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Higher expression levels of MSL2 in *D. virilis* lead to histone locus binding that is not seen in other *Drosophila* species.

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An abstract of
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Abstract

Higher expression levels of MSL2 in *D. virilis* lead to histone locus binding that is not seen in other *Drosophila* species.

By Mellisa Xie

Nuclear bodies are membraneless structures containing concentrated regulatory factors that coordinate nuclear processes such as gene expression. The histone locus body (HLB) is a nuclear body that is the main site of histone mRNA production. While many factors of the HLB are known, there are likely many unknown factors that contribute to histone gene regulation. In addition, while HLB function is highly conserved, it is also unknown how the HLB may function differently in different species. The histone locus (HL) of the model organism *Drosophila melanogaster* contains ~100 tandem arrays of the five histone genes. While *D. melanogaster* has one HL, the related species, *Drosophila virilis*, has two HL. We observe localization of male-specific dosage compensation proteins MSL2 and MSL3 to the major *D. virilis* histone locus using polytene chromosome immunofluorescence, which we do not observe in other *Drosophila* species. To confirm our immunofluorescence observations, we mapped existing MSL2 ChIP-seq data and discovered that when MSL2 from either species is overexpressed, it targets the *H2a-H2b* promoter. Finally, we performed RT-qPCR analysis on *D. melanogaster* and *D. virilis* to compare expression levels of *msl2*, observing higher levels of *msl2* in *D. virilis*, compared to *D. melanogaster*. Our results indicate that increased MSL2 expression leads to artificial HL targeting in *D. melanogaster* and natural targeting in *D. virilis*. In addition, our results caution against using *D. melanogaster* MSL2 overexpression systems to infer the role of this protein in dosage compensation.

Higher expression levels of MSL2 in *D. virilis* lead to histone locus binding that is not seen in other *Drosophila* species.

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Introduction

Conservation of gene regulation

Coordinated expression of genes is necessary for proper packaging and utilization of genetic material as the early embryo develops and grows (Duronio and Marzluff 2017). For example, the formation of nuclear bodies in eukaryotic cells creates micro-environments to concentrate proteins and related factors that allow functions, such as RNA processing and regulation of gene expression, to occur more efficiently (Tatomer et al. 2016; Mao, Zhang, and Spector 2011). The transcription of histone mRNA is tightly regulated by factors concentrated in a nuclear body called the histone locus body (HLB). In *Drosophila*, proteins such as Multisex Combs (Mxc) (White et al. 2011) and Chromatin-Linked Adaptor for MSL Proteins (CLAMP) (Rieder et al. 2017) target the histone locus to form the HLB. Mxc is a protein orthologous to the human protein Nuclear Protein of the ATM locus (NPAT) and is a core scaffolding protein (Kemp et al. 2021) required for HLB assembly and histone mRNA biosynthesis (White et al. 2011). CLAMP binds GA-rich sequences across the genome (Fig. 1), including in the histone gene array (Fig. 2), and regulates histone gene expression (Rieder et al. 2017). Although Mxc has retained the same function across species, Mxc is not well conserved, even in *Drosophila* (Fig. 3). CLAMP, on the other hand, is critical for Mxc localization to the histone genes in *Drosophila* (Rieder et al. 2017), and is very well conserved among *Drosophila* species (Fig. 3). Although CLAMP is critical in attracting Mxc, a protein found only at the histone locus body, CLAMP is not unique to the histone locus. It targets sites throughout the genome.

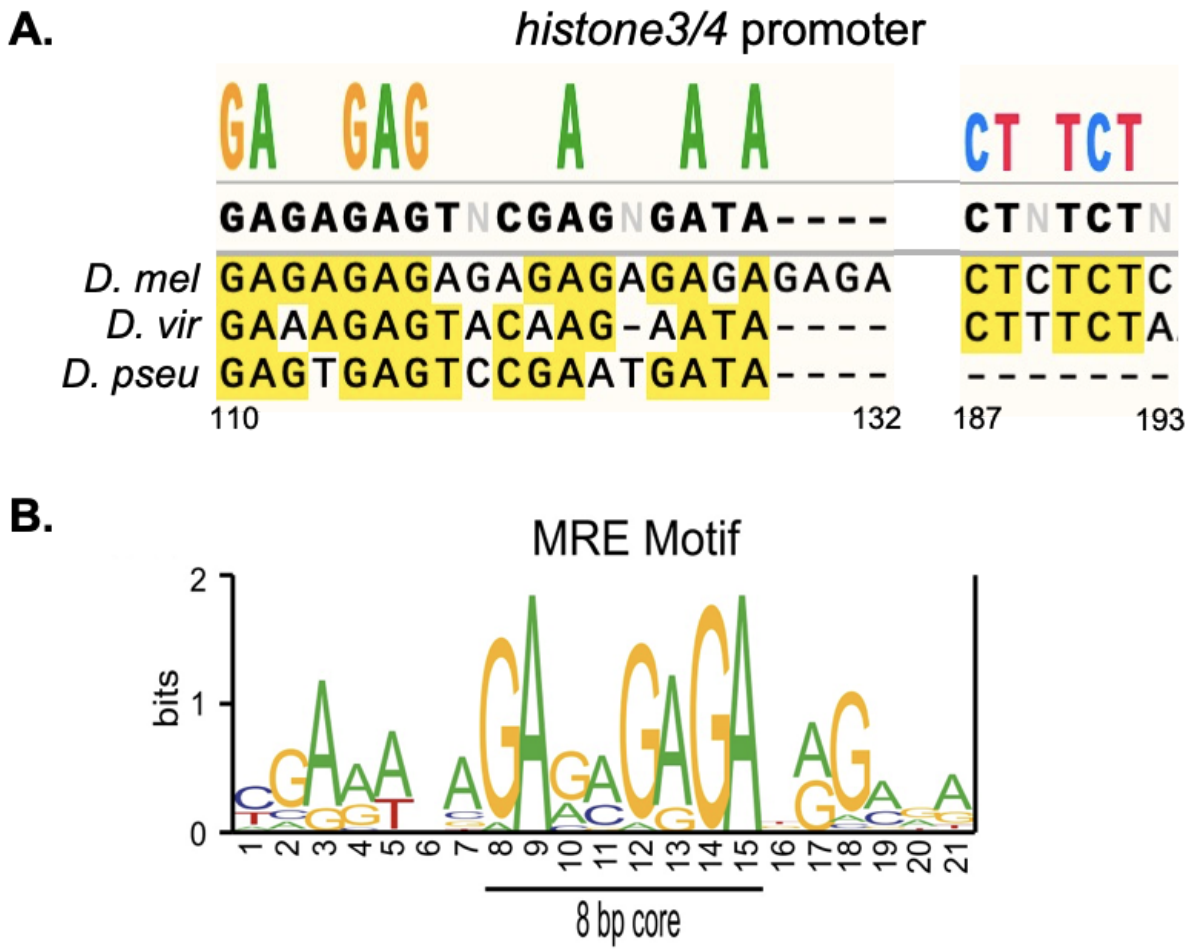

Figure 1: CLAMP targets GA-rich sequences across the genome
(A) CLAMP binds to the *D. melanogaster histone3/4* promoter by recognizing perfect GA repeats. Conservation of GA repeats is shown for *D. melanogaster* (D. mel), *D. virilis* (D. vir), and *D. pseudoobscura* (D. pseu). Adapted from Rieder et al. 2017. (B) CLAMP also targets GA-rich MSL recognition elements (MREs) on the X-chromosome. Figure from Soruco et al. 2013; adapted from Alekseyenko et al. 2008.

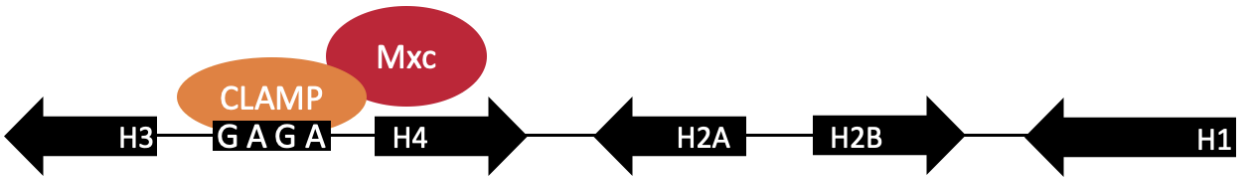

Figure 2: CLAMP targets GA-repeats at the *histone3/4* promoter, which promotes histone locus body formation.
Following CLAMP at the histone locus, scaffolding proteins, such as Mxc, initiate histone locus body formation (Rieder et al. 2017). There is no evidence that CLAMP and Mxc directly interact with each other, but Mxc forms a phase-separated body around the histone locus.

