleted of ARID1A, chromatin accessibility increased along with a deficiency of H3.3 and the continued presence of H3.1/3.2 and pSer2-RNAPII (an elongating form of RNA polymerase II) in the sex body. These data indicate that BAF-A helps define features distinguishing sex chromosomes from autosomes and that the absence of the BAF-A subcomplex during meiosis I results in the failure of MSCI during pachynema.

Although previous work described the association of H3.3 with the sex body (van der Heijden et al., 2007; Yuen et al., 2014) and its requirement for meiotic repression of sex-linked genes (Fontaine et al., 2022), our study revealed the need for BAF-A in the preferential localization of H3.3 to the sex body relative to autosomes. Interestingly, a similar role for BAF-A in promoting H3.3 incorporation occurs in human endometriotic cells (Reske et al., 2022). In this case, the genomic localization of H3.3 to super-enhancers required ARID1A. Our study describes the ARID1A-dependent localization of H3.3 at a chromosome-wide scale comprised of (i) local enrichment at intergenic loci and (ii) larger domains that resembled spreading much like that of *Xist* during female X chromosome inactivation (XCI) (Chaumeil et al., 2006). X chromosome inactivation (XCI) requires SWI/SNF subunits SMARCA4 and SMARCC1 in female mouse embryonic stem cells (mESC) (Keniry et al., 2022). SMARCC1 promotes XCI by increasing promoter accessibility of the inactive X chromosome (Xi). Interestingly, during MSCI, sex-linked chromatin displays enhanced accessibility (Maezawa et al., 2018b). In contrast, our studies showed that BAF-A limits rather than enhances sex-linked chromatin accessibility. These data highlight a mechanism that checks chromosome-wide relaxation during MSCI. The divergent outcomes of SWI/SNF-governed sex-linked chromatin accessibility during XCI and MSCI probably reflect noticeable sex-specific differences or arise from potentially perturbing distinct SWI/SNF subcomplexes.

Along with altered chromatin composition and structure, pachytene spermatocytes lacking ARID1A also displayed an abnormal association of pSer2-RNAPII (elongating form) with the sex body. Normally, total transcriptional output peaks towards the end of meiotic prophase-I, even with MSCI coinciding (Alexander et al., 2022; Ernst et al., 2019; MONESI, 1964). The dynamic switching from the paused (pSer5-RNAPII) to the elongating (pSer2-RNAPII) state of RNAPII drives this burst of transcription on autosomes (Alexander et al., 2022). The association of pSer2-RNAPII with the sex chromosomes without ARID1A renders them more

autosome-like, underlying the defect in sex-linked gene repression. The BAF-A-governed H3.3 dynamics on the sex chromosomes may limit the switch from the paused to the elongating state of RNAPII. In a human cell culture model, ZMYND11, a known tumor suppressor and reader of Lysine 36 trimethylation on H3.3 (H3.3K36me3), suppresses RNAPII elongation (Wen et al., 2014). Whether similar H3.3 readers affect RNAPII pausing during meiosis in mice remains unknown.

The initiation and maintenance of MSCI are known to be regulated by DNA damage response (DDR) factors, ATR, and MDC1, respectively (Ichijima et al., 2011; Royo et al., 2013; Turner et al., 2004). However, ARID1A deficient pachytene spermatocytes appeared proficient in sex-linked DDR signaling, evidenced by the normal enrichment of ATR, γH2Ax, and MDC1 on the sex body. Therefore, BAF-A-mediated transcriptional repression of the sex chromosomes occurs independently of DDR signaling. Given that the accumulation of ATR and MDC1 on the sex body precedes that of ARID1A, which only manifests late in diplonema, it is reasonable to hypothesize that DDR signaling might recruit BAF-A to the sex chromosomes.

ARID1A did not appear necessary for sex-linked DNA DSB formation. In contrast, the expected association of DMC1, the meiosis-specific DNA recombinase, with the asynapsed axes of the sex chromosomes do require ARID1A. Interestingly, sex-linked intergenic sites displaying an ARID1A-dependent enrichment of H3.3 appeared depleted for DMC1. Consistent with this antagonism, the loss of ARID1A resulted in the re-distribution of H3.3 to regions normally associated with DMC1. Although only correlative, these data suggest that regions displaying a localized enrichment of H3.3 might be unfavorable for DNA repair. Determining the extent to which a deficiency in BAF-A activity might impede sex-linked DNA repair is challenging. RAD51 binding to the sex chromosomes remained stable without ARID1A, suggesting that some sex-linked DNA repair remains. Our studies highlight a model in which BAF-A promotes meiotic progression by facilitating MSCI and DNA repair on the sex chromosomes.

