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Male-killing *Spiroplasma* Alters Behavior of the Dosage Compensation Complex during *Drosophila melanogaster* embryogenesis

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Summary

Numerous arthropods harbor maternally transmitted bacteria that induce the preferential death of males [1-7]. This sex-specific lethality benefits the bacteria because males are 'dead ends' regarding bacterial transmission and their absence may result in additional resources for their viable female siblings who can thereby more successfully transmit the bacteria [5]. Although these symbionts disrupt a range of developmental processes [8-10], the underlying cellular mechanisms are largely unknown. It was previously shown that mutations in genes of the dosage compensation pathway of *Drosophila melanogaster* suppressed male killing caused by the bacterium, *Spiroplasma* [10]. This result suggested that dosage compensation is a target of *Spiroplasma*. However, it remains unclear how this pathway is affected, and whether the underlying interactions require the male-specific cellular environment. Here we investigated the cellular basis of male embryonic lethality in *D. melanogaster* induced by *Spiroplasma*. We found that the dosage compensation complex (DCC), which acetylates X chromatin in males [11], becomes mis-localized to ectopic regions of the nucleus immediately prior to the killing phase. This effect was accompanied by inappropriate histone acetylation and genome-wide mis-regulation of gene expression. Artificially induced formation of the DCC in infected females, through transgenic expression of the DCC-specific gene *msl-2*, resulted in mis-localization of this complex to non-X regions and early *Spiroplasma*-induced death, mirroring the killing effects in males. These findings strongly suggest that *Spiroplasma* initiates male killing by targeting the dosage

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compensation machinery directly and independently of other cellular features characteristic of the male sex.

Results and Discussion

Spiroplasma are cell wall-less spirochetes belonging to the bacterial Mollicute class that infect the haemolymph and egg cytoplasm of their insect hosts [12,13]. Male killing strains of *Spiroplasma* are common within the Drosophila genus, having been detected in at least eight different Drosophila species including *D. melanogaster* [6]. In this latter species, males, but not females, infected with the male-killing MSRO (for *melanogaster* sex ratio organism) strain of *Spiroplasma* exhibit disrupted central nervous system (CNS) formation and heightened apoptosis in epithelial cells during the middle stages of embryogenesis, and they fail to reach the larval stages [14,15]. Previous work has suggested that *Spiroplasma* induces developmental defects in males by altering the male-specific process of dosage compensation [10]. In these studies, genetic crosses were utilized to test if strong loss-of-function mutations in several male specific lethal (*msl*) genes, including *msl-1, msl-2*, and *msl-3*, as well as *maleless* (*mle*), and *male absent on the first* (*mof*), could modulate male killing [10]. Normally *msl-2* is expressed only in males while the other genes are expressed in both sexes. In males the MSL-2 protein associates with the other proteins to form the DCC (also known as the MSL complex), which localizes preferentially to specific regions within the gene-containing euchromatin of the single X [16,17]. The presence of this complex induces acetylation of histone H4 at Lysine residue 16 (H4K16ac) within the body of compensated genes as a result of MOF acetylase activity [11,18,19]. This modification is accompanied by a ~2-fold transcriptional increase of most genes on the X chromosome so that the X-to-autosome (non-sex chromosome) ratio for gene expression in males matches that found in females, which have two active X chromosomes [20]. A substantial portion of *Spiroplasma*-infected males mutant for any of the DCC components were found to survive past the embryonic male killing phase [10]. This result demonstrated a genetic interaction between the dosage compensation components and the level of male killing. However, it remains to be determined whether this effect reflects a direct bacterial targeting of the dosage compensation machinery or instead an indirect suppression of male killing, perhaps because dosage compensation is downstream of another targeted cellular process.

