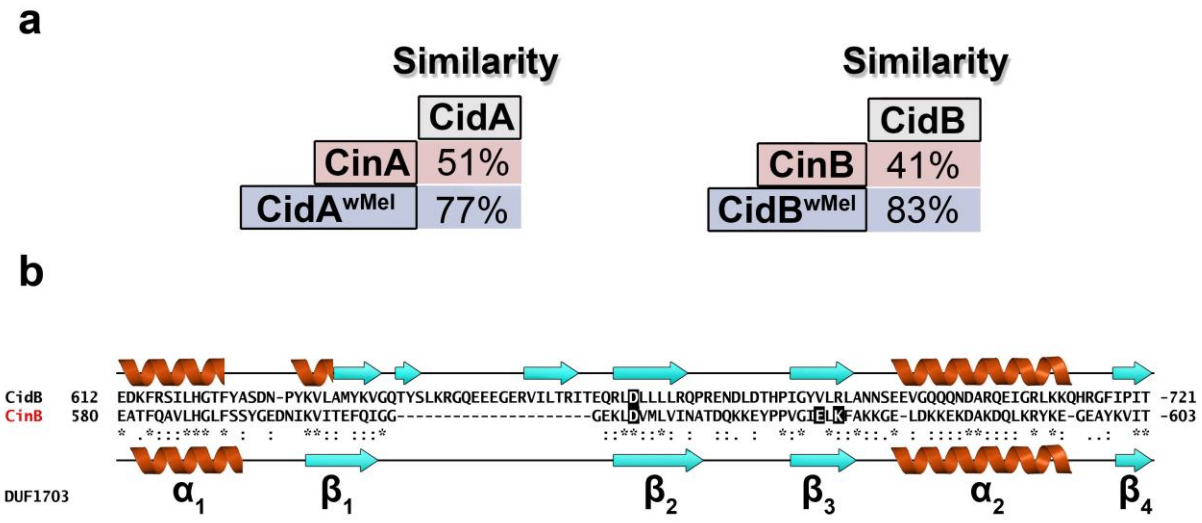
A *Wolbachia* Deubiquitylating Enzyme Induces Cytoplasmic Incompatibility

Supplementary Information

John F. Beckmann[1], Judith A. Ronau[1], and Mark Hochstrasser[1, 2, *]

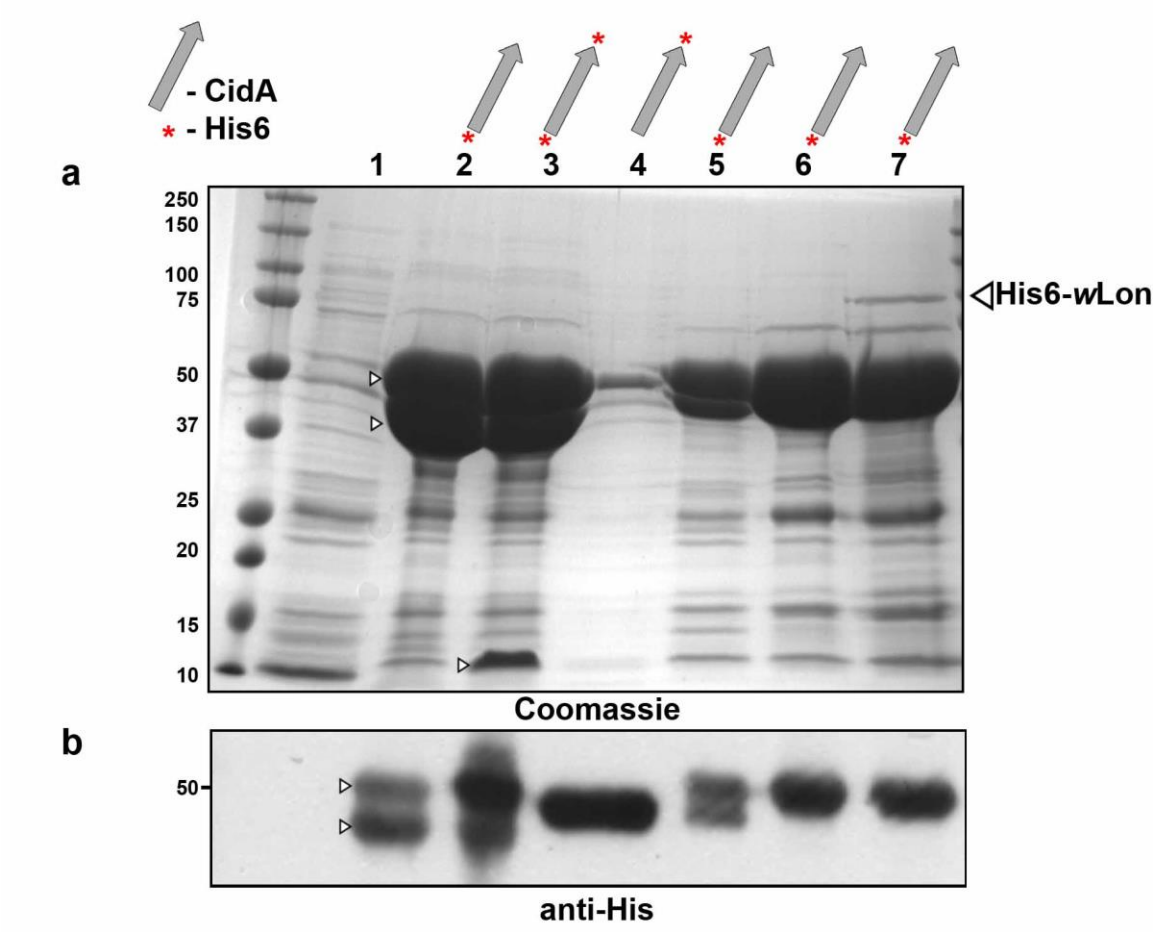
Departments of [1]Molecular Biophysics & Biochemistry and [2]Molecular, Cellular, & Developmental Biology, Yale University, 266 Whitney Avenue, New Haven, CT 06520. USA