Materials and Methods

Generation of *Arid1a* conditional mutant mice

Generation of the $Arid1a^{tm1Mag}$/Mmnc mutant allele was previously described (Chandler et al., 2015). The mice are available through the regional mutant mouse resource and research center (MMRRC; Stock number: 041418-UNC; www.mmrrc.org). *Arid1a* floxed mice ($Arid1a^{tm1Mag/}Mmnc$) exist on a CD1 genetic background. Crosses with testes-specific *Stra8-Cre* mice (expressed in P3 spermatogonia)(Sadate-Ngatchou et al., 2008) resulted in conditional knockouts. $Arid1a^{fl/fl}$; $Stra8\text{-}Cre^{Tg/0}$ females were crossed to either $Arid1a^{fl+}$or $Arid1a^{flfl}$ males to obtain $Arid1a^{flfl}$;$Stra8\text{-}Cre^{Tg/0}$($Arid1a^{cKO}$) and either $Arid1a^{flfl}$ or $Arid1a^{fl+}$ ($Arid1a^{WT}$) males. Haploinsufficiency associated with *Arid1a* negated the possibility of transmitting the Cre through the paternal germline. Genotyping primers: $Arid1a^{fl+}$ alleles – (F)-5' – CTAGGTGGAAGGTAGCTGACTGA -3'; (R) 5' – TACACGGAGTCAGGCTGAGC -3' (PCR product sizes-*fl*: 300 bp; +: 200 bp), and *Stra8-Cre* – (F) 5' -GTGCAAGCTGAACAACAGGA-3', (R) 5' -AGGGACACAGCATTGGAGTC-3' (PCR product size-*Cre*: 150 bp). Mice were housed with a 12hr light cycle (temperature – 20-24° C, humidity – 30-70%). All animal work followed approved UNC Chapel Hill IACUC protocols.

Histology

$Arid1a^{WT}$ and $Arid1a^{cKO}$ t2estes and cauda epididymites were fixed overnight in Bouins solution (Fisher scientific Ricca chemical; 11-201) at 4° C, followed by dehydration in ethanol

series (50%, 70%, and 100%) before embedding in paraffin. The animal histopathology core prepared stained tissue sections (hematoxylin and eosin for cauda epididymites; Periodic acid-Schiff for testes). Staining and morphology determined seminiferous tubule staging (Ahmed and Rooij, 2009; Meistrich and Hess, 2013). Summary of staging is provided (Supplementary table 1).

Immunofluorescence staining of testis cryosection and spermatocyte spreads

Testes cryosections and spermatocyte spreads from juvenile (P18, P19, and P26) and adult $Arid1a^{WT}$ and $Arid1a^{cKO}$ mice were examined with indirect immunofluorescence (IF). 8-10 µm thick cryosections of testes were prepared using published methods (Menon et al., 2021). Whole testes were fixed in 10% neutral buffered formalin (NBF) at 4° C for 20 minutes. They were cut in half, followed by 40 minutes of incubation in 10% NBF. Fixed tissues were washed three times (10 minutes per wash) in room temperature (RT) phosphate-buffered saline (PBS) pH7.4. The tissues were then treated with a series of sucrose washes -10% (30 min), 20% (30 min), and 30% (1 hr). Tissues were then incubated at 4° C overnight in a 1:1 ratio of a solution of 30% sucrose/optimum cutting temperature (OCT, Tissue-Tek; 62550-01), followed by embedding in OCT, sectioning, and storage at -80^0 C. Before immunostaining, cryosections were thawed on a heating block at 42^0 C, rehydrated for 10 minutes in PBS, and incubated in 10mM citric acid buffer (pH6.0) for 10 minutes at 80-90^0 C (antigen retrieval), during which addition of fresh citrate buffer (80-90 ^{0}C) occurred every 2 minutes, and then allowed to cool for 20 minutes. For immunostaining, cryosections were incubated overnight in a humidified chamber with primary antibodies at 4^0 C and then followed by a secondary antibody incubation at RT for 1 hr. Sera were diluted with antibody dilution buffer in PBS (ADB: 3% bovine serum albumin; 10% goat serum; 0.5% Triton-X 100). Two identical blocking steps preceded antibody incubations. These steps involved sequential incubations in (i) PBS, (ii) PBS/0.1% Triton-X 100, and (iii) blocking solution (1:10 dilution of ADB in PBS) for 10 minutes each at RT. Immunostained slides were washed twice in Kodak Photo-Flo 200 (PBS/0.32%), counterstained with DAPI, washed again in PBS/0.32% Photo-Flo 200, and then mounted in Prolong Gold antifade medium (P-36931; Life Technologies).

