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ORIGINAL ARTICLE

The essential *Drosophila* CLAMP protein differentially regulates non-coding *roX* RNAs in male and females

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Abstract Heterogametic species require chromosome-wide gene regulation to compensate for differences in sex chromosome gene dosage. In *Drosophila melanogaster*, transcriptional output from the single male X-chromosome is equalized to that of XX females by recruitment of the male-specific lethal (MSL) complex, which increases transcript levels of active genes 2-fold. The MSL complex contains several protein components and two non-coding *RNA on the X* (*roX*) RNAs that are transcriptionally activated by the MSL complex. We previously discovered that targeting of the MSL complex to the X-chromosome is dependent on the chromatin-linked adapter for MSL proteins (CLAMP) zinc finger protein. To better understand CLAMP function, we used the CRISPR/Cas9 genome editing system to generate a frameshift mutation in the *clamp* gene that eliminates expression of the CLAMP protein. We found that *clamp* null females die at the third instar larval stage, while almost all *clamp* null males die at earlier developmental stages. Moreover, we found that in *clamp* null females *roX* gene expression is activated, whereas in *clamp* null males *roX* gene expression is reduced. Therefore, CLAMP regulates *roX* abundance in a sex-specific manner. Our results provide new insights into sex-specific gene regulation by an essential transcription factor.

Keywords *Drosophila* · dosage compensation · gene regulation · transcription factor

Abbreviations

MSL complex	Male-specific lethal complex
CLAMP	Chromatin-linked adaptor for MSL proteins
roX	*RNA on the X*

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Introduction

Many species employ a sex determination system that generates an inherent imbalance in sex chromosome copy number, such as the XX/XY system in most mammals and some insects. In this system, one sex has twice the number of X-chromosome-encoded genes compared to the other. Therefore, a mechanism of dosage compensation is required to equalize levels of X-linked transcripts, both between the sexes and between the X-chromosome and autosomes (Lucchesi et al. 2005). Dosage compensation is an essential mechanism that corrects for this imbalance by coordinately regulating the gene expression of most X-linked genes.

In *Drosophila melanogaster*, transcription from the single male X-chromosome is increased 2-fold by recruitment of the male-specific lethal (MSL) complex. The MSL complex is composed of two structural proteins, MSL1 and MSL2, three accessory proteins, MSL3, males absent on the first (MOF), and maleless (MLE), and two functionally redundant non-coding RNAs, *RNA on the X* (*roX1*) and *roX2* (Meller and Rattner 2002; Lucchesi et al. 2005). We previously discovered that recruitment of the MSL complex to the X-chromosome requires the zinc finger protein chromatin-linked adapter for MSL proteins (CLAMP) (Soruco et al. 2013).

In addition to its role in male MSL complex recruitment, we suggested that CLAMP has an additional non-sex-specific essential function because targeting of the *clamp* transcript by RNA interference results in a pupal lethal phenotype in both males and females (Soruco et al. 2013). Further understanding of CLAMP function in the context of the whole organism required a null mutant. However, due to the pericentric location of the *clamp* gene, no deficiencies or null mutations were available. Using the CRISPR/Cas9 system, we introduced a frameshift mutation in the *clamp* gene, leading to an early termination codon before the major zinc finger binding domain. This frameshift mutation generated the $clamp^2$ allele, which eliminates detectable CLAMP protein production and is therefore a protein null allele. The majority of $clamp^2$ mutant males die prior to the third instar stage. On the other hand, females die at the third instar stage, suggesting sex-specific functions for CLAMP. Furthermore, CLAMP regulates the *roX* genes in a sex-specific manner, activating their accumulation in males and repressing their accumulation in females. Overall, we present a new tool for studying dosage compensation and suggest that CLAMP functions to assure that *roX* RNA accumulation is sex specific.

Results

Two clamp *alleles were generated using the CRISPR/Cas9 system*

The *clamp* gene is located within pericentric heterochromatin on the left arm of chromosome 2, 1 Mb from the centromere. Due to this chromosomal location, null mutants for the *clamp* gene were not previously available from *Drosophila* mutant collections. We therefore used the CRISPR/Cas9 genome editing system, which can introduce missense or frameshift mutations through the resolution of double-stranded breaks by non-homologous end joining (Sander and Joung 2014). To determine where to target the Cas9 endonuclease, we used the protein domain composition of CLAMP. There are two predicted domains in CLAMP: an amino-terminal glutamine-rich, low-complexity domain and a carboxy-terminal zinc finger domain consisting of six canonical zinc fingers (Fig. S1). We previously demonstrated that the zinc finger domain of CLAMP is sufficient for DNA interactions (Soruco et al. 2013). Therefore, in order to generate a *clamp* null allele, we used the CRISPR/Cas9 system to target specifically upstream of the zinc finger domain of the *clamp* gene (Fig. 1a) using the best available predicted guide RNA (Gratz et al. 2014).

We generated two different mutations using the CRISPR/Cas9 system, and we balanced each with a homozygous lethal *CyO* second chromosome balancer carrying a larval green fluorescent protein (GFP) marker to allow us to track both larval and adult genotypes. Visual inspection of the wing phenotype in adult animals revealed that one mutation was homozygous viable ($clamp^1$) while the other was not ($clamp^2$). Sequencing of the targeted region indicated that the $clamp^1$ homozygous viable animals carry a 6-bp deletion in the *clamp* locus, resulting in the loss of two amino acids and an in-frame shift of the amino acid sequence (Figs. 1a and S1). The homozygous lethal $clamp^2$ allele carries the same 6-bp deletion with an additional seventh base deleted (Figs. 1a

