A *Wolbachia* deubiquitylating enzyme induces cytoplasmic incompatibility

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*Wolbachia* are obligate intracellular bacteria that infect arthropods, including approximately two-thirds of insect species. *Wolbachia* manipulate insect reproduction by enhancing their inheritance through the female germline. The most common alteration is cytoplasmic incompatibility (CI), where eggs from uninfected females fail to develop when fertilized by sperm from *Wolbachia*-infected males. By contrast, if female and male partners are both infected, embryos are viable. CI is a gene-drive mechanism impacting population structure and causing reproductive isolation, but its molecular mechanism has remained unknown. We show that a *Wolbachia* deubiquitylating enzyme (DUB) induces CI. The CI-inducing DUB, CidB, cleaves ubiquitin from substrates and is encoded in a two-gene operon, and the other protein, CidA, binds CidB. Binding is strongest between cognate partners in *cidA-cidB* homologues. In transgenic *Drosophila*, the *cidA-cidB* operon mimics CI when sperm introduce it into eggs, and a catalytically inactive DUB does not induce sterility. Toxicity is recapitulated in yeast by CidB alone; this requires DUB activity but is rescued by coexpressed CidA. A paralogous operon involves a putative nuclease (CinB) rather than a DUB. Analogous binding, toxicity and rescue in yeast were observed. These results identify a CI mechanism involving interacting proteins that are secreted into germline cells by *Wolbachia*, and suggest new methods for insect control.

The mechanism of cytoplasmic incompatibility (CI) is frequently modelled as a modification–reactivation (or toxin–antidote) system in which sperm undergo a *Wolbachia*-mediated modification event that can be conditionally rescued in the egg by a *Wolbachia*-encoded factor. Normally, upon fertilization, the sperm-derived pronucleus undergoes nuclear envelope breakdown and exchanges proteins for maternal histones. Subsequently, male and female pronuclei juxtapose (but do not fuse) and undergo DNA replication before the first zygotic mitosis. Chromosomes from both pronuclei synchronously condense, align at metaphase and separate in anaphase. Ubiquitin is a small polypeptide that post-translationally modifies many proteins and has numerous functions. Protein ubiquitylation is highly dynamic and is reversed by cellular DUBs. Genetic evidence from diverse *Wolbachia* strains suggests that the modification and rescue functionalities of CI arise from at least two independent genes, similar to bacterial toxin–antidote systems. Most such toxin–antidote systems studied have simple two-gene operon structures. We therefore hypothesized that the *cidA-cidB* operon products might be the executers of CI.

As *Wolbachia* strains evolve within different host species, they accumulate mutations in their corresponding CI systems and become bidirectionally incompatible. This could potentially be due to their respective CI-regulating factors having evolved mutually exclusive binding specificities. Interestingly, *Wolbachia* genomes from *Culex pipiens* mosquitoes show extensive genetic duplication and divergence of the putative CI-inducing operons, possibly accounting for multiple incompatibilities. The *Wolbachia* strain wPip, for example, has two related operons (Fig. 1c,d). The second operon encodes proteins related to CidA and CidB, but the downstream gene encodes what is likely to be a functional PD-(D/E)XXK nuclease domain (DUF1703) rather than a DUB (Fig. 1d). We have provisionally named the two genes in this operon *cinA* (wPa*0294*) and *cinB* (wPa*0295*). CidB and CinB may share a common nuclease ancestor (Supplementary Fig. 1 and Fig. 1c, dotted lines), but the predicted nuclease active-site residues are not maintained in CidB. Importantly, the predicted functional status of the enzymatic components of these operons correlates with the ability of diverse *Wolbachia* strains to induce bidirectional CI (Supplementary Discussion).

In bacterial toxin–antidote systems, the two components bind one another. We therefore expressed recombinant tagged constructs of the *cidA-cidB* operon proteins (Supplementary Figs 2 and 3) and examined their interactions. Pull-down of His6-tagged CidA from extracts of *Escherichia coli* expressing both His6-CidA and CidB also brought down the CidB protein (Supplementary Fig. 3a,b). We observed similar binding of the cognate partners His6-CinA and CinB (Supplementary Fig. 3c,d).