Spermatocyte spreads were prepared as previously described (Gray et al., 2020) with minor modifications. Briefly, seminiferous tubules were incubated in hypotonic buffer (30mM Tris pH8.2, 50mM Sucrose, 17 mM Trisodium dihydrate, 5mM EDTA, 0.5mM DTT, 0.1 mM PMSF) for 10-20 minutes on ice. Tubules were minced in 100 mM sucrose (200 µL/testis) until the solution turned turbid. The resulting cell suspension was isolated, avoiding tissue debris, and held on ice. Cells were fixed and spread by dropping 20 µL of cell suspension directly onto glass slides coated with paraformaldehyde solution (1 % Paraformaldehyde; 0.15 % Triton X-100; 625 nM Sodium Borate, pH 9.2). Uniform spreading was ensured by tilting the slides along the near and opposite edges. Next, slides were incubated in a humidified chamber for 1 hr, following which they quickly air-dried and then washed two times in PBS/0.32% Photo-Flo 200 and once in H_2O/0.32% Photo-Flo 200. Air-dried slides were immediately processed for immunostaining as described above for cryosections or stored at – 80^0 C. Immunostained spreads were washed 2x with PBS/0.32% Photo-Flo 200, counterstained with DAPI, and washed again with Photo-Flo 200. Stained spreads were mounted in Prolong Gold antifade medium. The list of antibodies for IF is provided in supplementary table 2. The Leica Dmi8 fluorescent microscope was used to capture images. Z-stacks were deconvoluted using Leica Dmi8 image software (Huygens essentials version 20.04).

Fluorescence intensity measurements and object (foci) counting were performed with Fiji (Schindelin et al., 2012). RNAPII (pSer2) IF signal intensities were measured using the method

described previously (Menon et al., 2021). Briefly, pixel intensities were recorded from regions of interest (ROI) which include the sex chromosomes (sex body) and an area lacking cells (background fluorescence). Corrected total RNAPII-pSer2 fluorescence (CTRF) was calculated using method previously described (https://theolb.readthedocs.io/en/latest/imaging/measuring-cell-fluorescence-using-imagej.html), where CTRF= Integrated density associated with sex body – (Area of sex body x Mean fluorescence of background).

For counting axial DMC1 foci, images were cropped to an area encompassing the sex chromosomes. Cropped images were converted to 8-bit grayscale, and the DMC1 fluorescent channel selected for further processing. An ROI was set, manual thresholding was performed to select DMC foci, and noise (speckles) removed using Fiji median filter tool with a radius set to 2.0-4.0. Finally, DMC1 was enumerated with the count particles tool. Statistical significance was calculated using an unpaired student's t-test. Metadata associated with quantification of RNAPII (pSer2) fluorescence and DMC1 foci are provided (Supplementary table 3,4)

Preparation of acid-extracted histones and western blotting

Histones from P19 $Arid1a^{WT}$ and $Arid1a^{cKO}$spermatogenic cells were acid-extracted using published methods (Shechter et al., 2007). Seminiferous tubules were digested in 1 mL of Enriched Krebs-Ringer Bicarbonate (EKRB) Medium (EKRB salts – 1.2 mM KH_2PO_4, 1.3 mM $CaCl_2$, 119.4 mM NaCl, 4.8 mM KCl, 1.2 mM $MgSO_4$, 11.1 mM Dextrose; 25.2 mM $NaHCO_3$; 1X essential amino acids -Invitrogen, 11130051; 1X non-essential amino acids-Gibco,11140050) supplemented with 0.5 mg/mL collagenase (10 minutes, rotated at 32 °C). Digested tubules sedimented by gravity for 5 minutes at RT. The supernatant was removed, and tubules were resuspended in 1 mL EKRB medium and then settled for 5 minutes at RT. The sedimented tubules were transferred to 1 mL EKRB medium supplemented with 0.025% trypsin and 4 µg/mL DNase-I. The suspension was incubated for 10 minutes on a rotator at 32° C. Digestion was halted with 10% Fetal bovine serum (FBS), and single-cell suspensions were prepared by pipetting the slurry and then filtering it sequentially through 70 µm and 40 µm filters. Cells were pelleted at 600g for 5 minutes, washed once in PBS, and processed for histone extraction. Briefly, cell pellets were resuspended in 1 mL hypotonic lysis buffer (10mM Tris-Cl pH8.0, 1mM KCl, 1.5mM $MgCl_2$, 1mM DTT) and incubated on a rotator at 4° C for 30 minutes. Nuclei were pelleted at 10,000 g for 10 minutes at 4° C. Nuclear pellets were resuspended in 400 µL 0.2N HCl and incubated for 1 hr at 4 °C, followed by centrifugation at 14,000 g for 10 minutes at 4° C to isolate supernatants containing histones that were precipitated with Trichloroacetic acid (TCA), and then added to the nuclear suspension at a final concentration of 33% and incubated on ice for 30 minutes. Precipitated histones were pelleted at 14,000g for 10 minutes at 4° C and washed two times in 1 mL ice-cold acetone. Finally, washed histones were air-dried at RT for 20 minutes and resuspended in 100 µL ddH_2O. For western blots, histones were separated on a 15 % SDS-polyacrylamide gel and transferred to PVDF (Polyvinylidene Difluoride) membrane using a semi-dry transfer apparatus (Bio-Rad). Sample loading was assessed by REVERT^{TM} total protein stain (LI-COR, 926-11010). Blots were scanned on a LI-COR Odyssey CLx imager and viewed using Image Studio Version 5.2.5. Antibodies and their corresponding dilutions are listed (Supplementary table 2).