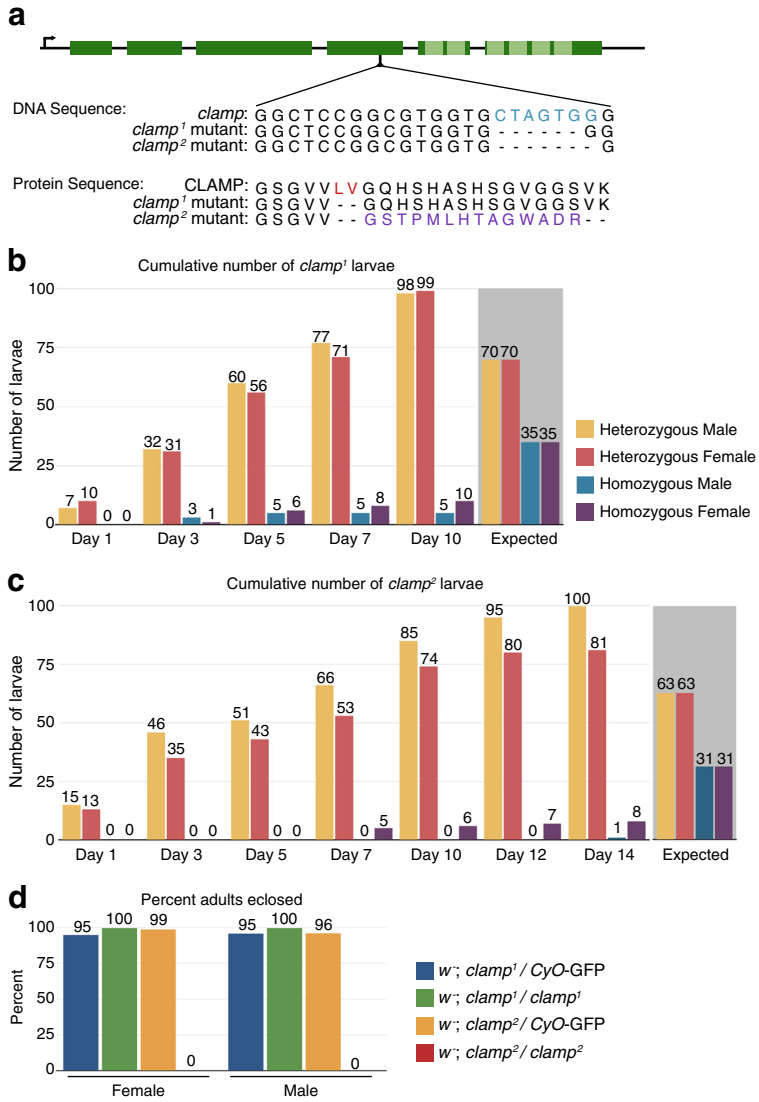

Fig. 1 The *clamp*^{2} mutation is homozygous lethal and the *clamp*^{1} allele is homozygous viable. **a** The CRISPR/Cas9-introduced frameshift is located in the fourth exon (*dark green boxes*) of the *clamp* gene, upstream of the DNA-binding domain containing six zinc fingers (*light green boxes*). The homozygous viable *clamp*^{1} mutation is a 6-bp deletion (*blue*), resulting in a deletion of two amino acids (*red*) and an in-frame shift of the protein sequence. The homozygous lethal *clamp*^{2} mutation consists of the same 6-bp deletion (*blue*), with an additional seventh base removed. This causes a frameshift of the protein sequence (*purple*) resulting in an early termination codon. **b** The cumulative number of larvae counted for both male and female *clamp*^{1} heterozygous and homozygous animals is shown. In total, 212 larvae were counted. *Day 1* indicates the first day in which wandering third instar larvae began emerging. Homozygous males (*blue*) and females (*purple*) began emerging 2 days after their heterozygous siblings. The expected number of larvae out of 212 for each sex and genotype is indicated with the *gray background*. **c** The cumulative number of larvae counted for both male and female *clamp*^{2} heterozygous and homozygous animals is shown. In total, 190 larvae were counted. *Day 1* indicates the first day in which wandering third instar larvae began emerging. Homozygous females (*purple*) began emerging after 7 days, while we observed a single homozygous male (*blue*) on day 14. The expected number of larvae out of 190 for each sex and genotype is indicated with the *gray background*. **d** The percent heterozygous and homozygous *clamp*^{1} and *clamp*^{2} adults that eclosed were counted from larvae collected in **b**, **c**. While almost all heterozygous and homozygous *clamp*^{1} mutants eclosed, only the heterozygous *clamp*^{2} mutants eclosed

and S1). The 7-bp deletion causes a frameshift in the amino acid sequence, leading to an early stop codon occurring 14 amino acids after the mutation (Figs. 1a and S1).

In order to quantify the viability of homozygous *clamp*^{1} and *clamp*^{2} mutants, we first scored for the presence (in heterozygotes) or absence (in homozygotes) of GFP fluorescence in larvae and then counted the number of adult flies that eclosed from each class. Over a period of 10 days, we would have expected to see 35 larvae each of homozygous *clamp*^{1} males and females out of the 212 larvae counted based on Mendelian ratios (Fig. 1b). However, a total of ten homozygous *clamp*^{1} females and five homozygous *clamp*^{1} males were observed (Figs. 1b and S2A, Table S2). Similarly, while we would have expected to see 31 homozygous *clamp*^{2} males and females out of the 190 larvae counted over a period of 14 days, a total of eight homozygous *clamp*^{2} females and only one homozygous *clamp*^{2} male larva were observed (Figs. 1c and S2B, Table S2). Using a chi-squared test, we calculated that both *clamp*^{1} and *clamp*^{2} homozygous mutant larvae occur at frequencies significantly lower than expected from Mendelian ratios (Table S2; *clamp*^{1} $\chi^2 = 66.82$, $p < 0.00001$ Table S3; *clamp*^{2} $\chi^2 = 74.30$, $p < 0.00001$ Table S4). Furthermore, we found that despite occurring at low frequencies, *clamp*^{1} homozygous mutants are not developmentally delayed. In contrast, the *clamp*^{2} homozygous mutants are delayed by approximately 7 days compared to their heterozygous siblings (Fig. 1b, c).

In order to quantify the adult viability defects caused by the *clamp*^{1} and *clamp*^{2} alleles, we compared the number of curly-winged (heterozygous) versus straight-winged (homozygous) adult flies that eclosed from previously genotyped larvae. We observed that almost all *clamp*^{1} heterozygous and homozygous mutants eclosed (Fig. 1d, Table S2). In contrast, homozygous *clamp*^{2} female larvae die as third instar larvae and only the heterozygous *clamp*^{2} mutants eclosed, suggesting that the *clamp*^{2} mutation is homozygous lethal in both males and females before adulthood (Fig. 1d, Table S2).

The *clamp*^{2} allele results in a homozygous lethal phenotype, suggesting that the mutation could be a recessive loss-of-function mutation. However, there are no deficiencies available to determine whether *clamp*^{2} is a genetic null. To determine if the homozygous lethality is due to the frameshift mutation in the *clamp* gene rather than another mutation in the genetic background, we generated a transgenic fly line containing a *clamp* transgene inserted on the third chromosome (Venken et al. 2006). The *clamp* transgene insertion stock contains a 12.5-kb region encompassing the *clamp* coding region and all putative upstream regulatory regions, but not any neighboring genes. We found that *clamp*^{2} homozygous lethality is rescued in both male and female flies when one copy of the *clamp* transgene rescue construct is present. Therefore, the lethality in the *clamp*^{2} homozygous mutants is caused by a loss of the *clamp* gene function and not a second site mutation.