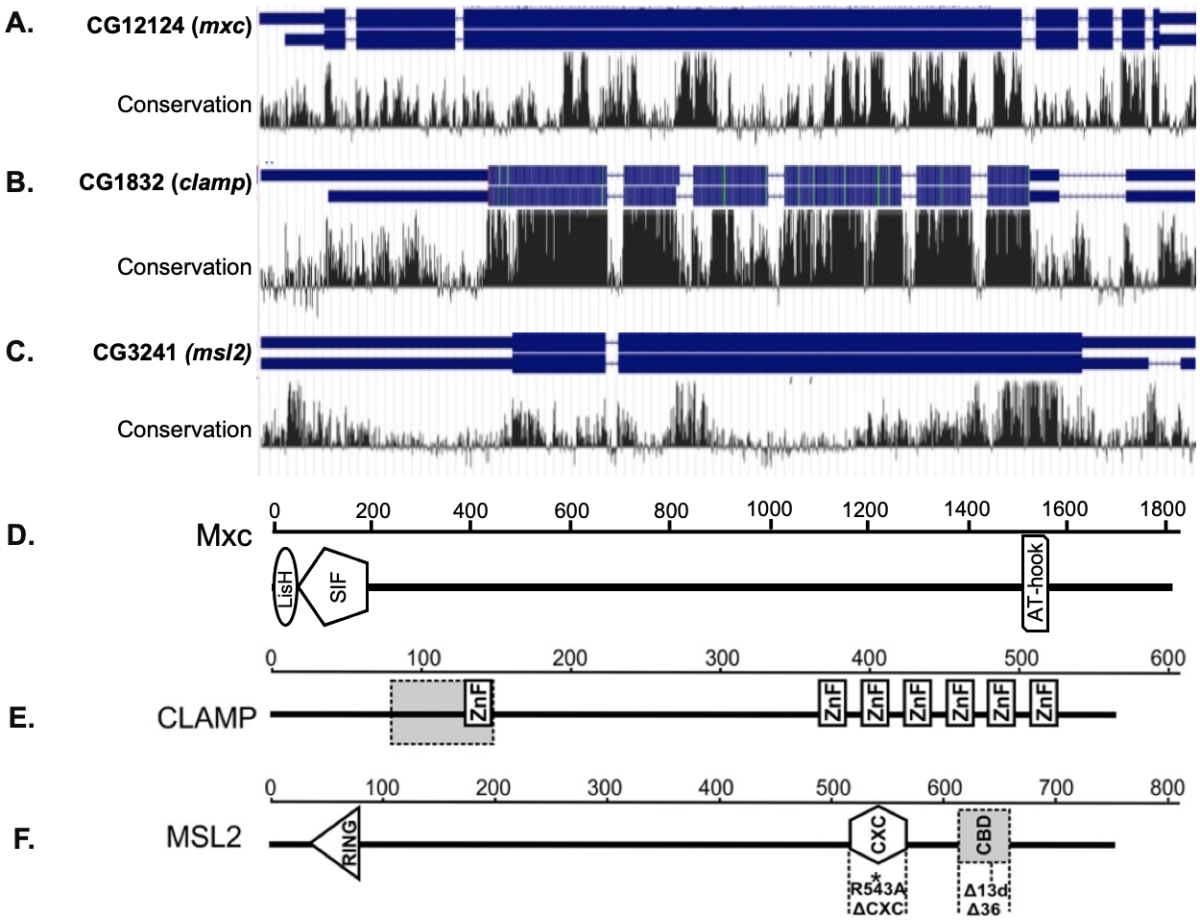

Figure 3: Conservation of Mxc, CLAMP, and MSL2.
(A)-(C) Conservation of *mxc*, c*lamp*, and *msl2* genes among 36 *Drosophila* species. (A) Conservation of *Drosophila mxc* (CG12124) gene, the *Drosophila* ortholog of NPAT. (B) Conservation of *Drosophila clamp* (CG1832) gene. *Clamp* is highly conserved among *Drosophila* and is the most conserved when compared to *mxc* and *MSL2*. (Adapted from Kuzu et al. 2016). (C) Conservation of *Drosophila msl2* (CG3241) gene. *Msl2* is the least conserved. Data from the UCSC Genome Browser. Exons are indicated in blue. (D)-(F) Mxc, CLAMP, and MSL2 protein domains. (D) Mxc domains include the LisH (amino acids 6-38) and self-interaction facilitator (SIF) domains (amino acids 39-185) near the N-terminus and an AT-hook motif (amino acids 1523-1535) near the C-terminus (from Terzo et al. 2015). (E) CLAMP is a zinc-finger protein. One zinc-finger domain includes amino acids 86-153 and the following 30 amino acids (shaded) are critical for MSL2's interaction with CLAMP (from Tikhonova et al. 2019). (F) MSL2 has a RING domain, CXC domain and CLAMP-Binding Domain (CBD). The CXC and CBD domains are responsible for recruiting DCC to the X-chromosome. Adapted from Tikhonova et al. 2019.

Dosage Compensation

CLAMP participates in the formation of another nuclear body: the dosage compensated male X-chromosome (Soruco et al. 2013). CLAMP recognizes GA-rich sequences called MSL Recognition Elements (MRE) sites (Fig. 2B) and recruits the (male-specific lethal) MSL complex to the X-chromosome (Soruco et al. 2013). Dosage compensation is a phenomenon that occurs in a broad range of species, including *Diptera*, roundworms (J. C. Lucchesi 1998), and mammals (Brockdorff and Turner 2015). It is required to balance sex-chromosome linked gene expression between males and females. In *Drosophila*, the dosage compensation complex (DCC) contains factors that play a role in upregulating gene expression from the single X chromosome (John C. Lucchesi 2018; Samata and Akhtar 2018) in order to equivalate the product amount of RNA synthesis from two X chromosomes in female flies (Mukherjee and Beermann 1965). Although the dosage compensation proteins such as MSL2 are less conserved than CLAMP (Fig. 3), consistently, they form the complex on the X-chromosome, regardless of the downstream mechanisms of dosage compensation.

In *Drosophila*, the DCC complex is formed by five proteins, MSL1, MSL2, MSL3, MOF, and MLE, and one of the two noncoding *roX* RNAs (Fig. 4). MSL1 is a scaffolding protein that connects MSL2, a DNA-binding protein, with the MSL3/MOF complex. MLE recruit *roX* RNA (Villa et al. 2021). In *D. melanogaster*, there was an expansion of GA dinucleotide repeats across the X-chromosome compared to autosomes. Subsequently, there was an enrichment of CLAMP, which in turn, enriches the MSL complex on the X-chromosome (Kuzu et al. 2016).

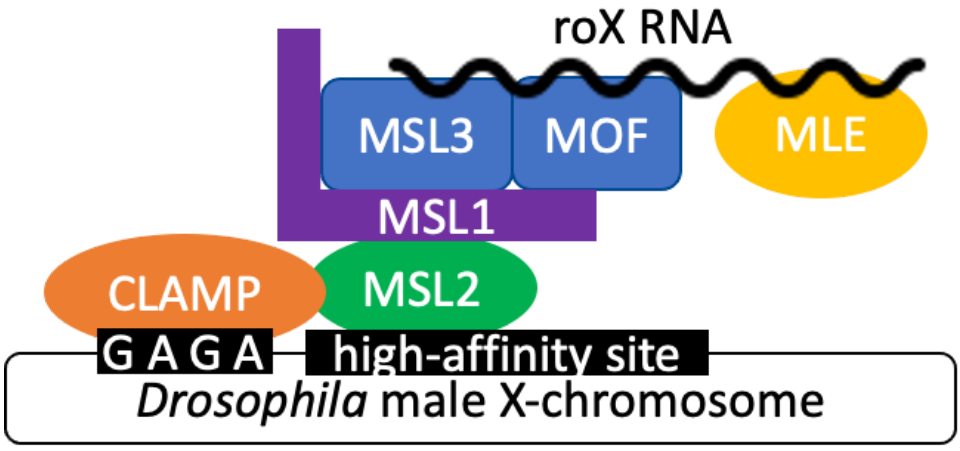

Figure 4: Arrangement of the dosage compensation complex (DCC) in *Drosophila*. The DCC complex in *Drosophila* contains five proteins, including MSL1, MSL2, MSL3, MOF, and MLE, and one of the two noncoding *roX* RNAs. MSL2 is the only DNA-binding protein, where it will bind to high-affinity sites to initiate dosage compensation (Alekseyenko et al. 2008). MSL2 is connected to the MSL3/MOF complex by scaffolding protein MSL1. MLE recruits *roX* RNA to the complex (Villa et al. 2021). CLAMP binds to GA-repeat rich sequences of the X-chromosome, and, in *D. melanogaster*, functions as the link between the DCC and X-linked sequences by directly interacting with MSL2 to open chromatin for DCC recruitment (Urban et al. 2017).

MSL2 is a male-specific dosage compensation protein (Zhou et al. 1995) that is not highly well conserved (Fig. 3). This may be because the MSL2 component of the DCC is only translated in males, although the other complex members are non-sex-specific. However, important domains for dosage compensation, such as the CXC domain, are conserved (Villa et al. 2021). In particular, MSL2 interacts with X-linked "PionX" sites, which distinguish the X chromosome from autosomal chromosomes (Villa et al. 2016). Villa et al. performed these MSL2 experiments in *Drosophila melanogaster* cell culture systems. For example, they expressed recombinant MSL2 CXC domains. They then used synthetic gDNA that represented the whole genome to search for potential MSL2 binding sites, such as the "PionX" sites, X-linked sites that include MREs (Fig.1B).