Isolation of Pachytene Spermatocytes by the Sta-Put method of Unit Gravity

Pachytene spermatocytes were purified from *Arid1a*^{WT}and *Arid1a*^{cKO} adult testis by the unit gravity sedimentation procedure using a Sta-Put apparatus (Bellvé, 1993; O'Brien, 1993). Testis from at least 12 adult males (>P60) were dissected into EKRB, decapsulated, and digested in 0.5mg/ml collagenase for 15 min at 32° C. The resulting tubules were gently washed with EKRB and digested with 0.25mg/ml trypsin supplemented with 4µg/ml DNase for 15 min at 32° C. Following trypsin incubation, additional DNase (4µg/mL) was added to reduce viscosity further. Samples were triturated for 3 minutes with a plastic transfer pipet to fragment flagella, break intercellular bridges between germ cells and further disperse cells into suspension. Trypsin digestion was stopped by adding 0.25mg/mL trypsin inhibitor (Sigma/EPRO, T9003), and cell aggregates were removed by filtration using 40 µm cell strainers. Cells from the flow-through were pelleted at 500g for 5 min at 4° C, resuspended in EKRB + 0.5% BSA, and adjusted to a 3.33x10^8 cells/mL density. 10^9 cells from this single-cell suspension were loaded onto a 2-4% linear BSA gradient and allowed to sediment in the Sta-Put apparatus at 4° C. After 2hr and 40min of undisturbed sedimentation, 10ml fractions were collected at 45 sec per tube. Fractions were pelleted at 500g for 5 min at 4°C and resuspended in 1ml EKRB + 0.5%BSA. Fractions containing >80% pachytene spermatocytes were identified by light microscopy, pooled, and stored in cryoprotective media (DMEM supplemented with 10%FBS and 20%DMSO) at -80° C pending further analysis.

RNA extraction and RT-PCR

Total RNA was isolated in TRIzol™ reagent (Invitrogen, 15596026) from Sta-Put purified fractions enriched for pachytene spermatocytes and round spermatids. The extracted RNA was purified using the Direct-zol RNA kit (Zymo, R2050). cDNA was synthesized using a random primer mix (New England Biolabs, S1330S) and ProtoScript® II reverse transcriptase (New England Biolabs, M0368L). RT-PCR was performed on 1:10 cDNA dilutions using Sso Fast EvaGreen supermix (Bio-Rad, 172-5280) on a thermocycler (BioRad, C1000). Amplicons associated with either floxed or excised *Arid1a* cDNA's were detected using primers: (F)-5' – TCCAGTAAGGGAGGGCAAGAAGAT -3'; (R) 5' – GTAGTTGGCGTTGGGCAAGGCATTA -3' (PCR product sizes-*fl*: 612 bp; *Δ*: 281 bp)

RNA-seq

RNA was extracted and purified in quadruplicate from frozen pellets of *Arid1a*^{WT} and *Arid1a*^{cKO} pachytene spermatocytes obtained bSalmony Sta-Put gravity sedimentation by methods described above. All samples displayed RNA integrity number (RIN) > 7.0 as determined by Agilent Tapestation (High sensitivity RNA screen tape). Sequencing libraries were prepared from 1 µg of total RNA using a Kapa mRNA HyperPrep kit (Roche, KK8580) per the manufacturer's instruction. Libraries were quantified using Qubit™ dsDNA Hs assay kit (Invitrogen, Q32854) pooled at equimolar concentrations, and sequenced on a single lane of the Illumina NovaSeq 6000 S Prime flow cell (50 bp reads, paired-end).