The *clamp*^{1} allele complements the homozygous null *clamp*^{2} allele

The *clamp*^{1} allele is a homozygous viable mutation, despite producing significantly fewer animals than expected from Mendelian ratios (Figs. 1b and S2A, Tables S2 and S3). We asked whether the delay in development could be explained by an impact of the *clamp*^{1} allele on CLAMP expression. To determine how *clamp*^{1} affects *clamp* messenger RNA (mRNA) accumulation, we measured the production of *clamp* transcript by an established qRT-PCR assay in third instar larvae by comparing homozygous *clamp*^{1} mutants with the w^{-}; *clamp*^{2}; *P*{CLAMP} transgenic line (Soruco et al. 2013). We normalized transcript abundance in male and female *clamp*^{1} mutant larvae to the respective sex of the transgenic rescue line to control for genetic background. In addition, we used three normalizing control genes (*gapdh*, *rpl32*, and *ras64b*). We analyzed the results first by ANOVA to test for differences in means, followed by a Tukey post hoc test to identify samples with statistically significant changes. We found no significant change in *clamp* transcript abundance in the *clamp*^{1} heterozygous or homozygous larvae compared to the rescue control (Fig. S3A). Therefore, the *clamp*^{1} mutation does not dramatically change abundance of the *clamp* transcript.

To determine if the *clamp*^{1} allele affects production of the CLAMP protein, we performed Western blotting of protein extracted from the salivary glands of third instar larvae, because whole larvae have large quantities of fat, making Western blotting difficult. Our analysis revealed that the deletion of two amino acids in the *clamp*^{1} mutants does not detectably affect CLAMP protein production compared to controls (Fig. S3B). To determine whether CLAMP protein produced from the *clamp*^{1} allele localizes to chromatin, we performed polytene chromosome immunostaining

for CLAMP in homozygous and heterozygous *clamp*^{1} male and female larvae. In wild type male and female animals, CLAMP localizes to many sites throughout the genome (Fig. S3C, $y^{-}w^{-}$ male and female). Localization of CLAMP in the *clamp*^{1} mutant animals is not measurably different from wild type CLAMP at the resolution of polytene chromosomes (Fig. S3C). We therefore concluded that the *clamp*^{1} mutants produce sufficient CLAMP protein to allow the animals to survive to adulthood.

Because our results suggest that the *clamp*^{1} allele produces functional *clamp* protein, we hypothesized that the homozygous viable *clamp*^{1} allele could complement the *clamp*^{2} homozygous lethal allele. To test this, we crossed the heterozygous *clamp*^{1} and *clamp*^{2} stocks to generate w^{-}; *clamp*^{1}/ *clamp*^{2} animals. We found that these animals are viable and survive to adulthood (data not shown) indicating that the *clamp*^{1} allele complements the homozygous lethality of the *clamp*^{2} allele.

While performing the complementation crosses, we discovered that *clamp*^{1} homozygous males are sterile. We hypothesized that if this phenotype is caused by the mutation in the *clamp*^{1} allele, we would expect the following two observations: (1) w^{-}; *clamp*^{1}/*clamp*^{2} heteroallelic males should also be sterile and (2) male sterility would be rescued by the CLAMP rescue transgene. We determined that w^{-}; *clamp*^{1}/*clamp*^{2} males are viable and fertile (data not shown), while w^{-}; *clamp*^{1}; *P*{CLAMP} males are sterile (data not shown). Therefore, we concluded that the *clamp*^{1} stock has a second site mutation that is linked to the *clamp*^{1} allele and results in male sterility. It is possible that this unknown second site mutation could contribute to the delay in development in the homozygous animals. Overall, we determined that the phenotypes we observed in the *clamp*^{1} mutants could not be attributed to the *clamp*^{1} allele. Therefore, we focused on characterizing the *clamp*^{2} allele because the homozygous lethal phenotype caused by this allele is rescued by the CLAMP transgene.

The *clamp*^{2} mutation is a protein null allele

Because our goal was to create a *clamp* protein null allele, we focused on characterizing the *clamp*^{2} allele that is homozygous lethal, a phenotype that is rescued by the CLAMP transgene (Fig. 1a). First, we determined the developmental stage when the last homozygous *clamp*^{2} male larvae die compared with females. However, it is difficult to phenotypically determine the sex of larvae prior to the third instar stage. Therefore, we developed a PCR assay to measure the presence of male larvae by amplification of the Y-chromosome gene *kl-5*. We extracted genomic DNA from ten first or second instar larvae that were either homozygous (GFP−) or heterozygous (GFP+) for the *clamp*^{2} allele, as determined by GFP fluorescence produced from the *CyO* balancer chromosome. We were unable to detect the *kl*-5 Y-chromosome gene in GFP− larvae after the second instar stage, indicating that the last *clamp*^{2} homozygous males die between the second and third instar developmental stages (Fig. S4A). In contrast, homozygous *clamp*^{2} females can survive to the third instar stage (Figs. 1c and S2B, Tables S2 and S4). Overall, we observed sexually dimorphic phenotypes caused by the *clamp*^{2} allele, suggesting that most homozygous males die earlier in development than females.

To determine how *clamp*^{2} affects *clamp* mRNA accumulation, we measured the production of *clamp* transcript in third instar larvae using qRT-PCR. We compared abundance of *clamp* mRNA in male and female *clamp*^{2} mutants to the same sex of the w^{-}; *clamp*^{2}; *P*{CLAMP} transgenic line. Although male homozygous *clamp*^{2} larvae are very rare, we were able to collect enough larvae to perform qRT-PCR due to the sensitive nature of the assay. We determined that there is no statistically significant change in *clamp* transcript abundance in *clamp*^{2} heterozygous or homozygous larvae (Fig. 2a). Therefore, the *clamp*^{2} mutation has no significant effect on the abundance of the *clamp* transcript.

To determine how the *clamp*^{2} mutation affects protein accumulation, we performed Western blot analysis on protein extracted from whole salivary glands of third instar larvae. We found that homozygous *clamp*^{2} female larvae do not produce full-length CLAMP (61 kDa), despite producing *clamp* mRNA (Figs. 2a, b and S4B). We could not test protein abundance from homozygous *clamp*^{2} males because we could not collect sufficient homozygous male larvae for Western blot analysis. The *clamp*^{2} frameshift mutation generates an early termination codon, which is predicted to result in a truncated protein with a molecular weight of 37 kDa. The CLAMP antibody is specific to the amino-terminus and therefore should detect truncated protein.