To better understand this phenomenon we cytologically examined the subcellular behavior of the DCC and its histone acetylation mark just before the lethal phase in male *D. melanogaster* embryos infected with MSRO *Spiroplasma* (from here onward referred to as *Spiroplasma*) [14]. This strain was lethal to 96% of male progeny in the *D. melanogaster* lines used in this study (Table 1). In control (uninfected) embryos, very little DCC signal was seen before 3hrs after egg deposition (AED), as indicated by hardly detectable levels of visible MOF or H4K16ac on chromatin (N=7 embryos; Figure 1A), consistent with previous studies showing that DCC formation occurs around this time [21,22]. By 4-6hrs AED, during embryonic germ band extension, MOF appeared as a distinct sub-nuclear focus that was highly enriched with H4K16ac within the epithelial (outer) cells of male embryos (N=13 embryos; Figure 1B). This enrichment of H4K16ac also co-localized with a single bright region of MSL-3 (N=11 embryos; Figure S1) and resided immediately adjacent to or

overlapping with X sequences (Figure S2). These signals became strongest and evenly distributed across the embryo's surface by the beginning of segmentation (7+hrs AED) (Figure 1C, Figure S1).

In *Spiroplasma*-infected male embryos, very little MOF and H4K16ac signals were visible before 3hrs AED (N=6 embryos), similar to control male embryos at this time (Figure 1D, compared to 1A). At 4-6hrs, AED MOF and H4K16ac accumulated into sub-nuclear foci corresponding to X sequences within a portion of nuclei (N=15 embryos; Figure 1E). However, these signals only weakly accumulated into foci or not at all, and in many regions of the embryo they were distributed broadly across the nucleus (N=13 of 15 embryos; Figure 1E). In contrast, overall nuclear morphology appeared normal in most cells at this time, although a small portion of nuclei appear hyper-condensed (Figure 1E). By 9+hrs AED, when embryos undergo body segmentation, many nuclei exhibited abnormal hyper-condensation (Figure 1F). Additionally, DCC and H4K16ac signals became very low (N=15 embryos; Figure 1F; Figure S1), which may stem from general chromatin mis-organization as nuclear morphology became more abnormal. No DCC foci were observed in wild type *Spiroplasma*-infected or uninfected female embryos (N=9 embryos; Figure S3) and, consistent with previous studies [14,15], all were normal in cellular morphology.

The observation of DCC localization and H4K16 acetylation in ectopic nuclear regions led us to ask if this effect causes abnormal genome-wide mis-regulation of gene expression, which could explain defective male development. In order to address this question, we collected male enriched embryo broods (see Supplemental Experimental Procedures) at both 2-3hrs and 5-6hrs AED, and performed transcriptional profiling of total mRNAs extracted from these samples (Table S1). Although there was a high congruence of gene expression between control and infected embryos, a subset of genes appeared to be mis-expressed with a bias toward over-expression in infected embryos at both developmental times (Figure 2A, Table S1). To further evaluate this trend, we plotted the distribution of expression ratios (infected/uninfected) for autosomal and X-linked genes at both time points and used the Kolmogorov-Smirnov (K-S) test to determine an overall deviation from a 1:1 ratio (*i.e.*, no change in expression between conditions). The distribution of X-linked expression ratios significantly deviated at 2-3hrs AED ($P<1\times10^{-4}$) while the autosomal distribution was found to significantly deviate at both 2-3hrs and 5-6hrs AED ($P<1\times10^{-7}$ and $P<1\times10^{-4}$, respectively; Table S1), confirming the observed bias toward global over-expression (Figure 2A).

Closer inspection of our data sets revealed that a modest number of both X and autosomal (non-sex chromosome) genes were significantly mis-expressed at each developmental time (Figure 2B,C; Table S1), although this number may be underestimated due to large variation in expression between replicates, potentially reflecting the stochastic nature of DCC mis-localization. Mis-expressed genes were located across each chromosome and not clustered at distinct region(s) (Figure 2B), consistent with the observed lack of DCC concentration at distinct non-X regions (Figure 1E,F). The finding of over-expressed genes on the X, however, was surprising given that the DCC normally becomes enriched on X chromatin and overall loss of DCC there would be expected to result in the opposite pattern. These findings are consistent with previous studies showing that dissociation of the DCC from the male X

results in MOF localization and H4K16 acetylation across the genome, as well as up-regulation of autosomal genes but not X-linked genes [23,24]. The majority of mis-expressed genes were over-expressed at both developmental times (Figure 2B,C; Table S1), consistent with the H4K16ac mark being associated with increased gene expression [25]. A portion of mis-expressed genes on the X and the autosomes exhibited greater than 2-fold expression level differences in infected embryos (Figure 2C), when, under normal conditions, the DCC transcriptionally compensates at only a ~2-fold level expression change on the X. These patterns may reflect an abnormal chromatin remodeling behavior of the DCC in the presence of *Spiroplasma*. This idea is consistent with previous studies suggesting that DCC activity is normally constrained by an unknown mechanism so that X gene up-regulation occurs at an ~2-fold level [24,26,27]; it is possible that the male killing effect alleviates this constraint so that the DCC becomes hyperactive. Alternatively, higher-than-2-fold expression changes may also occur from mis-regulation of loci involved in transcriptional repression.