Differential binding affinities of operon-encoded partners might account for the bidirectional incompatibilities noted above. This model would predict that proteins derived from the same operon associate in preference to their noncognate partners from other operons. To test this, we purified His6-tagged copies of CidB and CinB. These proteins were incubated with extracts of the corresponding *FLAG*-tagged CidA and CinB proteins and binding was assessed (Fig. 1f). Indeed, binding was much stronger between cognate proteins from the same operon. These results are consistent with a model in which operon-specific differences in partner binding affinities underlie the bidirectional
incompatibilities and partial rescues seen in genetic crosses with different Wolbachia strains.

When divergent Cl-causing Wolbachia strains are introduced into different insect species by microinjection, Cl is recapitulated20,21. This indicates that Wolbachia Cl factors can operate in a broad range of hosts (Supplementary Discussion). To test the modification–rescue model for Cl in a heterologous eukaryotic host, we expressed the Cid and Cin proteins in the yeast Saccharomyces cerevisiae (Fig. 2). Both CidB and CinB (but not CidA or CinA) caused temperature-sensitive growth inhibition when expressed in yeast. Growth was rescued by coexpression of the cognate partners, CidA and CinA, respectively. When the predicted cysteine protease active site in CidB was mutated from Cys to Ala (CidB* in Fig. 2a), temperature-sensitive lethality was lost. Similarly, upon mutation of the three predicted nuclese active-site residues in CinB (CinB*, Fig. 2a), temperature-sensitive lethality was again no longer observed. Changes in protein levels of the modifiers cannot account for the loss of toxicity, at least in the case of CidB* (Supplementary Fig. 4). Importantly, only the correctly matched cognate partners rescued growth when coexpressed with CidB or CinB (Fig. 2b). Toxicity and rescue for both operons was seen in two different yeast backgrounds (BY4741 and W303a). These results with yeast show that the cognate Wolbachia operon-encoded factors display toxicity and rescue, respectively; that toxicity depends on the (putative) enzymatic activities (see below) of the CidB and CinB proteins; and, finally, that suppression of toxicity in vivo correlates with cognate protein binding preferences in vitro.

Next we sought to characterize the enzymatic activity of CidB. We initially expected it would behave like a protease specific for the small ubiquitin-like modifier (SUMO) protein, because it bears a C48/Ulp1-like domain22. However, the purified protein did not cleave fluorogenic SUMO-7-aminomethylcoumarin (AMC) or SUMO-peptide fusions. By contrast, CidB reacted with a ubiquitin-based suicide inhibitor, HA-ubiquitin vinyl methyl ester (HA-UbVME); its reactivity was similar to that of a well-characterized DUB, UCH37 from Trichinella spiralis23 (Fig. 3a and Supplementary Fig. 5d). Enzyme activity was tested against polyubiquitin chains with isopeptide linkages (C-terminal ubiquitin and Supplementary Fig. 5d). Enzyme activity was tested against polyubiquitin chains with isopeptide linkages (C-terminal ubiquitin

Figure 2 | Testing of the modification–rescue hypothesis in S. cerevisiae. a. Expression of Wolbachia proteins from a galactose-inducible GALI promoter on minimal medium lacking uracil and containing galactose or glucose (three replicates). Control plasmids pYES2 (empty vector) and LacZ (negative control) cause no defects. Both CidB and CinB expression blocks yeast growth at high temperature. Inactivation of the Ulp1-like protease by a C1025A mutation (CinB*) or the putative DUF1703 nuclease by mutation of the D-E-K triad to A-A-A (CinB*) eliminates toxicity. b. Coexpression of CidB or CinB with different upstream operon components on minimal media lacking uracil and leucine shows growth rescue only with cognate partners (three replicates). Vector is pRS425.

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mutant (CidB*) was inactive against Ub-AMC. Despite the ability to cleave multiple substrates in vitro, CidB appears to have a restricted substrate range in cells, as bulk ubiquitin conjugates in yeast were not detectably altered by CidB expression (Supplementary Fig. 5c).

Because CidA binds CidB and suppresses CidB toxicity in yeast, we tested whether CidA inhibited CidB DUB activity in vitro. A 100-fold molar excess of CidA failed to inhibit UbVME reactivity. Therefore, CidA probably rescues toxicity in yeast by some other means, such as control of its localization. This would have the advantage that the related CidA and CinA proteins could interact in similar ways to enzymatically distinct cognate factors, such as those with DUB and nuclease domains.