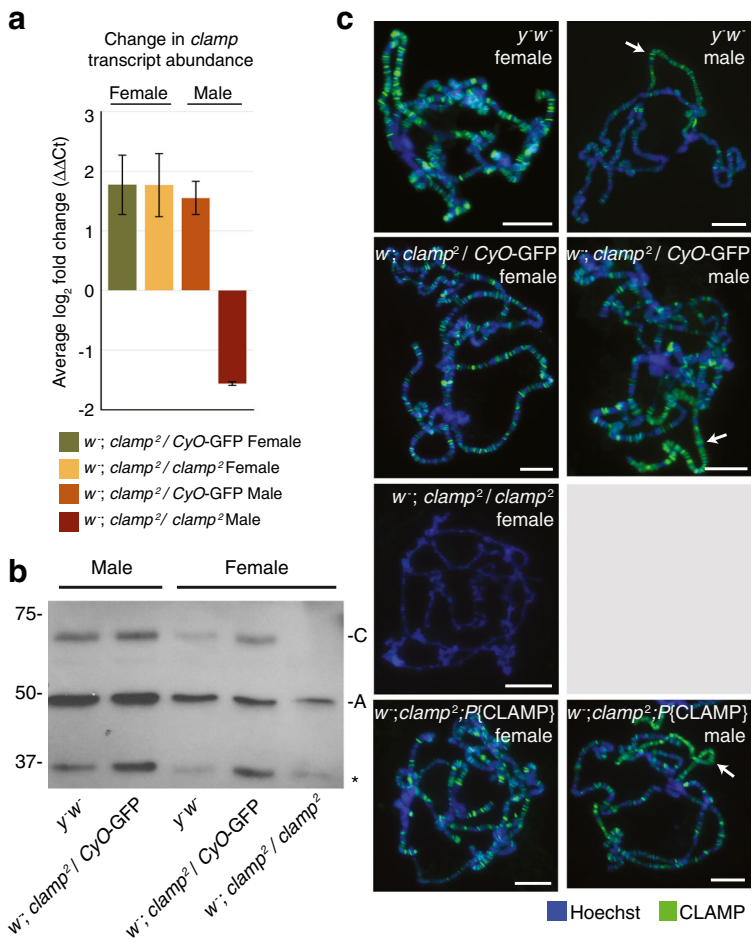

Fig. 2 The *clamp*^{2} mutation is a protein null allele. **a** Quantitative real-time PCR indicates no significant change in *clamp* transcript abundance in male or female third instar larvae heterozygous or homozygous for the *clamp*^{2} allele. Plotted is the average $\log_2$ fold change (ΔΔCt) from three biological replicates after internal normalization to three genes (*gapdh*, *rpl32*, *ras64b*). Female and male samples were normalized to the respective sex of w^-; *clamp*^{2}; *P*{CLAMP} transgenic larvae. *Error bars* show +/− 1 standard error of the mean (S.E.M., $^{**}p < 0.01$, $^{*}p < 0.05$). **b** Western blotting indicates that no full-length CLAMP protein ("*C*") is produced in homozygous *clamp*^{2} females. Loading control is actin ("*A*"). Although a background band is present in all samples at 37 kDa (*asterisk*), a truncated form of CLAMP is not apparent as a result of the *clamp*^{2} mutation. **c** There is no difference in CLAMP (*green*) localization on polytene chromosomes of heterozygous *clamp*^{2} male and female larvae compared to respective y^-w^- wild type controls. CLAMP does not localize to chromosomes in homozygous *clamp*^{2} females. The *clamp*^{2} homozygous mutant chromosomes are thinner than wild type and heterozygous *clamp*^{2} chromosomes. CLAMP immunostaining is rescued in *clamp*^{2} homozygotes when a 12.5-kb genomic region encompassing the *clamp* gene is inserted onto the third chromosome (w^-; *clamp*^{2}; *P*{CLAMP}). We did not perform polytene chromosome spreads from homozygous *clamp*^{2} male animals due to poor gland development (*gray box*). *White arrows* indicate the male X-chromosome. *Scale bars* are 0.02 mm

Although a background band is present in all samples around 37 kDa, we do not observe accumulation of truncated CLAMP specifically in *clamp*^{2} mutants (Fig. 2b). We previously observed this background band, and it is not ablated after *clamp* RNA interference (RNAi), suggesting that it is non-specific (Larschan et al. 2012). Therefore, it is likely that any CLAMP protein produced in the *clamp*^{2} mutant fails to accumulate. Furthermore, even if the truncated CLAMP protein is produced below the level of immunoblotting detection, it would not contain the zinc finger DNA-binding domain (Fig. 1a).

As an alternate approach to detect any remaining CLAMP protein in the homozygous *clamp*^{2} mutant, we examined the localization of the CLAMP protein on polytene chromosomes. CLAMP localizes to many sites throughout the genomes of both male and female wild type animals (Fig. 2c, y^-w^- male and female).

Localization of CLAMP in heterozygous mutant males and females is not visibly distinct from that in wild type controls (Fig. 2c, w^- ; $clamp^2$ / *CyO*-GFP). In contrast, CLAMP staining in $clamp^2$ homozygous female larvae indicated that if CLAMP is produced from the $clamp^2$ allele, it does not localize to polytene chromosomes (Fig. 2c, w^-; $clamp^2$/ $clamp^2$). Homozygous male polytene chromosomes could not be obtained due to lack of viable animals (Fig. 1c). Importantly, the CLAMP rescue transgene generates a functional CLAMP protein that localizes to polytene chromosomes in both male and female homozygous $clamp^2$ animals (Fig. 2c, w^-; $clamp^2$; *P*{CLAMP}). Polytene chromosome immunostaining further supports our conclusion that the $clamp^2$ mutation is a protein null allele. Additionally, we observed that the chromosomes in $clamp^2$ homozygous females are thinner than normal, a phenotype that has been previously observed in mutants for chromatin remodelers (Deuring et al. 2000).

To determine whether the disruption of chromosome morphology that we observed on interphase chromosomes also occurs on mitotic chromosomes, we performed mitotic chromosome spreads from third instar larval neuroblasts (Fig. S4C). Dramatic changes in mitotic chromosome morphology were not observed in the mitotic chromosome spreads (Fig. S4C). Therefore, it is likely that CLAMP is more important to maintain the chromatin organization of interphase chromosomes than mitotic chromosomes.

CLAMP differentially regulates roX *genes in males and females*

We originally identified CLAMP as a transcription factor essential for directly linking the MSL complex to the X-chromosome in males (Larschan et al. 2012; Soruco et al. 2013). However, CLAMP also localizes to thousands of promoters throughout the genome (Fig. 2c) and therefore has the potential to regulate additional transcripts (Soruco et al. 2013). Chromatin immunoprecipitation followed by sequencing (ChIP-seq) demonstrates that CLAMP localizes to the known regulatory regions of both *roX* genes, which are among the strongest MSL complex recruitment sites (Soruco et al. 2013). In addition, we previously determined that CLAMP positively regulates the transcription of *roX2* based on experiments performed in *Drosophila* (S2) cells and male larvae after *clamp* RNAi, likely because it recruits the MSL complex, which is known to activate *roX* transcription (Bai et al. 2004; Soruco et al. 2013).

Because we previously determined that CLAMP regulates *roX2* from *clamp* RNAi experiments, we determined the effect of the $clamp^2$ allele on *roX* accumulation in vivo in third instar larvae using qRT-PCR. The *roX* genes are not normally expressed in wild type female larvae due to the absence of the MSL complex, which activates their transcription in males (Meller et al. 1997; Meller 2003; Bai et al. 2004). We found that there was a large increase in the amount of both *roX1* and *roX2* transcripts in homozygous $clamp^2$ female larvae when normalized to female rescue controls (Fig. 3a, yellow bars). Consistent with our previous findings that CLAMP promotes transcription of *roX2* in male S2 cells, we found a large reduction in *roX2* transcript levels in $clamp^2$ homozygous males (Fig. 3b, red bars). In contrast, we did not see a significant decrease in *roX1* levels, consistent with our prior analysis of *roX* transcript abundance after *clamp* RNAi (Soruco et al. 2013). Therefore, CLAMP differentially regulates *roX* genes in males and females.