In *D. melanogaster*, both the male X-chromosome and histone locus are targeted by the CLAMP protein early during embryogenesis (Rieder et al. 2017; Rieder, Jordan, and Larschan 2019), indicating that CLAMP helps set up these nuclear bodies. Although the formation of these two nuclear bodies during embryogenesis is conserved across animals, the mechanisms of nuclear body formation differ between species. **How do poorly conserved proteins such as Mxc and MSL2 find their target loci? How does CLAMP, which is not specific to certain bodies/loci, recruit the correct co-factors Mxc and MSL2?** I hypothesize that this may be through slightly differing cis elements or context specific signals at the loci (Fig. 1).

Histone locus in D. melanogaster *versus* D. virilis

We find some clues regarding my hypothesis by comparing histone loci in different *Drosophila* species. The HLB is highly conserved across species. Humans have two dispersed histone loci (Albig and Doenecke 1997; Marzluff et al. 2002), and sea urchins have two sets of histone genes, one that is scattered and one that is clustered (Matsushita et al. 2017). Although HLB formation is conserved, there remains differences in histone loci between *Drosophila* species. For example, *D. melanogaster* has one histone locus while *D. virilis* has two histone loci (Berloco et al. 2001). *D. virilis* is estimated to have diverged from its common ancestor with *D. melanogaster* about 40 million years ago (Russo, Takezaki, and Nei 1995). In *D. melanogaster*, the single histone locus (HL) encompasses ~100 tandem quintet arrays of the five histone genes, including histone *H1*, *H2A*, *H2B*, *H3*, and *H4* on the left arm of Chromosome 2 (Fig. 5A) (Duronio and Marzluff 2017; Bongartz and Schloissnig 2019). In *D. virilis*, however, there are two unlinked histone loci, including a major locus, found on chromosome II (25F), and a minor locus, found on chromosome VI (43C). The major locus encompasses around 25-30 arrays while the minor locus

encompasses only around 6-8 repeats (Schienman, Lozovskaya, and Strausbaugh 1998). In addition to two loci, *D. virilis* also has two array configurations of the histone genes – a quintet and quartet. The quintet is composed of the five histone genes, including histone *H1*, *H2A*, *H2B*, *H3*, and *H4*. The quartet, however, lacks the *H1* gene but still has the other four histone genes, *H2A*, *H2B*, *H3*, and *H4*. While quintet configurations are found at both the major and minor loci, the quartet configuration is only found at the major locus (Fig. 5B) (Schienman, Lozovskaya, and Strausbaugh 1998). Despite these differences between species, conserved HLB factors, including CLAMP and Mxc, target both *D. virilis* and *D. melanogaster* histone loci (Berloco et al. 2001; Rieder et al. 2017).

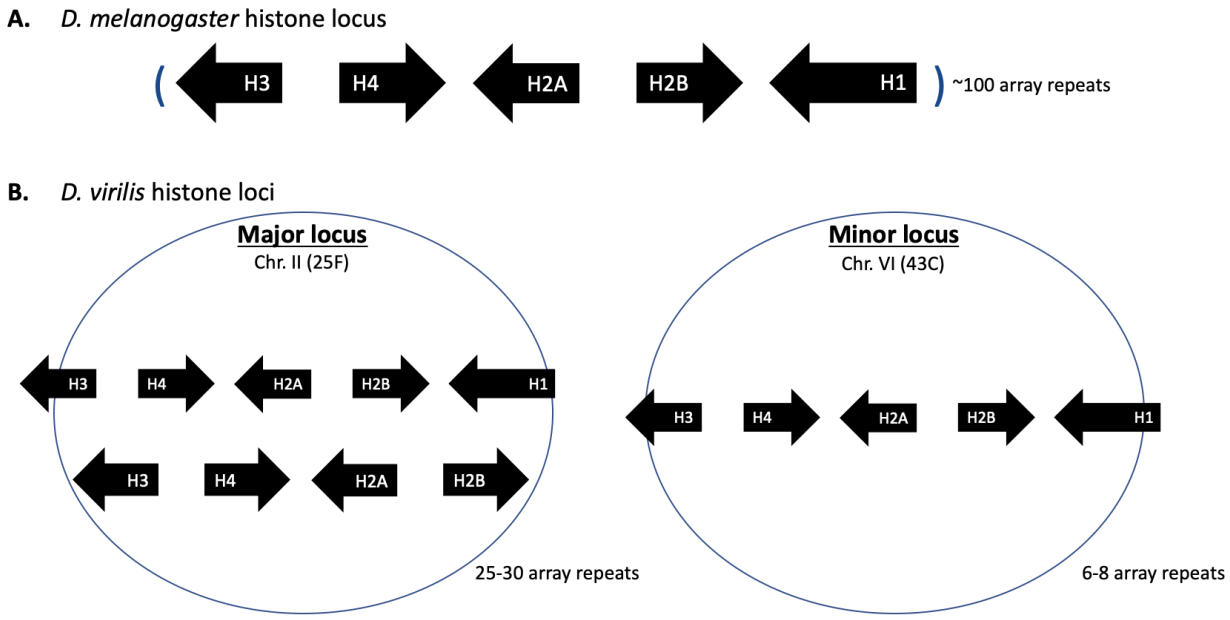

Figure 5: The *D. melanogaster* histone locus and *D. virilis* histone loci.
(A) In *D. melanogaster*, the histone locus, located on the left arm of Chromosome 2, includes approximately 100 repeats of the quintet histone gene array. (B) Unlike in *D. melanogaster*, *D. virilis* have two histone loci: the major locus (Chromosome II, 25F) and the minor locus (Chromosome IV, 43C). The major locus has both quintet and quartet arrangements of the histone genes while the minor locus only contains the quintet arrangement of histone genes. The major locus has around 25-30 histone array repeats, and the minor locus only has around 6-8 repeats of the quintet array (Schienman, Lozovskaya, and Strausbaugh 1998).

MSL2 targets one of the two D. virilis histone loci

CLAMP is common to both the male dosage compensated X-chromosome as well as the HLB. However, Mxc is specific to the HLB, while the DCC, including MSL2, is specific to the male X-chromosome in *D. melanogaster*. We were therefore surprised to observe localization of MSL2 to one of the two *D. virilis* histone loci using polytene chromosome immunofluorescence (Fig. 6), which we do not observe in *D. melanogaster*. The observation of MSL2 at the *D. virilis* histone locus provides an opportunity to study how MSL2 might influence histone gene expression through sex-specific mechanisms.

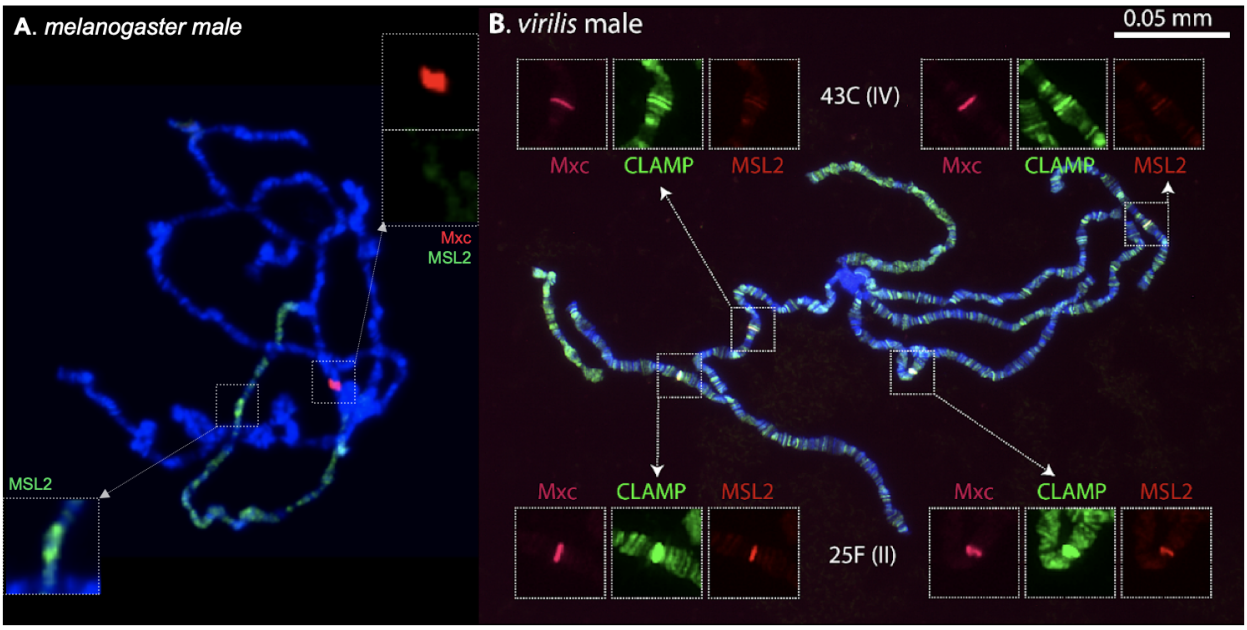

Figure 6: Localization of MSL2. (A) In *D. melanogaster*, the protein MSL2 targets the X-chromosome.
Immunofluorescence of Mxc reveals the histone locus. There is no colocalization of MSL2 with Mxc; MSL2 is not at the histone locus. (B) In *D. virilis*, MSL2 localizes to the major histone locus (25F) but not to the minor locus (43C). Further, the MSL2 antibody does not highlight the male X chromosome.