To test the ability of the cidA-cidB operon to induce CI in an insect in the absence of Wolbachia infection, we cloned expression constructs into the germline-optimized pUASp-attB vector\(^\text{24}\) for transgenic insertion into Drosophila melanogaster by the site-directed ΦC31 integrase\(^\text{25}\) (Supplementary Fig. 7). The multiple independent transgenic flies each had a fusion of the cidA-cidB open reading frames (ORFs) linked by a T2A viral peptide sequence that causes ribosomal skipping such that CidA and CidB are produced as separate proteins\(^\text{26}\) (Supplementary Fig. 7b). After transgenesis, we verified attB/P recombination by PCR, confirmed that our fly lines were not infected by native Wolbachia strains, and verified transgene expression by reverse-transcription (RT) PCR (Supplementary Fig. 7c–e). Strikingly, males expressing the transgenic operon displayed a fully penetrant sterility in matings with wild-type (WT) females (four biological replicates with two independent attP insertion sites; Fig. 4a and Supplementary Fig. 7). By contrast, females transgenic for the cidA-cidB operon were fertile, indicating that the operon caused embryonic lethality only if it was inherited from males. Mutational inactivation of the CidB DUB (CidB-C1025A) in transgenic inserts eliminated the ability of the insert to cause male sterility (Fig. 4a, ‘operon*’).

Attempts to rescue the CI-like phenotype with transgenic females expressing either CidA alone or the full operon were not successful. This precludes the unequivocal assignment of the ‘rescue’ component of CI to the cidA-cidB operon. Potential reasons for these negative results are provided in the Supplementary Discussion.

Previous reports have implicated genes in CI pathways without quantitative cytological analysis of the first mitotic cell cycle.

**Figure 3 | CidB is a DUB.** a, DUB reactivity with the N-terminally haemagglutinin (HA)-tagged suicide inhibitor, UbVME (three replicates). Shown is an anti-HA immunoblot analysis of 30 min reactions performed at room temperature. UbVME reacts with the wild-type CidB protein but not the C1025A catalytic mutant (CidB*). TsUCH37 is a positive control\(^\text{23}\). CidA at 100-fold molar excess does not inhibit UbVME reactivity. b, Cleavage by CidB of K48- and K63-linked ubiquitin chains assayed by anti-ubiquitin immunoblotting (three replicates). Usp2 is a positive control\(^\text{40}\). Enzyme and polyubiquitin chains were at 50 nM and 500 nM, respectively, and reactions were at 37 °C for 1 h. c, CidB has an approximately fourfold preference for K63-ubiquitin dimers compared to K48-linked dimers. Shown is a plot of initial velocity (divided by total enzyme concentration) as a function of substrate concentration from three independent experiments. Error bars are standard deviations.
Others have identified host genes that can induce CI-like sterility\textsuperscript{27}, but a \textit{Wolbachia} gene that can precisely mimic CI cytology at the first embryonic mitosis has never been identified. To verify that \textit{cidA-cidB} specifically induced CI rather than an alternative type of sterility, we determined whether embryos from crosses with \textit{cidA-cidB} transgenic males recapitulated CI-defining cytological

**Figure 4 | Induction of CI by transgenic \textit{cidA-cidB} males.**\textbf{a}, \textit{D. melanogaster} males carrying transgenic \textit{cidA-cidB} are sterile when mated to WT females (\(n = 30\) mating vials). Males with transgenic \textit{cidA-cidB}\textsuperscript{*} harbouring the CidB active-site mutation C1025A (operon\textsuperscript{*}) are fully fertile, as are females with the active transgenic operon. CidA, by itself, has no effect on fertility, and no strain singly transgenic for \textit{cidB} could be isolated. Enhanced green fluorescent protein (EGFP) is a negative control. Error bars are standard deviations. \textbf{b}, CI-like defects in the male pronucleus initially appear in late prophase, during the first division of the apposed female and male pronuclei, and accrue through mitosis. Abnormal cytology was observed in 56 transgenic CI embryos fixed after 18 min of development. \textbf{c}, Quantification of transgenic \textit{cidA-cidB} (CI) embryos’ mitotic defects, including uncondensed paternal chromosomes, delayed segregation of paternal chromosomes, or chromosomal bridging during the first zygotic cell cycle. Sample sizes of observed transgenic and WT embryos were 63 and 29, respectively. \textbf{d}, Quantification of developmental progress in transgenic (CI) embryos. At 24 h after egg laying, embryos were classified into three categories: early, pre-blastoderm formation; mid, blastoderm until segmentation stages; late, segmented stages. Quantification is based on three samples of approximately 200 embryos each. A total of 60\% of CI embryos arrested development in the early stage compared to 12\% from the WT control. Significant \(P\) values (<0.005) are indicated by an asterisk. Error bars are standard deviations.
and embryonic defects (Fig. 4b). These defects include impaired male pronuclear maturation at metaphase and delayed chromosome separation and bridging at anaphase. All were observed in the transgenic crosses. Of the embryos analysed during the first post-fertilization mitosis, 88% showed these Cid-like defects, compared to only 3% in WT crosses (Fig. 4c). Of the transgenic embryos that were left to develop for 24 h, 60% arrested before blastoderm formation (‘early’, Fig. 4d). Of the 20% of embryos that developed to segmentation, 69% showed segmentation deformities29 (Supplementary Fig. 8a,b). These specific developmental defects recapitulate those of Cid embryos3–5,28,29. Thus, the defects produced by cida-cidb expression in males replicate the established developmental abnormalities in CI-inducing crosses from Wolbachia-infected males. 