The large increase in abundance of the *roX* transcripts in $clamp^2$ homozygous female larvae (Fig. 3a) led us to ask how these levels compared to *roX* expression in wild type males. Therefore, we reanalyzed *roX* abundance by normalizing all transcript levels to $clamp^2$ homozygous males carrying the rescue *P*{CLAMP} transgene (Fig. 3c). We discovered that activation of *roX1* in $clamp^2$ homozygous mutant females leads to a similar abundance of *roX1* as in males (Fig. 3c, yellow bars). Therefore, the $clamp^2$ mutation results in *roX1* being expressed in females at similar level to that of males. The abundance of *roX2* in homozygous $clamp^2$ females is reduced compared to rescued males but is similar to that present in $clamp^2$ males. Therefore, the absence of CLAMP leads to similar basal levels of *roX2* abundance in both males and females and activates *roX1* to similar transcript abundance levels as seen in males.

The increase in *roX* expression in $clamp^2$ homozygous females compared to controls led us to hypothesize that homozygous $clamp^2$ females could be dying at the third instar larval stage due to this increase in *roX* expression. To test whether lethality in $clamp^2$ homozygous females is caused by the increase in *roX* expression (Fig. 3), we generated a triple mutant fly line that is homozygous null for both *roX* genes and homozygous for the $clamp^2$ allele. *RoX* null females are usually viable (Meller and Rattner

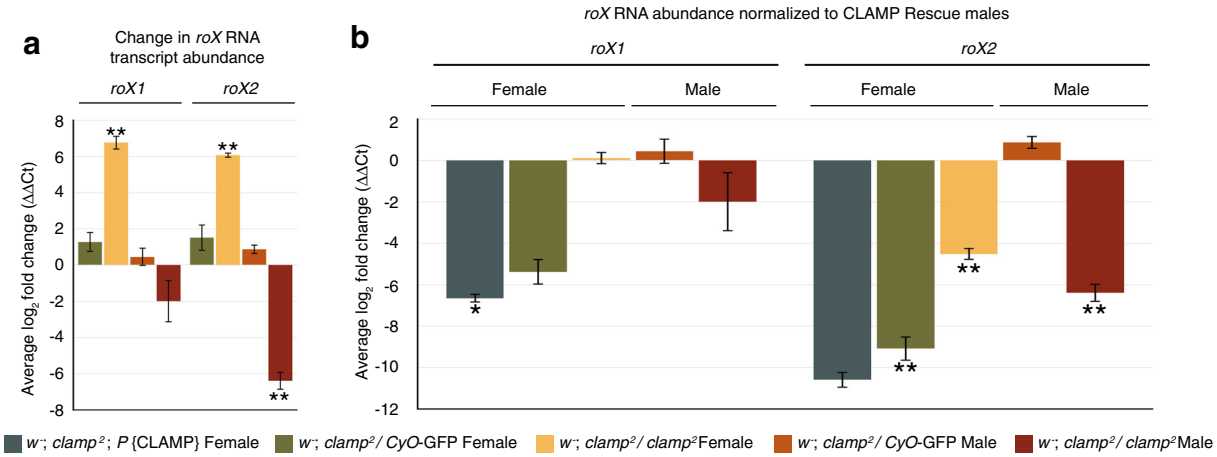

Fig. 3 CLAMP regulates transcript abundance of *roX* differentially in males and females. **a** The average $\log_2$ fold change for *roX1* and *roX2* abundance as measured by qRT-PCR in homozygous *clamp*^{2} females indicates that females have a significant increase in the abundance of both *roX1* and *roX2*, while homozygous *clamp*^{2} males have a significant decrease in *roX2* abundance. Shown is the average $\log_2$ fold change (ΔΔCt) of three biological replicates for *roX1* and *roX2* after normalization to three internal genes and compared to the respective sex of w^-; *clamp*^{2}; *P*{CLAMP} transgenic animals as in Fig. 2a. (*Error bars* are +/− 1 S.E.M., $^{**}p < 0.01$, $^{*}p < 0.05$). **b** The samples from Fig. 3a were normalized to w^-; *clamp*^{2}; *P*{CLAMP} rescue males and show that *roX1* transcript abundance in homozygous *clamp*^{2} females is statistically indistinguishable from males. The abundance of *roX2* in homozygous *clamp*^{2} females is statistically 16-fold depleted compared to w^-; *clamp*^{2}; *P*{CLAMP} rescue males. (*Error bars* are +/− 1 S.E.M., $^{**}p < 0.001$, $^{*}p < 0.005$)

2002). However, the combined loss of both *roX* genes does not rescue the homozygous lethality of the *clamp*^{2} allele (data not shown). Thus, we conclude that the increased expression of *roX* RNAs is not the sole cause of the lethality seen in *clamp*^{2} homozygous females. Because CLAMP occupies thousands of promoters genome-wide (Fig. 2c) (Soruco et al. 2013), the lethality of the *clamp*^{2} allele is likely caused by changes in regulation of multiple genes.

Ectopic MSL complex does not form in clamp^2 homozygous females

In males, the *roX* genes are targeted by the MSL complex for increased expression. To determine whether the increase in *roX* gene transcription in *clamp*^{2} homozygous females is caused by MSL complex component induction, we quantified transcript abundance of all MSL complex component genes: *msl1*, *msl2*, *msl3*, *mle*, and *mof*. We also compared transcriptional changes in mutant females with those in mutant males to determine if any changes are sex-specific. We found significantly increased *msl1*, *msl3*, and *mof* transcript abundance in *clamp*^{2} heterozygous and homozygous females compared to rescue controls (Fig. 4a, green and yellow bars). We also observed increased *msl2* transcript abundance in *clamp*^{2} homozygous females compared to controls (Fig. 4a, yellow bars). Interestingly, changes in *msl* transcript abundance are not sensitive to *clamp* gene dosage. Unlike the *roX* genes, the genes encoding MSL complex components do not have clear CLAMP binding sites in their regulatory regions (Soruco et al. 2013). Therefore, it is possible that changes in *msl* gene expression are due to other regulatory cascades that are altered in *clamp* mutant larvae. In contrast, the regulatory regions of the *roX* genes are two of the strongest CLAMP binding sites in the genome. Thus, it is unlikely that the regulation of *roX* RNA expression (Fig. 3a) occurs through an indirect mechanism.