In this thesis, I demonstrate that MSL2 localization to the HL is unique to *D. virilis*. I further show, using available ChIP-seq datasets, that both *D. virilis* and *D. melanogaster* MSL2 target the *D. melanogaster* histone gene array at the *histone 2a/2b* promoter when the proteins are overexpressed in cell culture systems. This is surprising because CLAMP, which interacts with MSL2, targets the *histone 3/4* promoter (Rieder et al. 2017). This may indicate that MSL2 is specifically interacting with DNA sequence, as hypothesized in Villa et al. 2021. Finally, I use RT-qPCR to demonstrate that *msl2* expression levels are higher in *D. virilis* compared to *D. melanogaster*. Overall, my observations suggest that increased MSL2 expression leads to artificial HL targeting in *D. melanogaster* and natural targeting in *D. virilis*. This hypothesis also suggests caution when using MSL2 over-expression systems, which have previously been used to determine dosage compensation mechanisms. Further studies should aim to overexpress *msl2* in *D. melanogaster* to determine if MSL2 is then driven to the *D. melanogaster* histone locus.

Methods

Drosophila *strains*

I used *Drosophila melanogaster* (Rieder lab stock number 44: y[1]w[1118]; +;+;+), *Drosophila virilis* (Rieder lab stock number 133; National Drosophila Species Stock Center (NDSSC) #15010-1051.88), *Drosophila pseudoobscura* (Rieder lab stock number 169; NDSSC #14011-0121.217), and *Drosophila willistoni* (Rieder lab stock number 168; NDSSC #14030-0811.15).

Immunofluorescence on polytene chromosomes

I performed immunostaining on polytene chromosomes from salivary glands dissected from third instar *Drosophila* larvae raised at 18°C. I used primary antibodies at the following concentrations: guinea pig anti-Mxc (1:5000; generous gift from Drs. Duronio and Marzluff), rabbit anti-MSL2 (1:150; generous gift from Dr. V. Meller (originally Dr. Ron Richmond)), and goat anti-MSL3 serum (1:500; generous gift from Dr. E. Larschan lab (originally Dr. M. Kuroda)). I used AlexaFluor secondary antibodies (Thermo Fisher Scientific) at a concentration of 1:1000. I used goat anti-guinea pig AF647 (catalog # A-21450) to stain for Mxc, and goat anti-rabbit AF488 (catalog #: A-11008) to stain for MSL2.

Bioinformatics

I used the computational biology tool, Galaxy (usegalaxy.org) (Afgan et al. 2018) to map MSL2 to the histone locus. First, I extracted overexpressed MSL2 ChIP-seq and DIP-seq FASTQ files

from Villa et al. 2021's Sequence Read Archive (SRA) list (NCBI GEO Accession: GSE165833) in Galaxy using the "Faster Download and Extract Reads in FASTQ" option. The extracted FASTQ files contain MSL2's sequence and quality information. Next, I normalized the reference genome, the histone array (HA) (McKay et al. 2015), using the "NormalizeFASTA" option. This will ensure that the HA genome will be compatible with MSL2's FASTQ files that were extracted. Afterward, I used Galaxy's "Bowtie2" to map MSL2's sequential data to the HA and produce a BAM file. Finally, I ran "bamCoverage" on the BAM file to produce a bigwig file, which contains better visualization of the alignment. I used the Integrative Genomics Viewer (IGV) (Robinson et al. 2011) to display the produced bigwig file. I formed the same steps for normal MSL2 expressed levels from MSL2 ChIP-seq FASTQ files from Schauer et al. 2017's Sequence Read Archive (SRA) list (NCBI GEO Accession : GSE94115).

Quantitative real-time PCR (RT-qPCR)

I conducted qRT-PCR using RNA extracted from sexed *Drosophila* larvae. I used two biological replicates for each *Drosophila* species and for each sex. For *D. melanogaster msl2*, I used *msl2* forward (GCCCTGTCCGTATGAATG) and reverse (CAACATGGGTAAACAACC) primers from Dr. E. Larschan lab. For *D. virilis msl2*, I designed a forward (GCATCCTTTGCCGATGATGACAC) and reverse (CCAGCGGGACTCAATGTAACC) primer that amplified 83bp of the *D. virilis msl2 rna* sequence.

Cloning tandem gRNA expression vectors with pCFD4

I followed the CRISPR Fly Design protocol

(http://www.crisprflydesign.org/wp-content/uploads/2014/06/Cloning-with-pCFD4.pdf) to design a plasmid that, when injected into a fly, would overexpress *msl2* in *D. melanogaster*. This draws from the TRiP-CRISPR Overexpression (TRiP-OE) protocol (https://fgr.hms.harvard.edu/vivo-crispr-0). Using an MSL2 gRNA forward (TATATAGGAAAGATATCCGGGTGAACTTCCGGCTCCACAATGCTCTCTGGTTTTAGAG CTAGAAATAGCAAG) and reverse (ATTTTAACTTGCTATTTCTAGCTCTAAAACAGCCCTTAAATGCCGTCCGGACGTTAAA TTGAAAATAGGTC) sequence, I ran a PCR reaction to amplify the pCFD4 plasmid insert (Addgene plasmid #49411). I then used Gibson Assembly to insert my *msl2* guide RNAs into pCFD4. Afterward, I transformed this plasmid into 5-alpha competent *E. coli* (NEB #C2988J) and used GeneWiz to Sanger sequence and verify the plasmid. Finally, I sent the plasmid to GenetiVision for injection into flies carrying the attP40 landing site.

Results

Immunostaining reveals MSL2 and MSL3 localize to the major D. virilis *histone locus.*

To verify the earlier immunostaining of MSL2 at one of the *D. virilis* histone loci (Fig. 6B), I immunostained *D. virilis* polytene chromosomes using anti-Mxc and anti-MSL2 antibodies. Consistent with earlier results (Fig. 6B), I observed MSL2 co-localizing with Mxc at the major histone locus (HL) in males. However, the X-chromosome did not light up under the MSL2 antibody (Fig. 7A). MSL2 is a male-specific DCC component and is not present in female *Drosophila* (Zhou et al. 1995). To determine if the antibody was recognizing MSL2, I also immunostained female *D. virilis* polytene chromosomes. I observed no co-localization of MSL2 with Mxc in either HL, suggesting that the anti-MSL2 antibody was correctly recognizing MSL2 (Fig. 7B).

To determine if the DCC, or just MSL2, targets the *D. virilis* HL, I stained *D. virilis* polytene chromosomes for another DCC member, MSL3. I observed that MSL3, like MSL2, targets the major *D. virilis* histone locus in males and the X-chromosome did not light up (Fig. 7C). In female *D. virilis*, the results were also consistent with MSL2 immunostaining. I observed no colocalization (Fig. 7D). This suggests that the DCC, not just MSL2, is co-localizing at the *D. virilis* major histone locus.

Next, to determine if localization of MSL2 to the histone genes is specific to *D. virilis*, I performed polytene chromosome immunostaining on *D. melanogaster* polytene chromosomes. In *D. melanogaster*, MSL2 does not colocalize with Mxc, which suggests that MSL2 is not at the histone locus. However, I did observe MSL2 binding to the male X-chromosome in *D. melanogaster* as the X-chromosome was lit up in the presence of the anti-MSL2 antibody (Fig.

8A). MSL2 is not present in *D. melanogaster* females (Fig. 8B). Following this, I also stained for MSL3 on *D. melanogaster* chromosomes, which showed staining results consistent with MSL2 (Fig. 8C-D). These observations show that DCC localization to the histone locus is not seen in *D. melanogaster*.