Some genes were consistently mis-expressed at both 2-3hrs and 5-6hrs AED (Figure 2D, Table S1). These genes represent a range of biological functions, with those involved cuticle formation being one of the more highly represented groups (Supplementary Figure 4; Table S1). These genes, and other—such as those involved in muscle assembly—are normally not expressed until late embryogenesis and afterward, suggesting that ectopic DCC mis-localization leads to activation of genes that are not normally expressed at these earlier times. While many of these genes may become mis-expressed due to ectopic DCC activity, others including the immune genes IM1, IM2-3, and IM4 (Table S1) could be induced as a general response to bacterial infection. The majority of immune response genes, however, were not induced at either developmental time analyzed (Table S1), a pattern that is consistent with previous work suggesting that *Spiroplasma* is capable of largely evading the host's immune system [28]. Of particular interest were two over-expressed regulators of apoptosis, *reaper* and *sickle*, at 2-3hrs AED, and just *sickle* at 5-6hrs AED (Table S1). The nature of our data does not allow us to address whether increased expression of these genes is a direct result of DCC mis-localization or instead a secondary response to broad gene mis-regulation. Nevertheless, elevated expression of these genes is consistent with previous cytological detection of increased cell death due to *Spiroplasma* infection at later embryonic stages [15].

Based on the above observations, *Spiroplasma* may initiate male killing by directly altering DCC localization as it forms during early embryogenesis, thereby leading to wide-scale gene mis-expression and developmental abnormalities. Although DCC-directed compensation is a distinguishing characteristic of the male sex during early development, other sex-specific differences exist at this time [29-31]. It is possible that *Spiroplasma* targets an earlier male-specific factor or process that leads to DCC mis-behavior or, alternatively, that other male factors could be essential for DCC targeting; either of these scenarios is consistent with suppression of male killing by mutational loss of the DCC [10]. To test if the DCC is the sole male-specific factor required for male killing, we assessed the viability of *Spiroplasma*-infected *D. melanogaster* females artificially expressing *msl-2* via a heat shock-inducible *hsp83* promoter [17]. Under heat shock this transgene expresses *msl-2* at a high level, an effect that is strongly lethal to females due to a proportionally high level of DCC formation

and activity [17]. However, without heat shock this transgene is leaky, expressing a low level of *msl-2* that does not affect female viability when in a genetic background that is heterozygous for an *msl-1* mutation [17]. DCC formation through leaky expression of this transgene was previously shown in female salivary gland nuclei [17]. Here we observed single foci of MOF in the nuclei of female embryos carrying one transgene copy (Figure 3), demonstrating that this transgene is also active during early development.

To assess the effect of DCC formation on the viability of infected females we crossed uninfected males carrying one copy of the *hsp-83 msl-2* transgene to wild type females that were either *Spiroplasma*-infected or uninfected and scored the viability of the resulting offspring (see Experimental Procedures for details of the crosses and genotypes). The ratio of uninfected, transgenic to uninfected, non-transgenic female progeny was close to 1 (Chi-square goodness of fit test, P=0.55) (Figure 3A), recapitulating that the leaky expression of the *msl-2* transgene alone has very little effect on female viability when in an *msl-1* heterozygous mutant background. However, the ratio of infected, transgenic to infected, non-transgenic female progeny was 0.411 (Figure 3A), a highly significant deviation from the expected 1:1 ratio of these genotypes that occurred in the absence of infection (Chi-square goodness-of-fit test, P<0.0001). Thus, the presence of the DCC is lethal to female embryos only when they carry the *Spiroplasma* infection. Consistent with this finding, *Spiroplasma*-infected female embryos expressing *msl-2* were nearly identical in abnormal sub-cellular phenotype to those of wild type infected male embryos: they exhibited mis-localized MOF at 4+hrs AED, and these signals diminished in intensity as nuclear morphology degenerated during later developmental times (Figure 3B). In contrast, transgene-carrying female embryos without infection showed well-formed MOF foci and normal nuclear morphology (Figure 3B).