In *clamp*^{2} homozygous males, most MSL complex transcripts did not show significant changes compared to controls (Fig. 4a). The strongest perturbation we observed was an 8-fold reduction in *mle* transcripts in *clamp*^{2} homozygous males compared to controls. However, reduction in *mle* transcript levels would not explain the complete loss of MSL complex recruitment we previously reported in males after constitutive *clamp* RNAi (Soruco et al. 2013) because significant MSL complex recruitment is observed in the absence of MLE (Kelley et al. 1999).

Increased transcripts of MSL complex components and *roX* RNAs in *clamp*^{2} homozygous females (Figs. 3

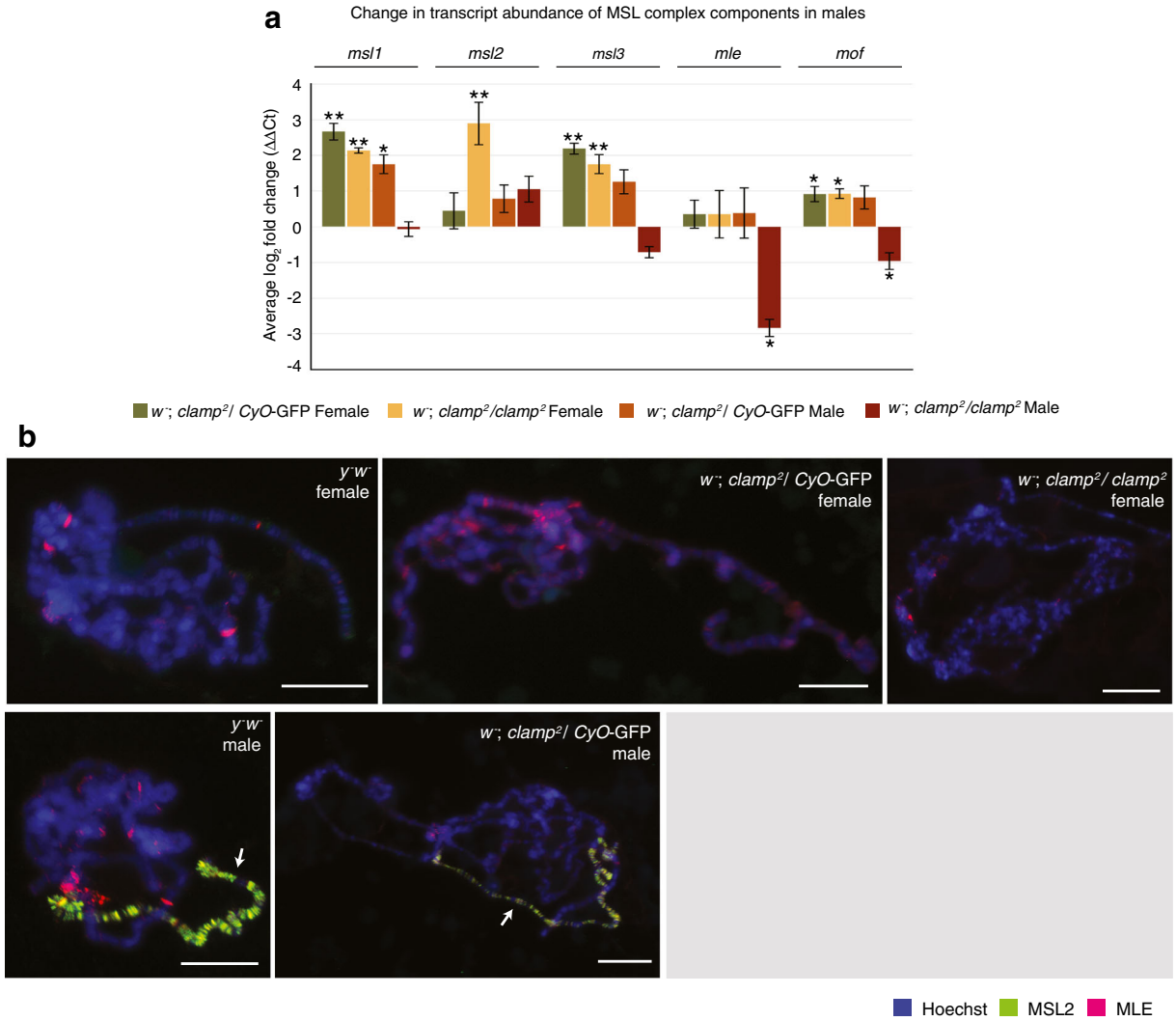

Fig. 4 Ectopic MSL complex is not formed in *clamp*^{2} females. **a** qRT-PCR shows that CLAMP regulates transcription of some MSL complex component genes in female larvae. In both heterozygous and homozygous *clamp*^{2} females, there are significant increases in abundance for the *msl1*, *msl3*, and *mof* transcripts. There is also a significant increase in *msl2* abundance in homozygous *clamp*^{2} females. There are significant decreases in transcript abundance of *mle* and *mof* in *clamp*^{2} homozygous males. Normalization was performed on three biological replicates using three internal normalization genes. Samples were normalized to the respective sex of the w^-; *clamp*^{2}; *P*{CLAMP} rescue animal. (*Error bars* are +/− 1 S.E.M., $^{**}p < 0.01$, $^{*}p < 0.05$). **b** Polytene chromosome immunostaining shows that the core MSL complex component MSL2 (*green*) is expressed only in males and localizes to the X-chromosome. The accessory protein, MLE (*red*), is expressed in both males and females and localizes throughout the genome. There is no change in localization of these proteins in heterozygous or homozygous *clamp*^{2} mutants. Reduced numbers of homozygous *clamp*^{2} male animals and their poorly developed salivary glands prevented generation of polytene chromosome spreads for this genotype (*gray box*). *White arrows* indicate the X-chromosome. *Scale bars* are 0.02 mm

and 4a) could promote ectopic MSL complex formation, a situation that is known to cause female lethality (Kelley et al. 1995). Therefore, we looked for ectopic formation of the MSL complex in *clamp*^{2} homozygous females by immunostaining polytene chromosomes for the MSL2 and MLE components of the MSL complex. We did not detect ectopic localization of MSL2 on either the X-chromosome or autosomes in *clamp*^{2} heterozygous or homozygous females (Fig. 4b, w^-; *clamp*^{2}/*CyO*-GFP and w^-; *clamp*^{2}/*clamp*^{2}). Furthermore, we detected MLE in similar non-X-specific patterns on *clamp*^{2} mutant and wild type female polytene

chromosomes, consistent with its known localization pattern (Fig. 4b, $y^{-}w^{-}$ and w^{-}; $clamp^{2}/clamp^{2}$) (Cugusi et al. 2015). Therefore, the large increases in *roX* transcripts observed in $clamp^{2}$ homozygous females are not likely to be due to MSL complex-mediated *roX* activation. However, it is possible that any MSL complex formed in $clamp^{2}$ homozygous females would be unable to localize to chromatin in the absence of CLAMP, leading to its destabilization and protein degradation.