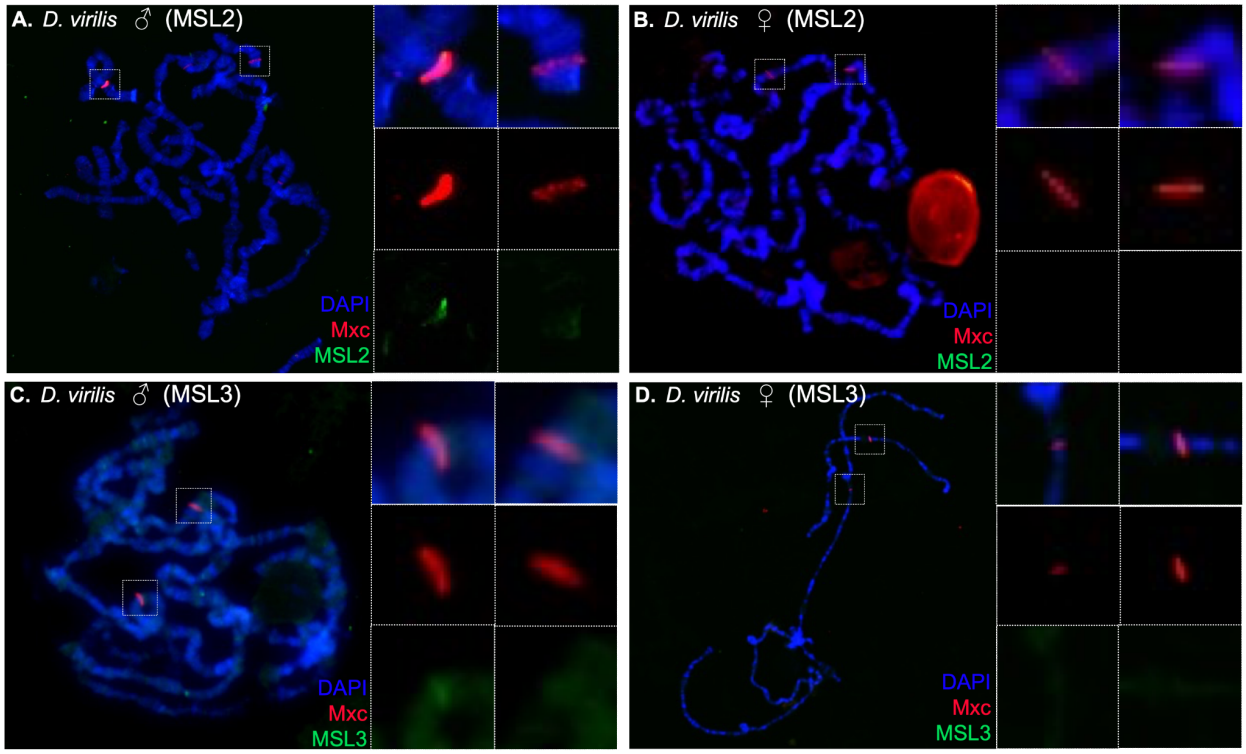

Figure 7: The DCC complex targets the major histone locus (HL) in *D. virilis*.
(A) Immunostaining of *D. virilis* chromosomes for Mxc and MSL2. Mxc reveals the HL. The major HL has faint banding for MSL2 indicating co-localization of Mxc and MSL2 at the HL. (B) MSL2 is not present at either HL in *D. virilis* females. (C) Immunostaining of *D. virilis* for Mxc and MSL3. The major locus has faint banding for MSL3, indicating MSL3 is also at the *D. virilis* major HL. This suggests the DCC is at the HL, rather than just MSL2. (D) MSL3 is not present at either HL in female *D. virilis*.

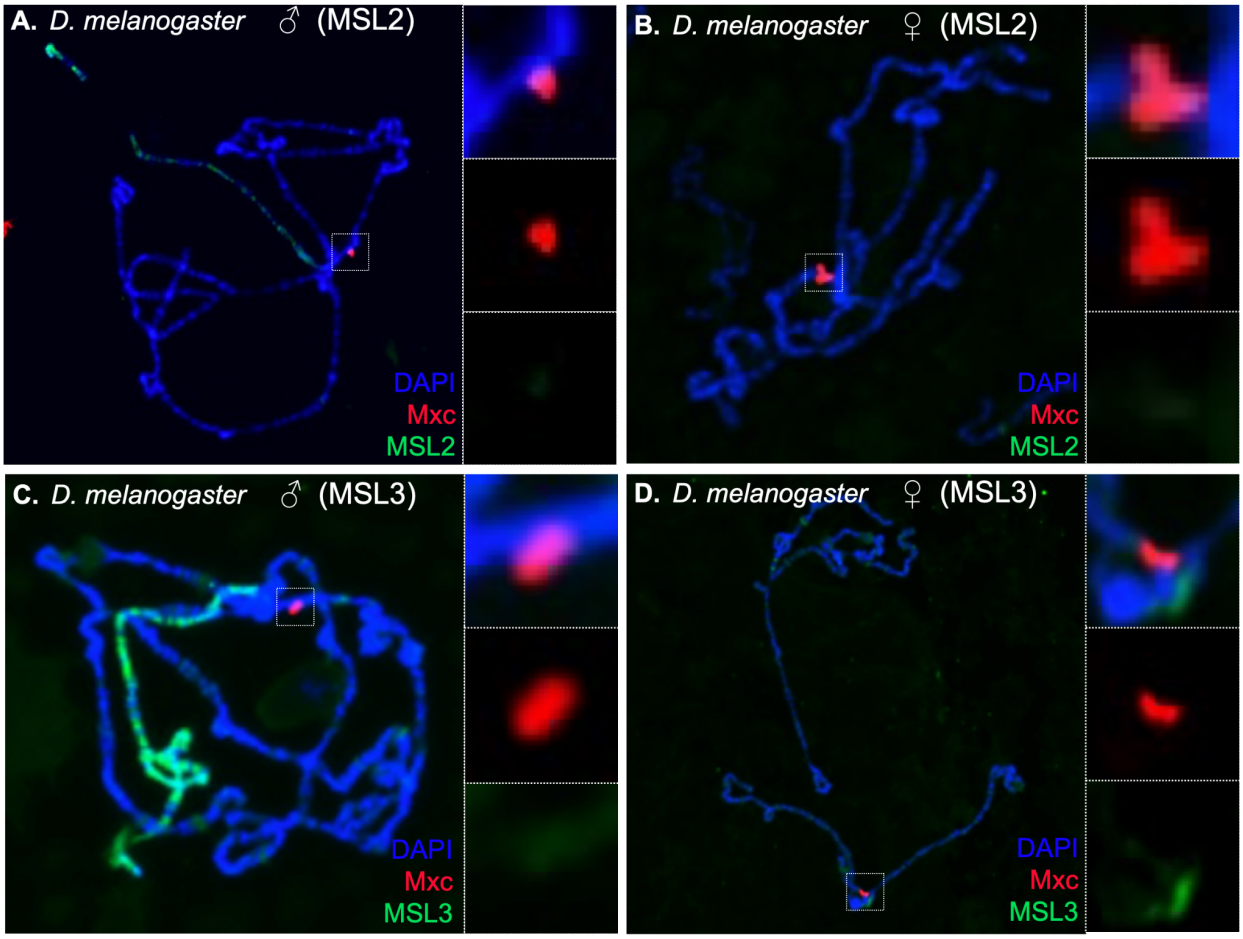

Figure 8: The DCC does not localize to the histone locus in *D. melanogaster*.
(A) Immunostaining of *D. melanogaster* for Mxc (red) and MSL2 (green) reveals MSL2 does not colocalize with Mxc at the single HL. This indicates that MSL2 is not at the HL. (B) MSL2 is not present in *D. melanogaster* females. (C) Immunostaining of *D. melanogaster* males for Mxc (red) and MSL3 (green). Consistent with MSL2 immunostaining, MSL3 is not seen at the HL. (D) MSL3 is not present in *D. melanogaster* females.

D. melanogaster and *D. virilis* diverged about 40 millions years ago (Russo, Takezaki, and Nei 1995). To determine if MSL2 localization is common in other *Drosophila* species that diverged from *D. melanogaster* more recently, I immunostained *D. pseudoobscura* and *D. willistoni* polytene chromosome spreads. Both *D. pseudoobscura* and *D. willistoni* possess a single histone locus. Similar to *D. melanogaster* immunostaining, when I stained *D. pseudoobscura* and *D. willistoni* with anti-Mxc and anti-MSL2 antibodies, I observed no

colocalization of MSL2 with Mxc at the histone locus. The male X-chromosome for both species lit up when stained for MSL2 (Fig. 9A, C). In *D. pseudoobscura* and *D. willistoni* females, MSL2 is not present (Fig. 9B, D). These results further suggest that the DCC is only present at the *D. virilis* major histone locus.