RNA-seq data analysis

Quantification of gene expression was done using Salmon (Patro et al., 2017). Transcript counts at the gene level were summarized using tximport (Soneson et al., 2016) and then imported to perform a differential gene expression analysis using DESeq2 (Love et al., 2014). The mouse (mm10) gene/transcript annotations were retrieved using AnotationDbi (Hervé

Pagès et al., 2021) and *TxDb.Mus musculus.UCSC.mm10.known gene (Bioconductor Core Team, 2019)* R packages from Bioconductor. Low-count genes (< 10 reads) were pre-filtered and significant differences in counts were called at a false discovery rate (FDR)≤ 0.05. Supplementary Table 5 lists differentially expressed genes.

CUT&RUN (Cleavage Under Targets and Release Using Nuclease)

CUT&RUN assays were performed on cryopreserved $Arid1a^{WT}$and $Arid1a^{cKO}$ pachytene spermatocytes (500,000 cells/genotype) obtained by Sta-Put, using a slightly modified version of previously described methods (Meers et al., 2019; Skene and Henikoff, 2017). For ARID1A, CUT&RUN was performed in triplicates per genotype with two different antibodies. H3.3 CUT&RUN was performed in duplicates on $Arid1a^{WT}$ and triplicates on $Arid1a^{cKO}$ samples. Aliquots of cryopreserved $Arid1a^{WT}$ and $Arid1a^{cKO}$ pachytene spermatocytes were quickly thawed in a water bath at room temperature (RT), pelleted at 600g for 5 minutes, and washed [wash buffer: 20mM HEPES(Na) pH7.5, 150 mM NaCl, 0.5 mM spermidine, EDTA-free protease inhibitor] three times. Cells were gently resuspended in wash buffer and bound to Concanavalin-A coated magnetic beads (20 µL beads/500,000 cells) by mixing on a rotator for 15 minutes. The bead slurry was separated using a magnet followed by permeabilization in 50 µL antibody buffer (wash buffer + 0.05% Digitonin + 2mM EDTA) per sample in 0.2 mL 8-strip PCR tubes. Primary antibody was added to the samples and incubated on a nutator at 4° C overnight. Cells were then separated using a magnet, washed two times in 200 µL Dig-wash buffer (wash buffer + 0.05% Digitonin), and incubated with a secondary antibody prepared in 50 µL of Dig-wash buffer/sample for 30 minutes on a nutator at 4° C. After antibody incubations, samples were washed twice in 200 µL Dig-wash buffer and then incubated on a nutator at 4° C with 50 µL Dig-wash buffer containing 1000ng/mL Protein A/G Micrococcal Nuclease (pA/G-MNase) for 1 hr. Following this, samples were washed twice in 200 µL Dig-wash buffer, resuspended in 50 µL Dig-wash buffer, cooled down to 0° C, and supplemented with $CaCl_2$ at a final concentration of 2 mM to activate MNase. Samples were digested at 0 °C for 40 minutes. The resulting chromatin fragments were extracted from the bead slurry by incubating in 2X STOP buffer (340 mM NaCl, 20mM EDTA, 4mM EGTA, 0.05% Digitonin, 100 µg/mL RNase A) at 37° C for 30 minutes. DNA was purified using ChIP DNA clean and concentrator kit (Zymo, D5205). Libraries were prepared using the Kapa Hyperprep kit (Roche, KK8504), examined for size, and quantified on an Agilent 2100 bioanalyzer using the high-sensitivity DNA kit (Agilent, 5067-4626). The libraries were pooled in equimolar amounts and sequenced on a single lane of the Illumina NovaSeq 6000 S Prime flow cell (50 bp reads, paired-end). Antibody details are listed in Supplementary table 2. The pA/G-MNase (Addgene ID: 123461) used for the CUT&RUN experiments was purified at the Protein Expression and Purification core at UNC Chapel Hill.

CUT&RUN data analysis

CUT&RUN data analyses were performed as previously described (Menon et al., 2021). Briefly, fastq files were analyzed with fastqc (version 0.11.9), then processed with TrimGalore (version 0.6.2, https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) keeping the --trim-n option. Trimmed reads were aligned to mm10 (mouse) reference genome using bowtie2 (Langmead and Salzberg, 2012) parameters: bowtie2 --very-sensitive-local --no-mixed --no-unal --dovetail --no-discordant. Alignments were outputted in BAM format using samtools (Li et al., 2009) and filtered for PCR duplicates using Picard tools, MarkDuplicates (https://broadinstitute.github.io/picard/). Deduplicated BAM files were used to generate bigWig files filtered for ENCODE mm10 blocked regions (Amemiya et al., 2019) with DeepTools, bamCoverage (Ramírez et al., 2016) with the following options: -bs 30 --