Discussion

We previously demonstrated that CLAMP has an essential role in MSL complex recruitment to the male X-chromosome. In addition, we suggested that CLAMP has an essential role in the viability of both males and females (Soruco et al. 2013). However, we could not perform in vivo studies to further investigate CLAMP function because there was no available null mutant line. In the current manuscript, we present a CLAMP protein null mutant and determine that this protein is essential in both sexes. This allele will provide a key tool for future in vivo studies on the role of CLAMP in dosage compensation, as well as identification of the essential function of CLAMP in both sexes.

Our initial characterization of the $clamp^{2}$ protein null allele revealed sexually dimorphic roles for CLAMP in regulation of the *roX* genes. We observed that CLAMP promotes *roX2* transcription in males but represses transcription of both *roX* genes in females. It is likely that recruitment of the MSL complex to the *roX2* locus by CLAMP promotes *roX2* expression in males. In females, where the MSL complex is not present, CLAMP may function to repress these loci as an additional mechanism to ensure that dosage compensation is male-specific. Additionally, we determined that most $clamp^{2}$ homozygous males die earlier in development than $clamp^{2}$ homozygous females. Earlier lethality in males is likely due to a misregulation of the dosage compensation process as a result of the loss of CLAMP-mediated MSL complex recruitment. However, CLAMP is enriched at the 5′ regulatory regions of thousands of genes across the genome. Therefore, it is likely that other non-sex-specific regulatory pathways are disrupted resulting in female lethality.

Furthermore, CLAMP is an essential protein because our CRISPR/Cas9-generated protein null *clamp* allele is homozygous lethal in both males and females. These results indicate that CLAMP has a previously unstudied non-sex-specific role that is essential to the viability of both males and females. An interesting observation that arose from our characterization is that polytene chromosome organization is disrupted in $clamp^{2}$ mutant females, suggesting that CLAMP may play a role in regulation of genome-wide chromatin organization of interphase chromosomes. A function in regulating chromatin organization provides one possible explanation for how CLAMP performs sexually dimorphic functions. For example, CLAMP may repress *roX* expression in females by promoting the recruitment of a repressive chromatin-modifying factor in the absence of the MSL complex. In contrast, CLAMP may activate *roX2* in males by creating a chromatin environment permissive for MSL complex recruitment in males. Although *roX1* and *roX2* are functionally redundant, our results suggest that CLAMP specifically activates *roX2* but not *roX1* in males. Interestingly, Villa et al. recently reported that *roX2*, but not *roX1*, is likely to be an early site of MSL complex recruitment (Villa et al. 2016), suggesting that CLAMP may function early in the process of dosage compensation.

Overall, our newly generated $clamp^{2}$ protein null allele provides an important tool to study how the essential CLAMP protein regulates its many target genes in vivo. The generation of the $clamp^{2}$ allele will facilitate future studies that will reveal a mechanistic understanding of how a single transcription factor can promote different sex-specific functions within an organism.

Materials and methods

Generation and validation of clamp *mutant fly line using CRISPR/Cas9 technology*

We used the flyCRISPR Optimal Target Finder tool available from the University of Wisconsin to design a CRISPR target sequence for *clamp* (Gratz et al. 2014). We cloned target sequence oligonucleotides for *clamp* (sense: 5′-CTT CGG CTC CGG CGT GGT GCT AGT-3′ and antisense: 5′-AAA CAC TAG CAC CAC GCC GGA GCC-3′) into the pU6-BbsI-chiRNA plasmid (Addgene no. 45946), following the protocol outlined on the flyCRISPR website. We validated correct ligation of the *clamp* CRISPR target sequence

into the pU6-BbsI-chiRNA plasmid by Sanger sequencing using universal M13 primers.

The commercial service Genetic Services, Inc., microinjected the validated pU6-BbsI-chiRNA plasmid containing the *clamp* target sequence into germline-expressing Cas9 flies (y^1, w^{1118};+; PBac{vas-Cas9, U6-tracrRNA}VK00027). Flies containing a single mutation were returned balanced over the *Curly of Oster* (*CyO)* second chromosome balancer. From these progeny, we identified the CRISPR/Cas9-generated mutations by PCR across the target region (forward: 5′-ACA ACT GAA GGG TTT GGA CGG-3′, reverse: 5′-CAT GCA GGC TGA ACA AAC AG-3′), followed by Sanger sequencing (forward: 5′-TCT GCA GGA CAA ACA CCT TG-3′; reverse: 5′-CCC AAG CAC AAC TTC AGC AAA-3′). From this validation, we isolated two independent *clamp* alleles: (1) y^1, w^{1118}; $clamp^1$/*CyO*; and (2) y^1, w^{1118}; $clamp^2$/*CyO;*.

Generation and validation of clamp *rescue transgene and fly line*

We generated a *clamp* rescue construct using the P(acman) system that utilizes a conditionally amplifiable bacterial artificial chromosome (BAC) clone, recombineering, and bacteriophage ΦC31-mediated insertion at a genomic attB site (Venken et al. 2006). We designed primers for two homology arms to capture a 12.5-kb region spanning the entire *clamp* locus (3.5 kb), including the presumed promoter (left homology arm (1.2 kb) forward: 5′-ACC GGC GCG CCG CAG AAG GAA GAG TTT CCG A-3′, reverse: 5′-CGC GGA TCC AAG TCC TGG CCT AAG CCC TA-3′; right homology arm (800 bp) forward: 5′-CGC GGA TCC TTT TGT GCA TGG TCA ACC ACG-3′, reverse: 5′-ACC TTA ATT AAG GGC AAA CAT ATT TCG CAC GAT AC-3′). We amplified homology arms off a conditionally amplifiable P(acman) BAC clone, Ch322 20C06 (BACPAC Resources), using CopyControl (Epicenter) reagent for vector amplification. We simultaneously cloned the arms into the PacMan vector 3XP3-eGFP-attB-Amp (gift from Koen Venken) at the multicloning site (MCS) using the engineered restriction sites *Asc*I-*Bam*HI (left) and *Bam*HI-*Pac*I (right) in a three-component ligation. We identified positive colonies via Sanger sequencing across the MCS. Using *Bam*H1, we linearized the intermediate vector and purified the product. Next, the linearized vector was transformed into *E. coli* that we had previously transformed with the *clamp* containing BAC clone Ch322 20C06 and expressing the mini-lambda vector encoding the ΦC31 recombinase (SW102, NCI BRB Preclinical Repository). We identified positive colonies via sequencing across the left and right homology arm junction.

Genetic Services, Inc., microinjected the full *clamp* rescue construct into *D. melanogaster* embryos containing the attB-docking site (VK33) on chromosome 3L band 65B2 (Venken et al. 2006). We identified *clamp* rescue construct transgenics using 3xP3-EGFP expression and maintained the subsequent stock in the homozygous state (y^1, w^{1118};+; P{3xP3-EGFP, *clamp* = CLAMP}).