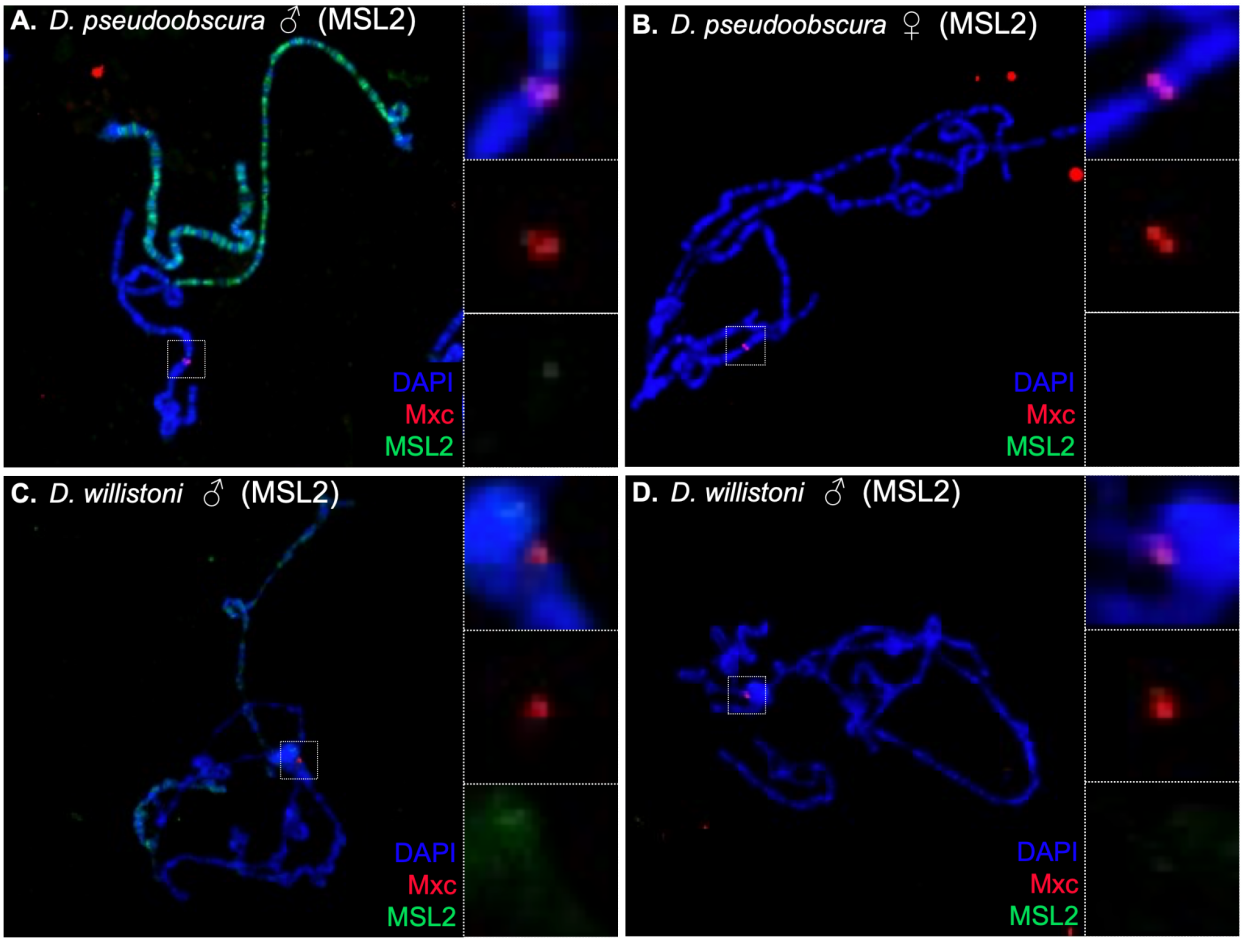

Figure 9: Immunostaining of MSL2 on polytene chromosomes from *D. pseudoobscura* and *D. willistoni*.
(A) In *D. pseudoobscura*, MSL2 does not colocalize with Mxc at the HL. However, MSL2 does target the X-chromosome in males. (B). MSL2 is not present in *D. pseudoobscura* females. (C) In *D. willistoni*, MSL2 does not colocalize with Mxc at the HL. However, MSL2 does target the X-chromosome in males. (D) MSL2 is not present in *D. willistoni* females.

Species	Gender	MSL2 at HLB?	X-chr. lights up from MSL2?	MSL3 at HLB?	X-chr. lights up from MSL3?
D. virilis	Male	***yes***	no	***yes***	no
	Female	no	no	no	no
D. melanogaster	Male	no	***yes***	no	***yes***
	Female	no	no	no	no
D. pseudoobscura	Male	no	***yes***	*not tested*	*not tested*
	Female	no	no	*not tested*	*not tested*
D. willistoni	Male	no	***yes***	*not tested*	*not tested*
	Female	no	no	*not tested*	*not tested*

Table 1: Table summary of polytene immunostaining results.
D. virilis polytene immunostaining reveals MSL2 and MSL3 at the major histone locus. The X-chromosome does not stain for MSL2 or MSL3, DCC proteins. This is inconsistent with other *Drosophila* species. MSL2 in *D. melanogaster*, *D. pseudoobscura*, and *D. willistoni* does not colocalize with Mxc, but MSL2 targets the male X-chromosome in all three species. I only tested MSL3 in *D. melanogaster* but results are consistent with MSL2 staining. These results are in contrast with earlier literature, which suggest that MSL2 targets the X-chromosome in *D. virilis* but not in *D. pseudoobscura* and *D. willistoni* (Copps et al. 1998).

MSL2 interacts with the histone2a-histone2b *promoter.*

Next, I wanted to determine if MSL2 targets specific DNA sequences within the histone gene array (Fig. 5A). I hypothesized that MSL2 targets the *histone3/histone4* promoter, since this region is targeted by the CLAMP protein (Rieder et al. 2017). Villa et al. 2021 overexpressed both MSL^{mel} and MSL^{vir} from cell culture and performed ChIP-seq and DIP-seq on the cells. Chromatin immunoprecipitation (ChIP)-seq reveals the genomic binding locations of proteins and histone modifications by fragmenting DNA until only protein-bound DNA remains (Park 2009). Similarly, DNA immunoprecipitation (DIP)-seq utilizes naked genomic that a particular purified recombinant protein binds, and it is sequenced to reveal the DNA-binding sites (Gossett and Lieb 2008). I mapped Villa et al. 2021's overexpressed $MSL2^{vir}$ ChIP-seq data from Kc cells (Fig. 10A), $MSL2^{vir}$ DIP-seq data from S2 cells (Fig. 10B), and $MSL2^{mel}$ ChIP-seq data from Kc cells to the *D. melanogaster* histone locus (Fig. 10C). From all mapping results, I observed peaks

between the *histone2a-histone2b* genes, suggesting that MSL2 interacts with the *histone2a-histone2b* promoter sequence (Fig. 10A-C). These observations are inconsistent with my original hypothesis. However, when I mapped MSL2 from *D. melanogaster* at normal expression levels, I observed no peaks at the *histone2a-histone2b* promoter or anywhere in the histone array. I mapped normal MSL2 expression levels first from Schauer et al. 2017's ChIP-seq data from MSL^{mel} from S2 cells (Fig. 11A). I also mapped MSL^{mel} ChIP-seq data from salivary glands from Figueiredo et al. 2014 (Fig. 11B). Because Figueiredo et al. 2014's ChIP-seq data was done in salivary glands, I anticipated similar results as the immunostaining on polytene chromosomes because they come from salivary glands. However, I observed no peaks at the histone array. These observations suggest that when MSL2 is overexpressed, it targets the *histone2a/histone2b* promoter, but it does not do so at normal expression levels.