Although compensation of most X-linked genes involves the DCC, a sub-set of genes involved in early embryogenesis become compensated as much as one hour before DCC formation, thus likely occurring through a DCC-independent mechanism [31]. Although the details of this process are currently unknown, it is clear that gene dosage compensation in Drosophila is more complex than previously thought and extends beyond the DCC pathway. Moreover, this complexity infers that the embryonic cytotype begins to differ substantially between males and females as early as one hour after fertilization. Our ability to reconstitute the killing effect in female embryos by expression of *msl-2* alone strongly suggests that DCC mis-localization is a direct effect of *Spiroplasma* and does not result secondarily from targeting of the earlier, DCC-independent compensation. More generally, DCC mis-localization by *Spiroplasma* does not require interactions with other cellular processes that are characteristic of male embryos. Thus, DCC mis-localization can be viewed as a sex-independent phenomenon that may arise from a specific interaction of this complex with a bacterium-produced factor in the cells of whichever sex these components are present. This interaction could also involve other host cellular components that must be present in both male and female embryos.

Taken together, our results support a model in which *Spiroplasma* initiates male killing by altering DCC behavior on both the X and ectopically on non-sex chromosomes, thereby leading to abnormal histone acetylation and gene mis-expression across the genome. The

mis-expression of certain genes – such as those involved in apoptosis – may elicit severe cellular responses at even slightly elevated levels [32,33]. Thus, perturbations of several key genes may have profound deleterious effects on early male development. Wild type infected females would be spared this effect because these individuals do not normally express the MSL-2 protein and, therefore, harbor no integral DCC [17,34]. Knowledge that the DCC itself may be a direct target of *Spiroplasma* in *D. melanogaster* will help to identify the specific interacting molecules of host and symbiont that underlie this phenomenon through further experimentation.

Experimental Procedures

Fly lines and infection

Embryo collections for cytology were obtained from two different fly lines. One was a *Canton-S* line carrying the MSRO strain of *Spiroplasma* that was used previously in [14]. A second was a line homozygous for a transgenic copy of *msl-3-GFP* (A gift from M. Kuroda). This line was infected for this particular study by haemolymph transfer from the infected *Canton-S* line (protocol provided by T. Haselkorn). Fly lines were propagated by crossing virgin infected females from each fly line to males from an uninfected stock of the same genetic background. Before crossing, infected females were held to 1 week in order to insure strong penetrance of male killing. All embryo collections, crosses, and propagation lines were maintained at 25°C.

For testing the effects of *msl-2* expression in infected females, we crossed *w ; msl-1; P{w+ hsp83-msl-2}* females (stock obtained from M. Kuroda) to *w / Y ; + ; TM3 Ser Sb / TM6 Tub* males. F1 Male progeny of the genotype *w / Y ; msl-1 / + ; P{w+ hsp83-msl-2} / TM3 Ser Sb* were then crossed to either *Spiroplasma*-infected or uninfected *Canton-S* females aged 1 week. The resulting progeny from this cross carried either the *P{w+ hsp83-msl-2}* transgene or the *TM3 Ser Sb* balancer chromosome and were easily distinguished by the presence or absence of the two co-inherited dominant markers.

Embryo collection and fixation

Mated females of the desired genotype and infection status were allowed to lay on collection plates containing grape agar medium and embryos were aged to the desired developmental stage at 25°C. Embryos were dechorionated in 50% bleach, fixed for 10 minutes in 4% paraformaldehyde/heptane, and devitellinized in methanol. Embryos were either kept in methanol at −20°C for long-term storage or rehydrated for cytolological purposes. Hydration was conducted in a series of methanol:water solutions in order of the following ratios: 9:1, 5:5, and 1:9, followed by three 5min washes in 1xPBT (1xPBS and 0.1%Tween-20).