smoothLength 60 -bl -- scaleFactor. The option --scaleFactor was used to normalize for composition bias between CUT&RUN libraries generated from $Arid1a^{WT}$ and $Arid1a^{cKO}$ pachytene spermatocytes. csaw (Lun and Smyth, 2015) derived normalization factors (nf) were used to calculate the effective library size (library size X nf). The inverse of the effective library size per million was supplied to --scaleFactor to generate normalized bigWig files. Peak calling was performed with MACS2 (version 2.1.2) with options: -f BAMPE --broad --broad-cutoff 0.05 --keep-dup all, on filtered BAM files associated with ARID1A and H3.3 relative to an IgG control previously generated from pachytene enriched populations obtained from P18 testes (Chakraborty and Magnuson, 2022). Peak overlaps across replicates were determined using bedtools intersect (Quinlan and Hall, 2010). Peak calls are provided in supplementary table 6. Peaks were annotated using HOMER (version 4.9.1), annotatePeaks.pl. Replicate bigWig files were averaged using WiggleTools (Zerbino et al., 2014). Averaged bigWig files generated heatmaps with DeepTools, computeMatrix, and plotHeatmap. Data was visualized on Integrative Genomics Viewer (Robinson et al., 2011).

ATAC-seq

Cryopreserved aliquots of $Arid1a^{WT}$ and $Arid1a^{cKO}$pachytene spermatocytes were processed in quadruplicate for ATAC-seq using the standard Omni-ATAC protocol (Corces et al., 2017) with minor changes. These involved increasing the cell number to 100,000 per replicate, using Diagenode tagmentase (loaded Tn5 transposase, C01070012) and Diagenode 2X tagmentation buffer (C01019043). Libraries were quantified using NEBNext® kit for Illumina® (New England Biolabs, E7630S), pooled at equimolar amounts, and sequenced on a single lane of the Illumina NovaSeq 6000 S Prime flow cell (50 bp reads, paired-end).

ATAC-seq data analysis

After analyzing the quality of fastq files with fastqc (version 0.11.9), they were processed with TrimGalore (version 0.6.2), keeping the --trim-n option. Alignments to mm10 reference genome were performed with bowtie2 (Langmead and Salzberg, 2012) parameters: bowtie2 --very-sensitive -X 2000, and outputted in BAM format using samtools. Resultant BAM files were filtered for PCR duplicates using Picard tools, MarkDuplicates, and mitochondrial reads using samtools view (code: samtools view -h Dedup.bam | grep -v 'chrM' | samtools view -b -h -f 0x2 – | samtools sort > Dedup_noChrM.bam). Filtered BAM files were used to call peaks with MACS2 (parameters: -f BAMPE -n de -q 0.05 --nomodel --nolambda --keep-dup all) and generate normalized bigWig files with DeepTools, bamCoverage (options: -bs 30 --smoothLength 60 -bl -- normalized using RPKM). Peak overlaps across replicates were determined using bedtools intersect (Quinlan and Hall, 2010). Peak calls are provided in supplementary table 7. Peak annotation (annotatePeaks.pl) and motif analyses (findMotifsGenome.pl) were performed with HOMER (version 4.9.1). PRDM9 motif frequency near ATAC-seq peaks was determined using HOMER annotatePeaks.pl with options: -size 20000 -hist 10. Replicate bigWig files were averaged using WiggleTools (Zerbino et al., 2014). Averaged bigWig files generated heatmaps with DeepTools, computeMatrix, and plotHeatmap. Data was visualized on Integrative Genomics Viewer (Robinson et al., 2011).

Data availability

RNA-seq, CUT&RUN, and ATAC-seq data generated in this study are deposited with GEO (Gene Expression Omnibus) under accession number GSE225612. DMC1 SSDS (GSE35498), RAD51 SSDS (GSE143582), and H3K4me3 ChIP-seq (GSE89502) datasets were previously

published (Brick et al., 2012; Hinch et al., 2020; Maezawa et al., 2018a) and are available on GEO.

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Author contributions

D.U.M and T.M conceptualized and designed the project. D.U.M. and N.M. carried out experiments. Data curation and validation are done by D.U.M and N.M. The writing was performed by D.U.M and NM, reviewed and edited by T.M. Project supervision and funding acquisition by T.M.

Competing interests

The authors declare no competing interests.