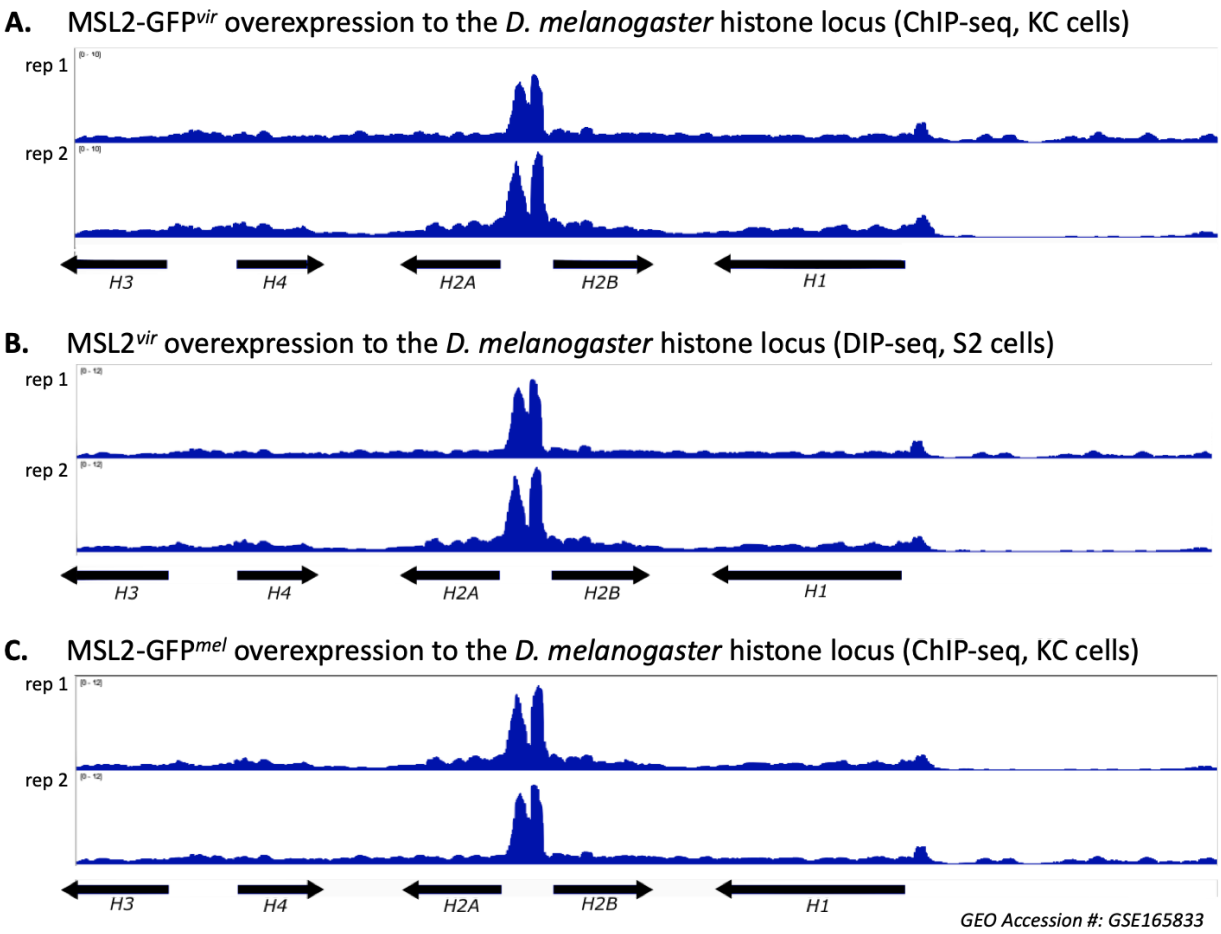

Figure 10: Overexpression of MSL2 in *D. melanogaster* leads to peaks at the *histone2a-histone2b* promoter.
(A) Overexpressed MSL^{vir} in Kc cells (female) results in peaks at the *histone2a-histone2b* promoter. ChIP-seq data from Villa et al. 2021. (B) Overexpressed MSL^{vir} in S2 (male) cells results in peaks at the *histone2a-histone2b* promoter. DIP-seq data from Villa et al. 2021. (C) Overexpressed MSL^{mel} in Kc cells results in peaks at the *histone2a-histone2b* promoter. ChIP-seq data from Villa et al. 2021 (GEO Accession #: GSE165833).

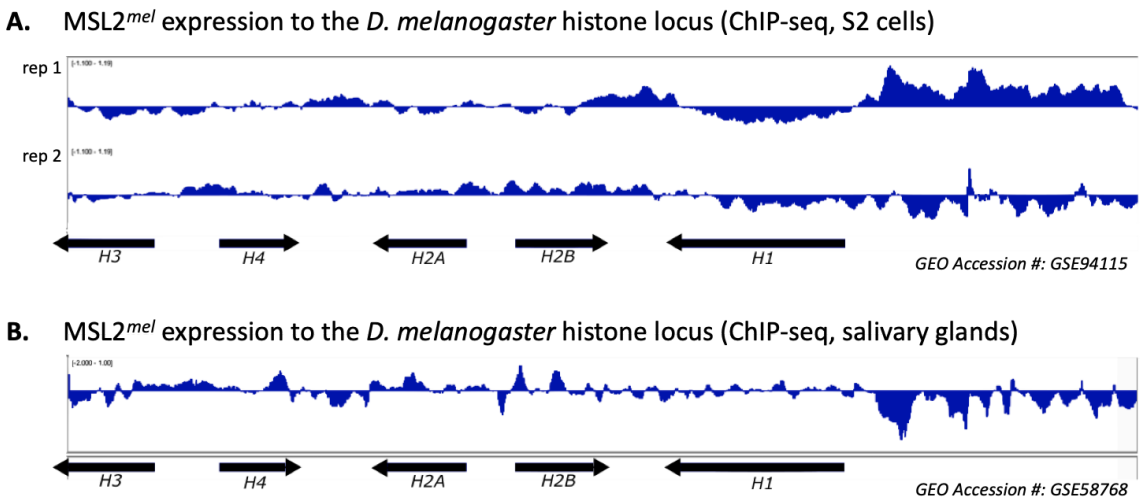

Figure 11: No MSL2 peaks are observed in the histone array upon normal MSL2 expression in *D. melanogaster*.
(A) MSL^{mel} from S2 cells depict no peaks in the histone array. ChIP-seq data from Schauer et al. 2017 (GEO Accession #: GSE94115). (B) MSL^{mel} from salivary glands, which are the same glands polytene chromosomes are stained from, still depict no peaks in the histone array. ChIP-seq data from Figueiredo et al. 2014; only one replicate was performed. (GEO Accession #: GSE58768).

D. virilis *expresses higher levels of MSL2 than* D. melanogaster.

Finally, I wanted to determine if higher levels of MSL2 were responsible for histone array targeting. I performed quantitative real-time PCR (RT-qPCR) on RNA from both *D. melanogaster* and *D. virilis* larvae to determine the relative *msl2* RNA levels. When compared to male *D. melanogaster*, female *D. melanogaster* have lower *msl2* RNA levels, consistent with previous observations that *msl2* is not expressed in females (Zhou et al. 1995). However, both male and female *D. virilis* have much higher *msl2* RNA expression levels than observed in *D. melanogaster* (Fig. 12). These observations suggest that higher levels of MSL2 result in histone locus targeting. Consistent with my previous observations (Fig. 10, 11), overexpression of MSL2 may also result in artificial targeting.

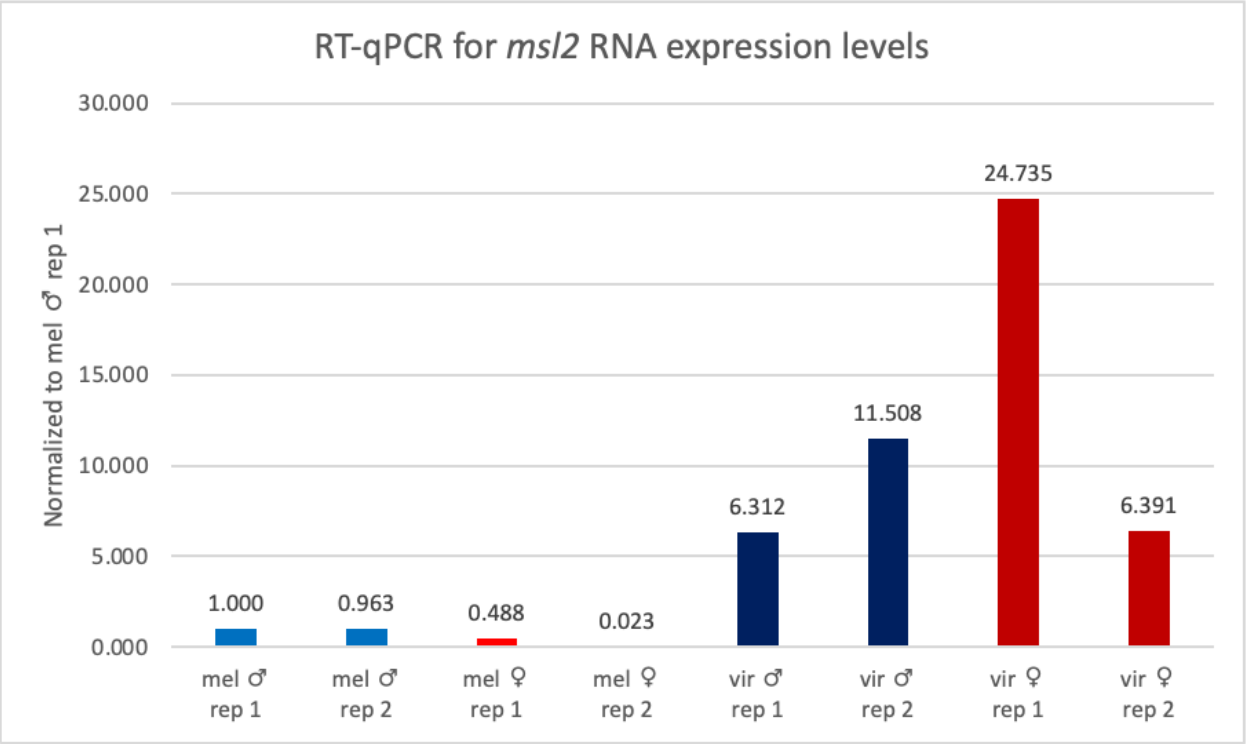

Figure 12: RT-qPCR results on the relative *msl2* RNA expression levels in *D. melanogaster* (mel) and *D. virilis* (vir).
Both male and female *D. virilis* produce higher levels of *msl2* RNA than *D. melanogaster*. I analyzed two biological replicates (rep #) for all samples. Male *D. melanogaster* samples are shown in blue and female samples in red. Male *D. virilis* samples are shown in dark blue and female samples in dark red. Results are normalized to the first *D. melanogaster* male replicate.