Immunohistochemical staining and fluorescence in situ hybridization

Hydrated, fixed embryos were incubated in the following primary antibodies overnight at 4°C: mouse anti-GFP (1:50, clone number 4C9, Developmental Studies Hybridoma Bank); goat anti-MOF (1:100, clone number dn-17, Santa Cruz Biotech); rabbit anti-acetyl-Histone H4 Lysine 16 (1:100, catalog number 07-329, Millipore). Following incubation with primary antibodies, embryos were washed 3×15min in 1xPBT before secondary antibody staining.

Anti-mouse, anti-rabbit, and anti-goat secondary antibodies were conjugated with either Alexa555 or Alexa633 (Molecular Probes-Invitrogen) and used at a dilution of 1:300. Secondary staining was conducted out of light for 1hr at room temperature and washed three times subsequently.

Fluorescence *in situ* hybridization was conducted with an oligonucleotide probe specific for a satellite DNA sequence unique to the Y chromosome [35]. The probe consisted of the sequence 5'-AAT ACA ATA CAA TAC AAT ACA ATA CAA TAC-'3 and was synthesized commercially (IDT, Inc.) with Cy2 conjugated at the 5' end. Whole mount embryo hybridizations were conducted as described [35], except that embryos used for this purpose were stained first with antibodies, re-fixed in 4% paraformaldehyde for 45 minutes, and washed 3X in 2xSSCT buffer before probe hybridization.

Confocal Imaging and data processing

Fluorescence images were collected on a Leica TCS SPE confocal microscope. Images were collected by using identical laser, gain, and offset settings for both uninfected and infected tissues for direct comparison between these conditions. The specific setting values of the original Leica-formatted images can be obtained at request from the authors. After image capture, image files were exported in TIFF format and processed in Adobe Photoshop CS5 version 12. Images were enhanced with the brightness and contrast settings being manipulated identically between infected and uninfected conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Highlights

- The DCC becomes mis-localized in *Spiroplasma*-infected male fruit fly embryos

- Infected males exhibit ectopic H4K16 acetylation and genome-wide gene mis-expression