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Editors

Reviewer #1 (Public Review):

The work by Debashish U. Menon, Noel Murcia, and Terry Magnuson brings important knowledge about histone H3.3 dynamics involved in meiotic sex chromosome inactivation (MSCI). MSCI is unique to gametes and failure during this process can lead to infertility. Classically, MSCI has been studied in the context of DNA Damage repair pathways and little is known about the epigenetic mechanisms behind maintenance of the sex body as a silencing platform during meiosis. One of the major strengths of this work is the evidence provided on the role of ARID1A, a BAF subunit, in MSCI through the regulation of H3.3 occupancy in specific genic regions. This is well supported by a combination of immunofluorescence, RNA seq, CUT&RUN and ATAC-seq.
The mouse model in this study is a conditional Stra8 Cre mouse. Loss of ARID1A in this mouse, caused up regulation of XY linked genes in prophase I spermatocytes and ingression of RNA pol II to the sex body, indicating a role for this chromatin remodeller in MSCI. Using RNA seq and CUT&RUN and ATAC-seq, the authors show that ARID1A regulates chromatin accessibility of the sex chromosomes. ARID1A interacts with gene transcription start sites of sex-linked genes, and loss of ARID1A increased promoter accessibility of XY linked genes with concomitant gene up regulation.

This work suggests that ARID1A regulates chromatin composition of the sex body relative to the autosomes. In the absence of ARID1A, spermatocytes show less enrichment of H3.3 in the sex chromosomes and stable levels of the canonical histones H3.1/3.2. By overlapping CUT&RUN and ATAC-seq data, authors show that changes in chromatin accessibility in the absence of ARID1A are given by redistribution of occupancy of H3.3. Gained open chromatin in mutants corresponds to up regulation of H3.3 occupancy at transcription start sites of genes regulated by ARID1A.

Interestingly, ARID1A loss caused increased promoter occupancy by H3.3 in regions usually occupied by PRDM9. PRDM9 is a protein with histone methyltransferase activity that catalyzes histone H3 lysine 4 trimethylation during meiotic prophase I, and positions double strand break (DSB) hotspots. Lack of ARID1A causes reduction in occupancy of DMC1, a recombinase involved in DSB repair, in non-homologous sex regions. These data suggest that ARID1A might indirectly influence DNA DSB repair on the sex chromosomes by regulating the localization of H3.3. This is very interesting given the suggested role for ARID1A in genome instability in cancer cells (Nacarelli et al 2020: 10.1080/23723556.2019.1690923, Zhang et al. 2023: 10.1093/carcin/bgad011 and others). It raises the question of whether this role is also involved in meiotic DSB repair in autosomes and/or how this mechanism differs in sex chromosomes compared to autosomes.

It is worth mentioning that authors show that there are Arid1a transcripts that escape the Cre system. This might mask the phenotype of the Arid1a knockout, given that many of the

sequencing techniques used here are done on a heterogeneous population of knockout and wild type spermatocytes. In relation to this, I think that the use of the term "pachytene arrest" might be overstated, since this is not the phenotype truly observed (these mice produce sperm). ARID1A is present throughout prophase I and it might have pre-MSCI roles that impact earlier stages of Meiosis I and cell death might be happening in these earlier stages too.

Overall the research presented here is solid, adds new knowledge on how the sex chromatin is silenced during meiosis and has generated relevant databases for the field.

Reviewer #2 (Public Review):

The authors tried to characterize the function of the SWI/SNF remodeler family, BAF, in spermatogenesis. The authors focused on ARID1A, a BAF-specific putative DNA binding subunit, based on gene expression profiles. The study has several serious issues with the data and interpretation. The conditional deletion mouse model of ARIDA using Stra8-cre showed inefficient deletion; spermatogenesis did not appear to be severely compromised in the mutants. Using this data, the authors claimed that meiotic arrest occurs in the mutants. This is obviously a misinterpretation. In the later parts, the authors performed next-gen analyses, including ATAC-seq and H3.3 CUT&RUN, using the isolated cells from the mutant mice. However, with this inefficient deletion, most cells isolated from the mutant mice appeared not to undergo Cre-mediated recombination. Therefore, these experiments do not tell any conclusion pertinent to the Arid1a mutation. Furthermore, many of the later parts of this study focus on the analysis of H3.3 CUT&RUN. However, Fig. S7 clearly suggests that the H3.3 CUT&RUN experiment in the wild-type simply failed. Thus, none of the analyses using the H3.3 CUT&RUN data can be interpreted. Overall, I found that the study does not have rigorous data, and the study is not interpretable. If the author wishes to study the function of ARID2 in spermatogenesis, they may need to try other cre-lines to have more robust phenotypes, and all analyses must be redone using a mouse model with efficient deletion of ARID2.