Research on CI was pioneered over 60 years ago using intraspecific crosses of the mosquito C. pipiens3. The Wolbachia–CI link was made in 1971 (ref. 4), but the molecular mechanism has remained obscure. Our data provide strong evidence that the Wolbachia cida-cidb operon is responsible for CI. The most parsimonious interpretation of our yeast and transgenic fly data is an adaptation of the modification–rescue framework first proposed by Hurst12 and Werren30, in which CidB is the modifier and CidA would function as the rescue factor. Wolbachia bacteria have a type IV secretion system that could translocate the CidA and CidB proteins into the host cytoplasm. The cida-cidb sequence resides in a Wolbachia prophage (Supplementary Discussion), so virus-induced cell lysis would be another potential route of transmission. In analogy to many toxin–antidote systems in free-living bacteria, we propose that within the fertilized egg of an incompatible cross, CidA is rapidly inactivated or degraded. Unless CidA is supplied by a maternal Wolbachia infection in the egg’s cytoplasm, the paternally supplied CidB enzyme would become active. CidA alone might also not be sufficient for rescue in the egg; additional Wolbachia or host factors might be required, possibly for co-localization of the cognate partners. The exact targets of the CidB DUB enzyme (and putative CinB nuclease) and the detailed molecular pathway of cida-cidb-induced CI also remain to be determined.

Regardless of these outstanding mechanistic questions, our results suggest immediate potential practical benefits. The complete sterility induced by cida-cidb in male insects and the lack of obvious harmful effects on their fitness suggest that release of such transgenic sterile males could be highly effective for population control of many insect pests or human disease vectors. An obvious application would be in limiting the mosquito vectors responsible for transmission of dengue and Zika viruses or malarial parasites.

Methods
DNA manipulation. DNA was purified from Wolbachia-infected insects according to ref. 31. Genes from cid and cin operons were cloned from DNA of wPip-infected C. pipiens Buckeye mosquitoes33 and from YW wMel-infected D. melanogaster flies. PCR products were amplified with the primers listed in Supplementary Table 1 using PhusionHF DNA polymerase (New England Biolabs), gel-purified and ligated into various plasmid vectors, including the pBAD (ThermoFisher; arabinose induction), pET (ThermoFisher; isopropyl-β-D-thiogalactoside (IPTG) induction), pGOLD (gift from Chittaranjan Das; IPTG induction) and pGEX (GE Healthcare; IPTG induction) E. coli expression vectors. All plasmid inserts were fully sequenced at the Yale Keck Foundation DNA sequencing facility. Point mutations were introduced by QuickChange mutagenesis (Stratagene). Further modifications such as truncations or tag additions were carried out using the site-directed, ligase-independent mutagenesis method SLIM32.