- Infection leads to death of females artificially expressing the DCC
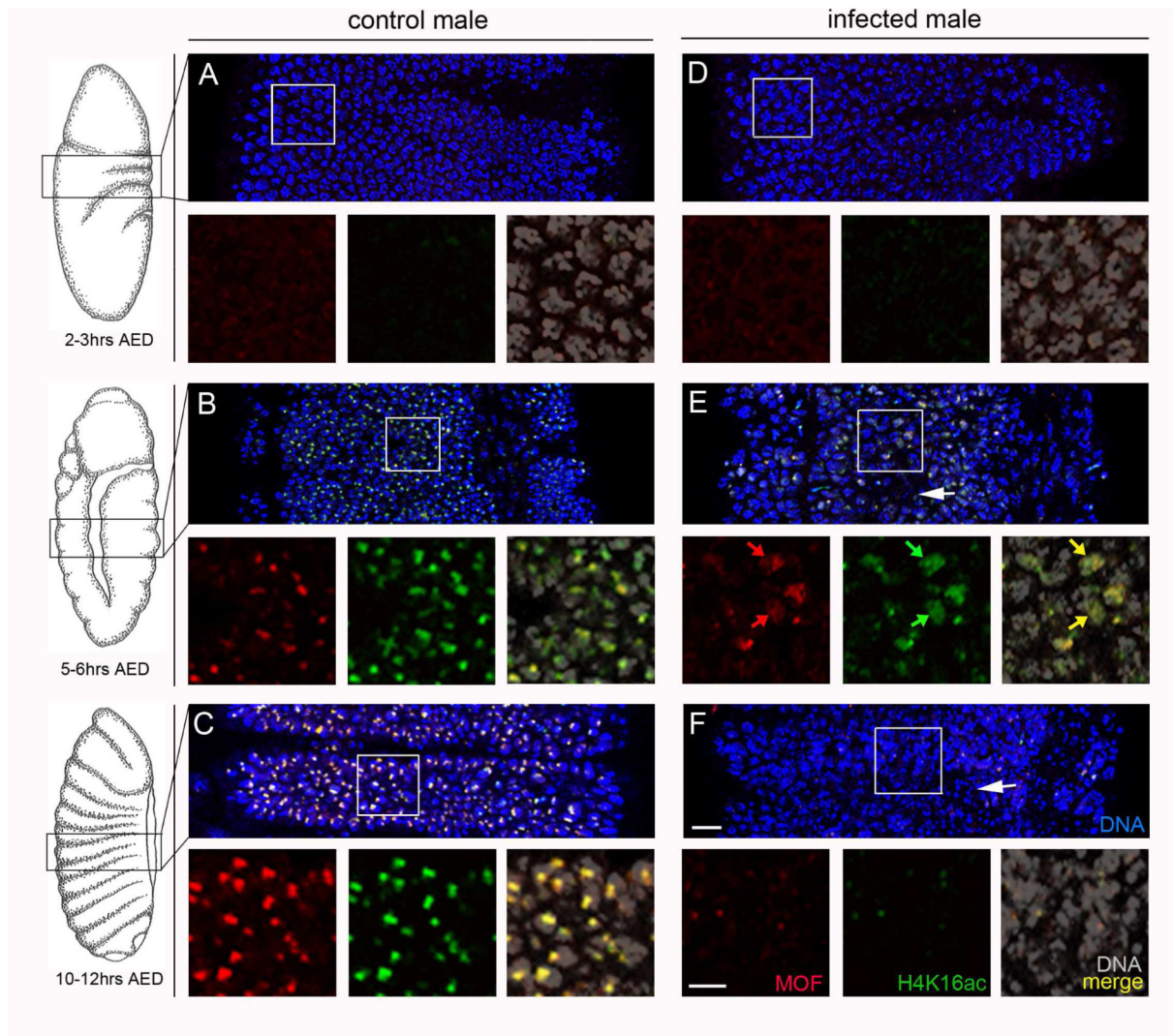
Figure 1. *Spiroplasma*-infected male embryos exhibit mis-localized MOF and H4K16ac
(A,B) Both control (uninfected) and infected embryos at 2-3hrs after egg deposition (AED) show very little MOF or H4K16ac signals. (C,D) At 5-6hrs AED, MOF and H4K16ac signals become bright and co-localize to a single focus in each nucleus of control embryos. The H4K16ac signals co-localizes with X sequences and overlap with MSL3 (see Figures S1 and S2 respectively). In contrast, these signals, although still overlapping, become