*corresponding author (mark.hochstrasser@yale.edu)



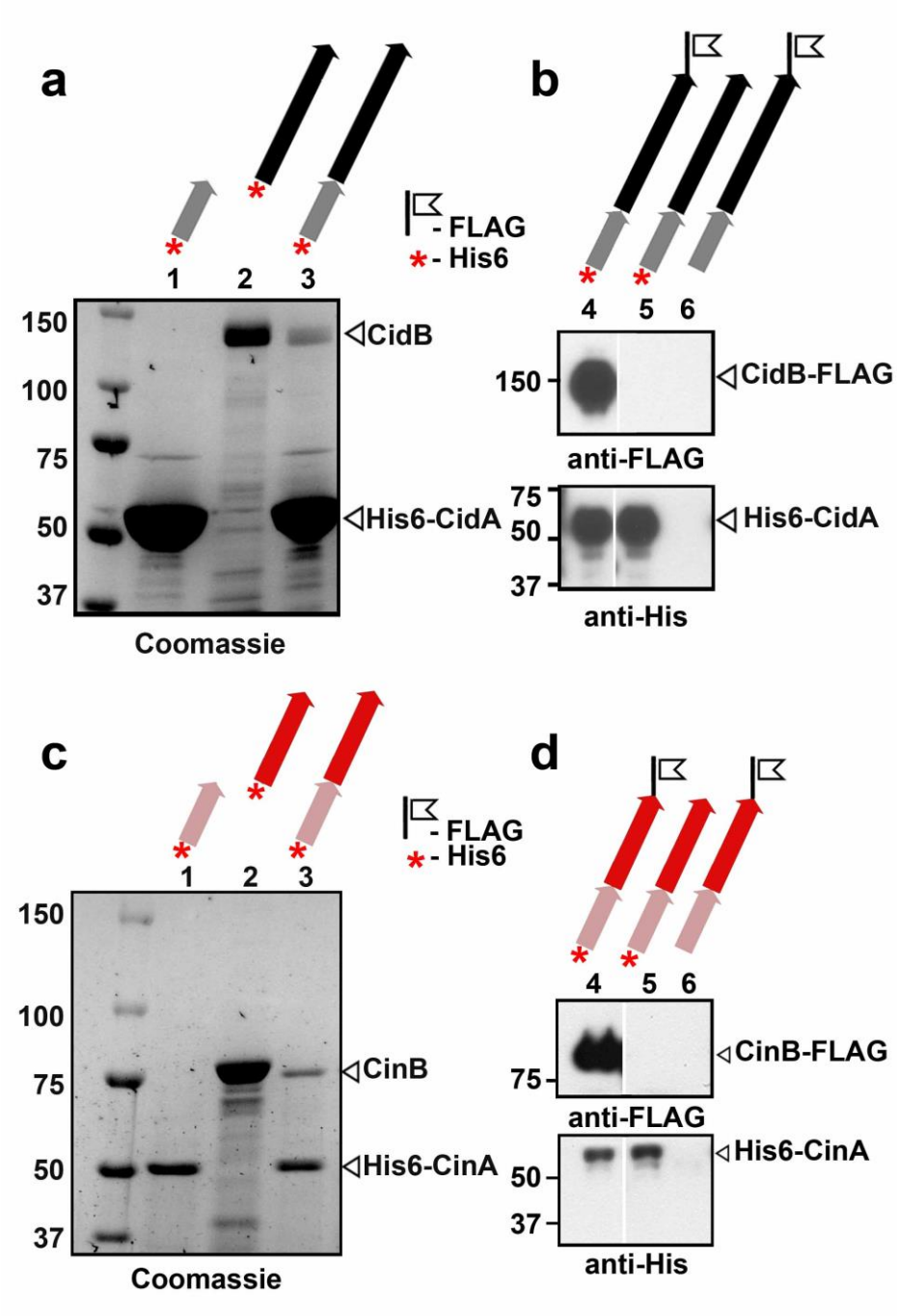
Supplementary Figure 1.