Protein purification for pull-down analysis of His6-tagged proteins. The procedure followed was a slight modification of the Dynabeads manufacturer's protocol (Novex). Recombinant proteins were initially expressed in the E. coli TOP10F’ strain, but because they were prone to proteolytic cleavage by Lon protease (Supplementary Fig. 2), we switched to expression in BL21-AI (ThermoFisher) or Rosetta DE3 (Novagen) E. coli strains, which lack Lon. Large (2 L) or small (100 ml) cultures were grown in Luria broth (LB) at 37 °C with vigorous shaking to an optical density at λmax,ass of 0.5 and induced with either 0.02% arabinose (pBAD) or 1 mM IPTG (pET). Protein induction in most cases was allowed to proceed overnight at 18 °C or at 30 °C in the case of a mycobacterial panicyclic (pBAD) or at 37 °C in the case of the pET. After induction at 37 °C for 4 h, cells were collected and lysed via French press. Proteins were purified by GST-affinity chromatography using glutathione-agarose (Thermo Scientific). After removal of the GST tag with PreScission protease (GE Biosciences), the protein was further purified by size-exclusion chromatography using a HiLoad Superdex S75 PG column (GE Biosciences) in a buffer consisting of 50 mM Tris-HCl (pH 7.6), 150 mM NaCl and 1 mM diethiothreitol (DTT). All reactions were performed in triplicate, and quenched, flash-frozen and stored at −80 °C until use. Point mutations were introduced by site-directed mutagenesis using a Thermofisher QuickChange mutagenesis kit (Stratagene). Further modifications such as truncations or tag additions were carried out using the site-directed, ligase-independent mutagenesis method SLIM32.

Diubiquitin cleavage assays. Cleavage assays were carried out using CidB (762–1143) following a previously published protocol33. Briefly, 250 nM CidB was incubated in a reaction buffer of 50 mM Tris (pH 7.6), 20 mM KCl, 5 mM MgCl2, and 1 mM diubiquitin concentration. All diluent was 20 to 120 μM. In assays using Lys48 diubiquitin, 400 nM CidB was used. All reactions were carried out at room temperature for 10 min (lys63 reactions) or 15 min (lys48 reactions) and were quenched by the addition of the Diubiquitin cleavage reaction in Image software44. To account for the release of two ubiquitin moieties (P and P') from a single reaction, the initial rates of each reaction were divided by 2. All kinetic data were analysed with Kaleidagraph Version 4.1.3b1 and could be fit to the Michaelis–Menten equation: 

\[ V = \frac{V_{\text{max}} [S]}{K_m + [S]} \]

where \( V \) is the reaction velocity, \( V_{\text{max}} \) is the maximum velocity, \( K_m \) is the Michaelis constant and [S] is the concentration of substrate. Error bars are standard deviations. We also tested the reaction of full-length CidB with all seven possible ubiquitin lysine linkages by incubating 1 μM enzyme with 1 μM diubiquitin for 1 h, 4 h or overnight at 37 °C using the Ub Explorer Panel (LifeSciences). Finally, we incubated 50 nM CidB with 500 μM Lys63-linked polyubiquitin (in size range from two to seven ubiquitin) in BioXcel (762–1143) following a previously published protocol33. Brie...
Ubiquitin-AMC and UBL-AMC hydrolysis assays. Ubiquitin (Ub) and ubiquitin-like (UBL) proteins were prepared using the iScript cDNA Synthesis Kit (BioRad) and the cDNA was used as a template for PCR reactions with primers that amplified either cidd or rp3. 

Flies were maintained at room temperature on a standard diet. For CI analysis, two males (<3 days old) were mated to 10 virgin females in an individual tube. One tube of twelve flies was one N. Adult flies were removed after 10 days of egg laying, and fecundity was assessed by counting eclosed adult progeny. In the case of the crosses that led to sterility, flies were allowed to lay eggs until they died in the tube, and they never produced offspring. To assess the cytology of early embryos resulting from an incompatible cross with cidd-cidB transgenic males, ~300 virgin female "CS flies were placed in a collection container with ~100 transgenic cidd-cidB males and put on apple juice plates with yeast paste for 2 days. Embryos were then collected by a squirt, let it lie every 15 min, dechorionated in 50% bleach and acidified in a solution of 5 ml heptane, 2 ml 2.5× PBS, 500 µl 0.5 M EDTA and 1 ml 37% fresh formaldehyde. The fixing solution (10 ml) was kept in a glass centrifugation tube to allow visualization of liquid-phase layers and eggs. Vitelline membranes were removed by replacing the heptane top layer with two volumes of methanol and vigorous shaking.

Sunkin de-vitellinated embryos were collected with a Pasteur pipette, washed three times with methanol, and stored overnight at 4°C before they were rehydrated with PBTA solution (1x PBS, 1% BSA, Albumin, 0.05% Triton X-100, 0.02% sodium azide) and stained with Hoechst 33342 dye (ThermoFisher Scientific) at 1:1,000 in PBTA. Stained embryos were washed and mounted on glass slides and sealed under a coverslip with nail polish. Microscopic analysis of the embryos was performed on a Zeiss Axioskop microscope using a x100/1.4 NA objective lens.