distributed across nuclei in infected embryos (yellow arrows in right panel). Some nuclei in infected embryos appear hyper-condensed (white arrow). (E,F) At 9-11hrs AED, MOF and H4K16ac signals persist as bright foci in the nuclei of control embryos. However, in infected embryos these signals are barely visible in most nuclei, and more nuclei are hyper-condensed (white arrow). Scale bar equals 15 μm in wide panel F and 5 μm in the high magnification panel under F.
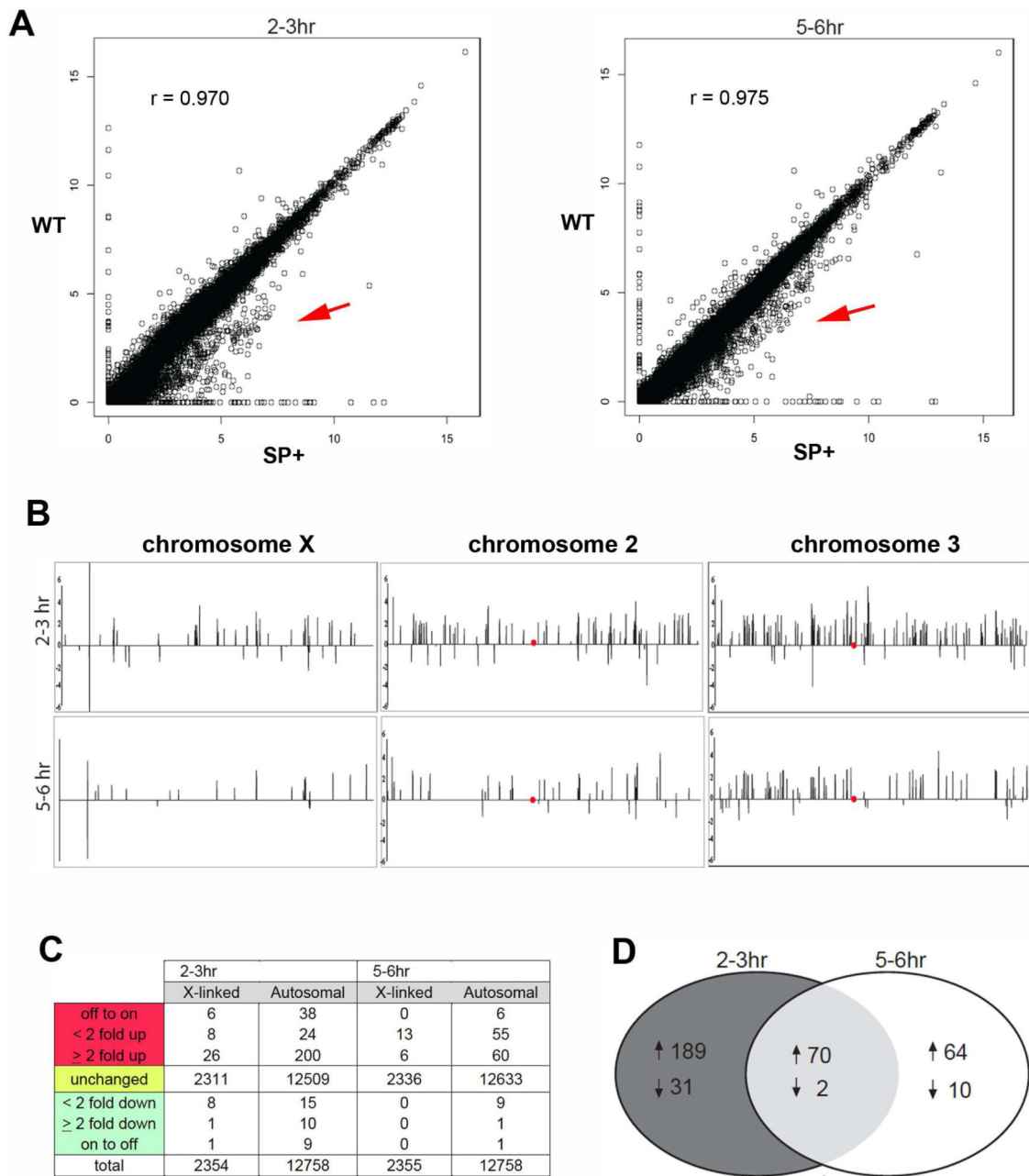
Figure 2. *Spiroplasma*-infected male embryos exhibit genome-wide misregulation of gene expression