Reviewer #3 (Public Review):

In this manuscript, Magnuson and colleagues investigate the meiotic functions of ARID1A, a putative DNA binding subunit of the SWI/SNF chromatin remodeler BAF. The authors develop a germ cell specific knockout mouse model using Stra8-cre and observe that ARID1A-deficient cells undergo pachytene arrest, although due to inefficiency of the Stra8-cre system the mice retain ARID1A-expressing cells that yield sperm and allow fertility. Because ARID1A was found to accumulate at the XY body late in Prophase I, the authors suspected a potential role in meiotic silencing and by RNAseq observe significant misexpression of sex-linked genes that typically are silenced at pachytene. They go on to show that ARID1A is required for exclusion of RNA PolII from the sex body, consistent with a meiotic sex chromosome inactivation (MSCI) defect. The authors proceed to investigate the impacts of ARID1A on chromatin accessibility and H3.3 deposition genome-wide. H3.3 is known be regulated by ARID1A and is linked to silencing, and here the authors find that upon loss of ARID1A, overall H3.3 enrichment at the sex body as measured by IF failed to occur, but H3.3 was enriched specifically at transcriptional start sites of sex-linked genes that are normally regulated by ARID1A. The results suggest that ARID1A normally prevents H3.3 accumulation at target promoters on sex chromosomes and based on additional data, restricts H3.3 to intergenic sites. Finally, the authors present data implicating ARID1A and H3.3 occupancy in DSB repair, finding that ARID1A KO leads to a reduction in focus formation by DMC1, a key repair protein. Overall the paper covers a lot of ground, provides

important new insights into the process of MSCI from the perspective of chromatin composition and structure, and raises many interesting questions. In general the paper is well written and the data are clear. Specific points to address are as follows:

1. A challenge with the author's CKO model is the incomplete efficiency of ARID1A loss, due to incomplete CRE-mediated deletion. The authors effectively work around this issue, but they don't state specifically what percentage of CKO cells lack ARID1A staining. This information should be added. They refer to cells that retain ARID1A staining in CKO testes as 'internal controls' but this reviewer finds that label inappropriate. Although some cells that retain ARID1A won't have undergone CRE-mediated excision, others may have excised but possibly have delayed kinetics of deletion or ARID1A RNA/protein turnover and loss. Such cells likely have partial ARID1A depletion to different extents and therefore in some cases are no longer wild-type. In subsequent figures in which co-staining for ARID1A is done, it would be appropriate for the authors to specify if they are quantifying all cells from CKO testes, or only those that lack ARID1A staining.

2. The authors don't see defects in a few DDR markers in ARID1A CKO cells and conclude that the role of ARID1A in silencing is 'mutually exclusive to DDR pathways' (p 12) and 'occurs independently of DDR signaling' (p30). The data suggest that ARID1A may not be required for DDR signaling, but do not rule out the possibility that ARID1A is downstream of DDR signaling (and the authors even hypothesize this on p30). The data provided do not justify the conclusion that ARID1A acts independently of DDR signaling.

3. After observing no changes in levels or localization of H3.3 chaperones, the authors conclude that 'ARID1A impacts H3.3 accumulation on the sex chromosomes without affecting its expression or incorporation during pachynema.' It's not clear to this reviewer what the authors mean by this. Aside from the issue of not having tested DAXX or HIRA activity, are they suggesting that some other process besides altered incorporation leads to H3.3 accumulation and if so what process would that be?

4. The authors find an interesting connection between certain regions that gained chromatin accessibility after ARID1A loss (clusters G1 and G3) and presence of the PRDM9 sequence motif. The G1 and G3 clusters also show DMC1 occupancy and H3K4me3 enrichment. However, an additional cluster with gained accessibility (G4) also shows DMC1 occupancy and H3K4me3 enrichment but unlike clusters G1 and G3 has modest H3.3 accumulation. The paper would benefit for additional discussion about the G4 cluster (which encompasses 960 peak calls). Is there any enrichment of PRDM9 sites in G4? If H3.3 exclusion governs meiotic DSBs, how does cluster G4 fit into the model?

5. The impacts of ARID1A loss on DMC1 focus formation (reduced sex chromosome association) are very interesting and also raise additional questions. Are DMC1 foci on autosomes also affected during pachynema? The corresponding lack of apparent effect on RAD51 implies that breaks are still made and resected, enabling RAD51 filament formation. A more thorough quantitative assessment of RAD51 focus formation will be interesting in the long run, enabling determination of the number of break sites and the kinetics of repair, which the authors suggest is perturbed by ARID1A loss but don't directly test. It isn't clear how a nucleosomal factor (H3.3) would influence loading of recombinases onto ssDNA, especially if the alteration is not at the level of resection and ssDNA formation. Additional discussion of this point is warranted. Lastly, there currently are various notions for the interplay between RAD51 and DMC1 in filament formation and break repair, and brief discussion of this area and the implications of the new findings from the ARID1A CKO would strengthen the paper further.