Discussion

The DCC localizes to the major D. virilis *histone locus, which is not seen in other* Drosophila *species*.

The anti-MSL2 and anti-MSL3 antibodies we used did not light up the *D. virilis* male X-chromosome (Fig. 7A, C), which is unexpected since MSL2 and MSL3 are both DCC proteins. Additionally, previous literature suggests MSL2 targets the *D. virilis* X-chromosome (Copps et al. 1998). I was cautious about interpreting this result as MSL2 in *D. virilis*, especially because the anti-MSL2 and anti-MSL3 antibodies are raised against MSL2 protein from *D. melanogaster*, and the proteins are not well conserved (Fig. 3). However, because I did not observe MSL2 or MSL3 co-localization in female *D. virilis* polytene chromosome spreads, this suggests that the antibody is recognizing the correct proteins. Additionally, because the MSL3 immunostaining results were consistent with the MSL2 immunostaining results, these observations suggest that the DCC is present at the locus and not just the MSL2 protein. Future studies should include western blots from both *D. melanogaster* and *D. virilis* samples to confirm that the MSL2 antibody recognizes the correct sized protein (85 KDa).

D. melanogaster and *D. virilis* diverged from their common ancestor about 40 million years ago (Russo, Takezaki, and Nei 1995). Many other fly species are more closely related to *D. melanogaster* than *D. virilis*, such as *D. pseudoobscura* and *D. willistoni*. *D. pseudoobscura* is most closely related to *D. melanogaster*, having diverged from their common ancestor about 25 million years ago while *D. willistoni* diverged from its common ancestor with *D. melanogaster* about 35 million years ago (Fig. 13) (Bhutkar et al. 2007). Like *D. melanogster*, *D. willistoni* and *D. pseudoobscura* both have one histone locus. I did not observe MSL2 colocalizing with Mxc in

either *D. pseudoobscura* or *D. willistoni*, although I did observe X-chromosome localization (Fig. 9). These observations suggest that the DCC localization to the histone locus is not common in other *Drosophila* species and might only occur naturally in *D. virilis*.

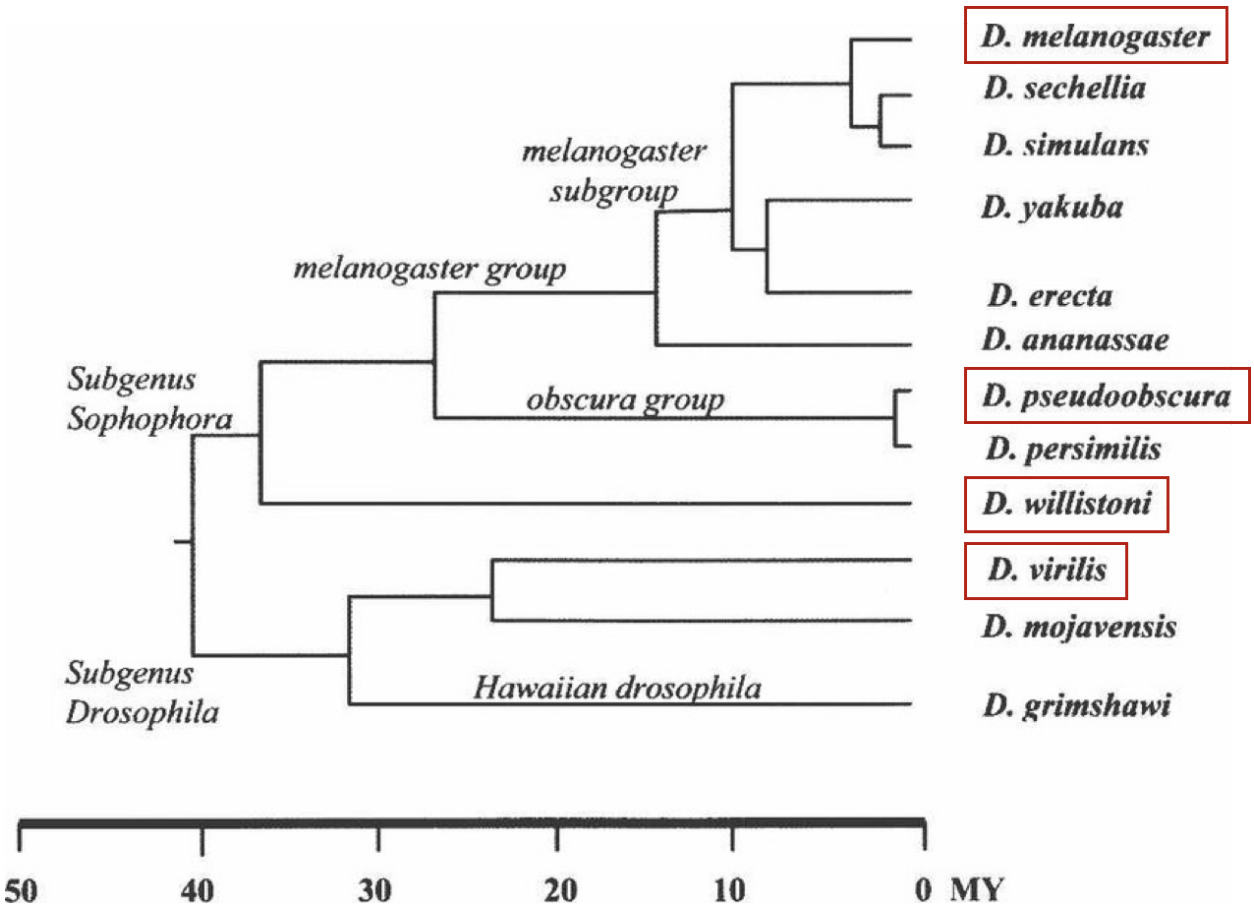

Figure 13: Phylogenetic tree of *Drosophila* species.
D. virilis and *D. melanogaster* diverged from their common ancestor 40 million years ago. Two other species, *D. willistoni* and *D. pseudoobscura*, are more closely related to *D. melanogaster* than *D. virilis* is to *D. melanogaster*. *D. willistoni* and *D. melanogaster* diverged from their common ancestor 35 million years ago, while *D. pseudoobscura* and *D. melanogaster* diverged from their common ancestor 25 million years ago. From Bhutkar et al. 2007.

Under artificial overexpressed conditions, MSL2 targets the D. melanogaster *histone gene array.*

Although I only observed MSL2 targeting the HL in *D. virilis*, it is not a property unique to the MSL2 ortholog from *D. virilis*. When I mapped over-expressed MSL2^{mel} ChIP-seq data from Kc (female) cells to the *D. melanogaster* histone locus, I observed peaks at the *histone2a-histone2b* promoter sequence (Fig. 10C). However, I did not see these peaks in normal MSL2 expression levels from S2 (male) cells (Fig. 11A), suggesting that the *D. melanogaster* MSL2 ortholog will target the *D. melanogaster* histone gene array only when there are higher levels of MSL2. Because *D. virilis* has higher expression levels of *msl2* (Fig. 12), this may lead the *D. virilis* MSL2 ortholog to naturally target the *D. virilis* major locus (Fig. 7A). Finally, in all the overexpressed MSL2 ChIP-seq and DIP-Seq data, I observed peaks only at the *histone2a-histone2b* promoter sequence (Fig. 10). This is also surprising because CLAMP, which interacts with MSL2 on the male X-chromosome, targets the *histone3-4* promoter (Rieder et al. 2017).

Previous work from Villa et al. 2021 used overexpressed *D. melanogaster* MSL2 to draw conclusions about the role of this protein in dosage compensation. For example, they determined that *D virilis* MSL2 doesn't intrinsically distinguish the X-chromosome from the autosomes but overexpressed *D. melanogaster* MSL2 does. Further, they found that the CXC protein domain of MSL2 lacks MRE specificity. However, their studies used MSL2^{vir} in *D. melanogaster* cell culture, such as in female Kc cells. To perform experiments in female cells, they created higher expression levels of MSL2, which can lead to artificial targeting, as I observed in Fig. 10A, C. Additionally, *D. virilis* naturally has higher levels of *msl2* than *D. melanogaster* (Fig. 12). Therefore, using MSL2^{vir} in *D. melanogaster* may lead to surplus amounts of MSL2 in their cell culture, which may produce artificial results. Overall, my data caution against drawing

conclusions using MSL2 overexpression systems, which have previously been used to determine dosage compensation mechanisms. When MSL2 is overexpressed, this can lead to artificial genome targeting, such as the artificial histone locus targeting in *D. melanogaster* ChIP-seq data (Fig. 10C), as this was inconsistent with immunostaining results (Fig. 8A).

In summary, my studies indicate that the DCC targets the *D. virilis* major histone locus, which does not occur in other *Drosophila* species. This may be due to *D. virilis* producing higher levels of MSL2 leading to its targeting at the histone locus. Our future work will build on this by overexpressing *msl2* in *D. melanogaster* to determine if higher expression levels drive MSL2 to the *D. melanogaster* histone locus on polytene chromosomes, similar to what I observed in *D. virilis*.

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