(A) Scatterplots show averaged FPKM values between corresponding replicates for expressed genes in wild type embryos plotted against those of *Spiroplasma*-infected embryos for both time points. Values are shown in log scale. Red arrow indicates a higher clustering of genes that appear over-expressed in infected embryos. (B) Chromosome maps depicting the positions of significantly mis-expressed genes in *Spiroplasma*-infected embryos relative to uninfected embryos. Peaks above the horizontal lines represent genes that are significantly over-expressed in *Spiroplasma*-infected embryos, and peak height reflects expression ratio on the log scale. Red dot corresponds to the chromosome

centromeres (C) The number of significantly mis-expressed genes on the X vs. the autosomes (non-sex chromosomes) and their general fold change values between *Spiroplasma*-infected and uninfected embryos. The patterns in B and C reflect an enrichment of over-expressed genes on all chromosomes and for both developmental times. (D) A Venn diagram depicting the number of over-expressed genes (up arrow) and under-expressed (down arrow) in *Spiroplasma*-infected embryos that are unique to each developmental time point and those common between these times. See Table S1 for raw data, and Figure S4 for ontogeny groupings of mis-expressed genes common between 2-3hrs and 5-6hrs AED.
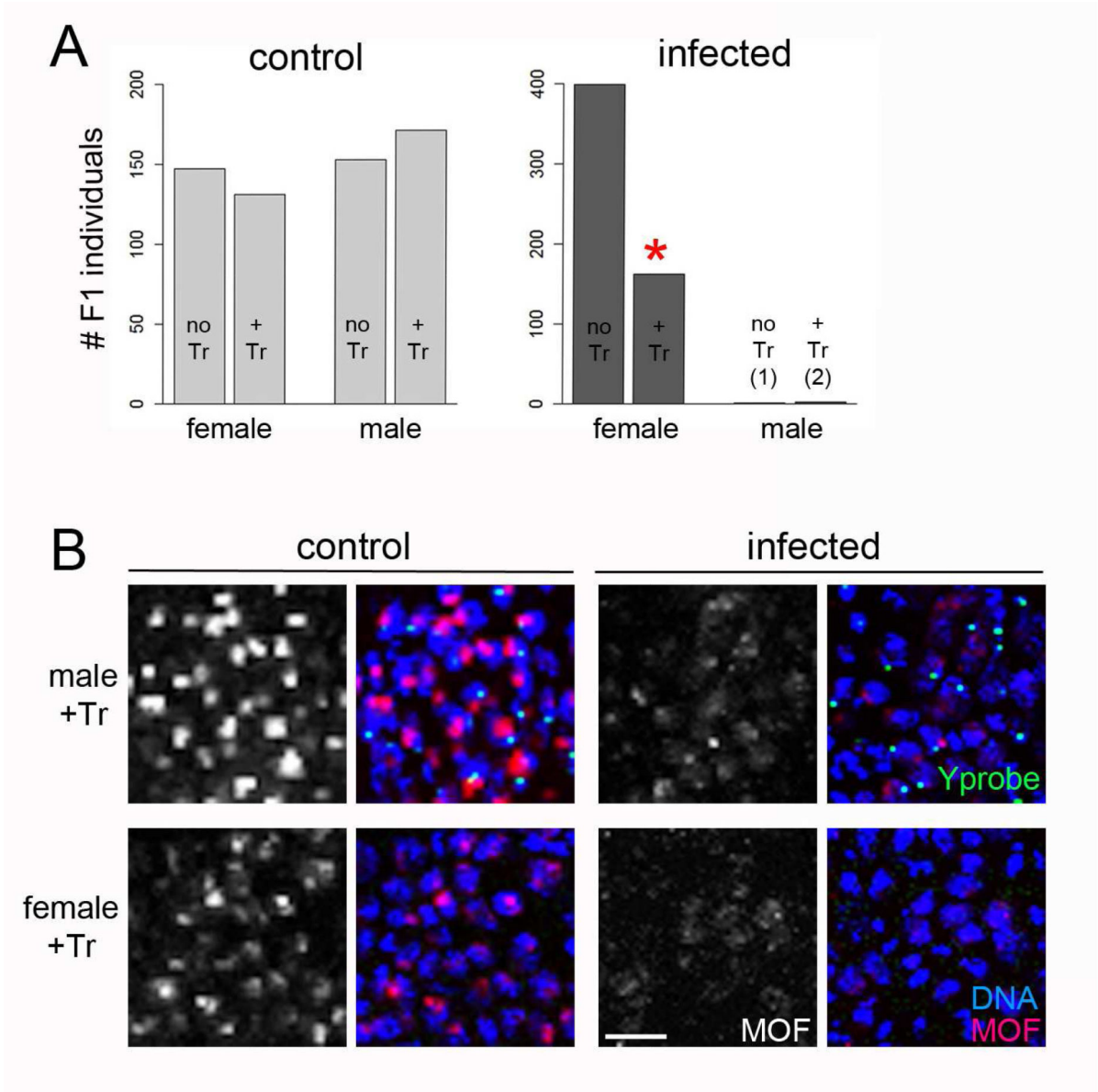
Figure 3. *Spiroplasma* infection causes death of females expressing low levels of MSL-2 and mis-localized DCC during female embryogenesis

(A) The number of F1 progeny carrying the *hsp83-MSL-2* transgene (+Tr) and their siblings without the transgene (no Tr) produced from either uninfected (control) or *Spiroplasma*-infected mothers. Red asterisk indicates a significant difference between transgenic and non-transgenic females infected by *Spiroplasma*. (B) Transgenic male and female embryos that are either uninfected or *Spiroplasma*-infected. Uninfected embryos of both sexes show distinct MOF localization, whereas in infected embryos MOF fails to concentrate and becomes distributed lightly across nuclei. Uninfected females do not exhibit foci of MOF or H4K16ac (see Figure S3). Scale bar equals 5 μm.

Table 1

Adult sex ratios for *Spiroplasma*-infected and uninfected fly lines

Fly line	Number females	Number males	Total	% males
Canton-S	117	99	216	46
Canton-S (Sp+)	178	0	178	0
w;msl-3-GFP	233	248	481	52
w;msl-3-GFP (Sp+)	265	5	270	2