Variations in the cytological quantifications are shown as the standard deviation of the mean of triplicate samples of 200 embryos (Fig. 4d). Polar bodies were used as a landmark where polar bodies were not observed were excluded from the data in Fig. 4c. Images were captured by AxioVision Re.4.8 software and adjusted for contrast and assembled in Photoshop (Adobe). The images confirmed that the cidd-cidB transgenic males, while sterile, mated and successfully fertilized eggs. In cases where nuclei were not well visualized in a single plane of focus, a Z-stack maximum projection was created in ImageJ.

Crosses at testing rate of cidd-cidB-induced lethality were performed by first creating various heterozygous [gal4; UAS-cidA] flies. These were generated by crossing yw; UAS-cidA homozgyous virgin females with male driver strains that are expected to express Gal4 during oogenesis: #4442, nanos-Gal4; #32551, ubiquitin-Gal4; #44241, oskar-Gal4; #7662, MTAe-Gal4 (all transgenes on the second chromosome) #31777, MTD-Gal4, which flask multiple GAL4 inserts on all three large chromosomes including nanos-Gal4, nanos-Gal4VP16 and otu-Gal4. These double heterozygotes were then mated with cidd-cidB males to test fecundity. Fly stocks were obtained from the Bloomington Stock Center or were gifts.

Data availability. All yeast and fly strains and all DNA reagents described here will be made available upon publication. The data that support the findings of this study are available from the corresponding author upon request.

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References

1. Drosophila genetic analysis. An initial cidA-cidB-attP-cidB operon construct was synthesized and cloned into plasmid pUC57 (vector) (Supplementary Fig. 7B). Genes were then subcloned from the mother vector by restriction digestion or PCR amplification and ligated into yeast vectors (Supplementary Table 1). The 2-micron plasmids pYES2 (Araaf) and pRS450 (LEU2) both had the GAL1 promoter and CYC1 terminator and were used for expression of WOBLIC genes in yeast12. Expression from the low-copy CEN vector pRS451GAL1 was also used. For serial dilutions of yeast cells, cultures were grown overnight in non-inducing minimal synthetic media lacking either uracil, leucine or both depending on the plasmid(s) used for expression. Cells were pelleted by centrifugation, washed with sterile water, and spotted in fivefold serial dilutions. Internal OD595 of 0.05 was counted on solid minimal defined media containing either 2% galactose or glucose and lacking either uracil, leucine or both. Plates were placed at 30, 32, 34 and 37°C for 3 days.

Drosophila genetic analysis. An initial cidA-cidB-attP-cidB operon construct was synthesized and cloned into the pUC57 vector (Supplementary Fig. 7B). Genes were then subcloned from the mother construct into the pUASP-attB vector by PCR and restriction digestion. The full-length operon construct pUASP-attB-cidA-cidB was unstable in TOP10F bacteria and prone to degradation. The plasmid was stabilized in CopyCat Epicenter cell lines (Bacterial Cell Line Expression System). All constructs for transgenesis in the pUASP-attP vector were fully sequenced and verified to lack spurious mutations.

DNA constructs were sent to BestGene for microinjection of D. melanogaster embryos. Fly backgrounds #9744 and #9750 (containing different attP insertion sites on the third chromosome) were chosen for site-directed attP/B integration by the 9C3i integrase. Red-eyed flies were selected and screened for BestGene. Following receipt of transgenic lines, we independently verified attP/B integration by PCR using primers 509 and 510 (97944, 0.5 kb product) or 509 and 511 (97950, 0.7 kb product; Supplementary Table 1), which amplified a product only if site-specific recombination had occurred. We also verified that our #9744, #9750 and "CS (WT) strains were uninfected with native Wolbachia bacteria as responders that might affect an assay for sterility on this crossing data. This was done using PCR to amplify the cidd/wol gene. As a positive DNA control, we amplified an ~200 bp product of D. melanogaster rps3. The basal P-element promoter in pUASP-attP induced sufficient expression to induce phenotypes without a Gal4 driver. This was confirmed by RT-PCR analysis carried out using primers P1 and P2 (Supplementary Table 1). The cidd transgene does not influence the female-associated reproductive strategy of the host or the wild-type plasmid's expression.

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