Genetic manipulation of clamp *mutant alleles and quantification of phenotypes*

To generate $clamp^1$ and $clamp^2$ mutant lines with a larval phenotypic marker, we used standard methods to cross the original balanced stocks to a *CyO*-GFP stock that expresses GFP at all stages of larval development (w^{1118}; sna^{scl}/*CyO*, P{ActGFP.w$^-$}CC2). The resulting w^{1118}; $clamp^1$/*CyO*-GFP and w^{1118}; $clamp^2$/*CyO*-GFP stocks (referred to in text as $clamp^1$ and $clamp^2$, respectively) express both larval and adult phenotypic markers and were used for all remaining experiments. In addition, we generated a balanced $clamp^2$ stock that expresses GFP under regulation of the *twist* promoter (Bloomington stock no. 6662: w^{1118}; In(2LR)Gla, wg^{Gla-1} /CyO, P{w[+mC]=GAL4-twi.G}2.2, P{UAS-2xEGFP}AH2.2)). The resulting w^-; $clamp^2$/*twi*-GFP stock was used to generate the mitotic spreads. All other experiments utilized the *CyO*-GFP stock expressing GFP under the regulation of actin.

To assess larval viability, we collected third instar larvae from either a w^{1118}; $clamp^1$/*CyO*-GFP (212 larvae collected) or a w^{1118}; $clamp^2$/*CyO*-GFP (190 larvae collected) heterozygous cross. For each larva, we visually determined the sex and *clamp* genotype. From these larvae, we monitored eclosion of the pupae into adult flies.

To test if the $clamp^2$ mutation can be rescued by a *clamp* transgene, we crossed the w^{1118}; $clamp^2$/*CyO*-GFP stock to the y^1, w^{1118};+; *P*{CLAMP} rescue line and scored viability at the adult stage by wing phenotype. The resulting w^{1118}; $clamp^2$; *P*{CLAMP} line (referred to as w^-; $clamp^2$; *P*{CLAMP} in text) was maintained as a stock in the homozygous state and used for all quantitative analyses.

Sample collection for western blotting and PCR for kl-5 gene

We tested for the presence of the Y-chromosome gene *kl-5* in first, second, and third instar larvae of the following animal genotypes: (1) y^1, w^{1118};+; (referred to as $y^- w^-$), (2) w^{1118}; $clamp^2$/*CyO*-GFP, and (3) w^{1118}; $clamp^2$/$clamp^2$. We collected and pooled 10 larvae each of the first and second instar developmental stages. For third instar larvae, we dissected 10 salivary glands of sexed males and females of each genotype in cold PBS. As an additional control, we tested 10 adult male and adult female whole flies. We flash froze all samples in liquid nitrogen and homogenized with a steel bead using a Retsch MM300 TissueLyser Mixer Mill. Next, we suspended the homogenized samples in 30 μL of lysis buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 25 mM NaCl, 0.2 mg/ml proteinase K, 1 ng/μL RNase) and incubated them at 37 °C for 30 min, followed by a 5-min incubation at 90 °C. We purified genomic DNA by standard phenol/chloroform extraction using Phase Lock tubes (5 PRIME) per the manufacturer's instructions, followed by ethanol precipitation.

We tested purified genomic DNA for the presence of the *kl-5* gene by PCR using the following primers: forward 5′-ATC GCA AAC GAG TGG TCT CA-3′; reverse 5′-TGT ATC AAG GGC AGG CAT CC-3′. As a genomic DNA loading control, we amplified the *clamp* locus with the PCR primers used to identify the mutation.

Quantitative real-time PCR

To analyze transcript abundance, we used TRIzol (Thermo Fisher Scientific) following the manufacturer's instructions to extract total RNA from three biological replicates of five third instar larvae from each genotype. We reverse-transcribed 1 μg of total RNA using the SuperScript VILO cDNA Synthesis Kit (Life technologies) by following the manufacturer's protocol. Three technical replicates for each target transcript were amplified using SYBR Green (Life Technologies) on an Applied Biosystems StepOnePlus™ Real-Time PCR System. Primers were used at a concentration of 200 nM to amplify targets from 2 ng of cDNA. Primer sequences for qRT-PCR are in Table S1. We calculated transcript abundance using the standard ΔΔCt method using *gapdh*, *rpl32*, and *ras64b* as internal controls (Livak and Schmittgen 2001). We normalized female mutant samples to the female w^{1118}; $clamp^2$; *P*{CLAMP} transgenic rescue, except where specified. We normalized male mutant samples to the male w^{1118}; $clamp^2$; *P*{CLAMP} transgenic rescue. We tested statistical significance by performing an ANOVA multiple comparison test on the mean ΔCt values, followed by a Tukey post hoc analysis for multiple comparison correction.

Western blotting

We dissected salivary glands from third instar larvae in cold PBS and froze samples in liquid nitrogen. We extracted total protein from the samples by homogenizing in lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% SDS, 0.5× protease inhibitor) using a small pestle. After a 5-min incubation at room temperature, we cleared the samples by centrifuging at room temperature for 10 min at 14,000×*g*. To blot for CLAMP and actin, we ran 5 μg of total protein on a Novex 10% Tris-Glycine precast gel (Life Technologies). We transferred proteins to PVDF membranes using the iBlot transfer system (Thermo Fisher Scientific) and probed the membranes for CLAMP (1:1000, SDIX) and actin (1:400,000, Millipore) using the Western Breeze kit following the manufacturer's protocol (Thermo Fisher Scientific).

Relative expression of protein for CLAMP was quantified using the gel analysis tool in ImageJ software following the guidelines outlined on the website (Schneider et al. 2012). For each genotype, we first internally normalized the amount of CLAMP protein to actin. Next, we determined relative expression of protein by comparing the actin-normalized quantities to sex of the respective y^1, w^{1118};+; ($y^- w^-$) sample.

Chromosome squashes and immunostaining

We prepared larval polytene chromosome squashes as previously described (Cai et al. 2010) and mitotic chromosome spreads from larval neuroblasts following method no. 3 as described (Pimpinelli et al. 2000). We stained polytene chromosomes with anti-CLAMP (rabbit, 1:1000, SDIX), anti-MLE (rabbit, 1:500, gift from M. Kuroda), or anti-MSL2 (rat, 1:500, gift from P. Becker) primary antibodies. We used DAPI to stain mitotic chromosomes. For detection, we used all Alexa Fluor secondary antibodies at a concentration of 1:1000. We visualized polytene chromosome slides at

40× on a Zeiss Axio Imager M1 Epifluorescence upright microscope with the AxioVision version 4.8.2 software. We visualized mitotic spreads at 60× on a Zeiss LSM 800 confocal microscope with Airyscan using Zen Blue software.

Data availability

Drosophila stocks are available upon request. Table S1 contains primer sequences for qRT-PCR.

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