Supplementary Figure 1. Sequence comparison of putative modifier and rescue factors. **a.** Percent similarity using BLAST of the *w*Pip CidA and CidB as queries against related factors. DUB-based operons from *w*Pip and *w*Mel *Wolbachia* strains share higher similarity and are more closely related than they are to the nuclease-type operon from *w*Pip. **b.** Secondary structure predictions by Psipred[30] suggest an underlying conserved structure between CidB and CinB. Both factors have the αββββαβ-fold of the predicted DUF1703 nuclease. However, only CinB maintains a complete catalytic D-E-K triad (black boxes), and in CidB the αββββαβ fold is interrupted by an insertion. In CidB this nuclease-like fold is N-terminal to the additional DUB catalytic domain (**Fig. 1c**, dotted lines). The evidence to date is most consistent with a duplication and divergence from a common CinB-like ancestral operon, although the converse cannot be ruled out, namely, that an ancestral CidB-like operon picked up a nuclease domain and later lost the DUB domain.



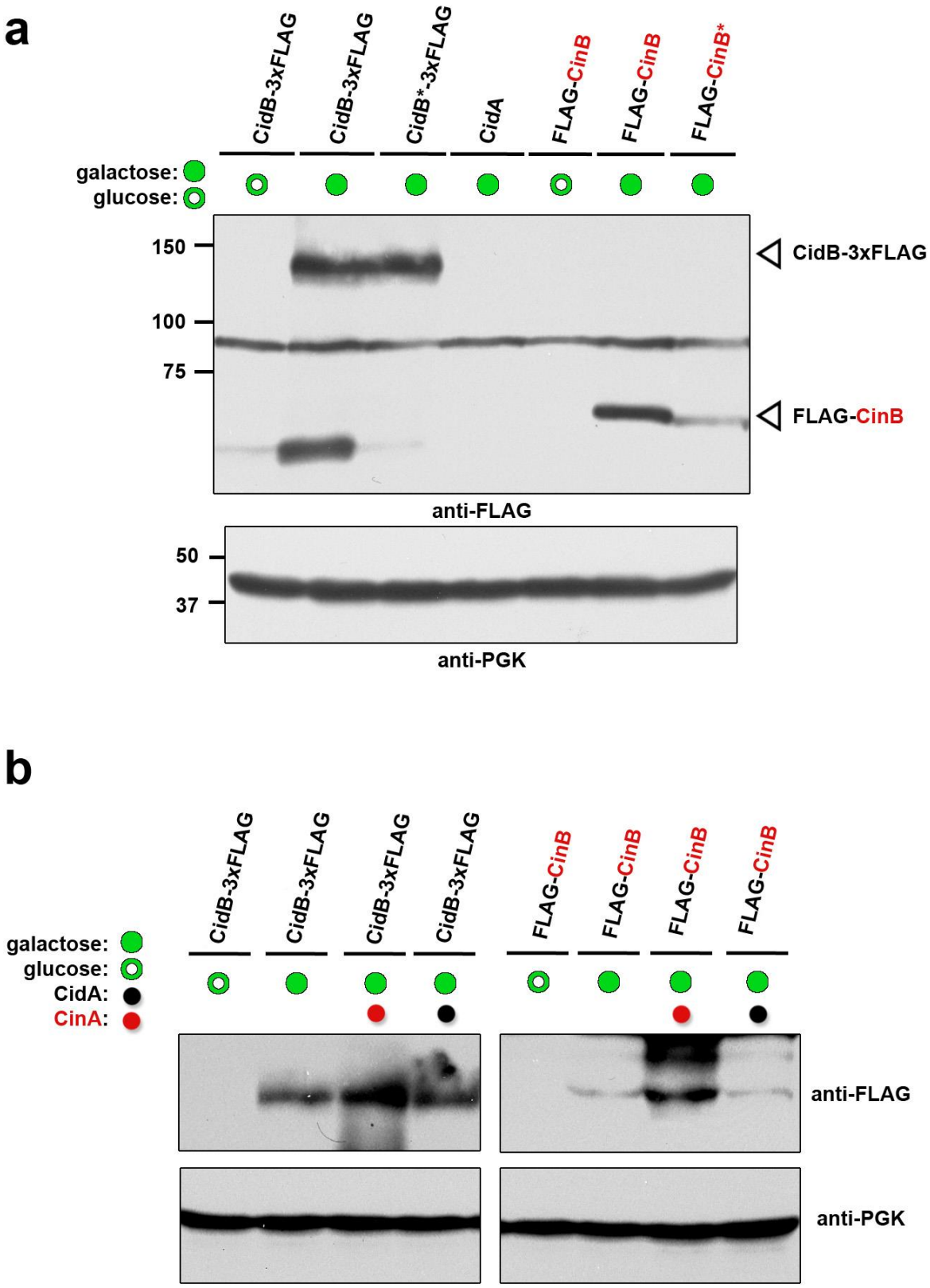
Supplementary Figure 2.

Supplementary Figure 2. His6-mediated pull-downs of recombinant CidA shows C-terminal cleavage by *E. coli* Lon protease (3 replicates) but not *Wolbachia* Lon protease (2 replicates). **a.** Coomassie SDS-PAGE analysis of recombinant CidA protein expression. Lane 1, pBadB vector; 2, N-terminally tagged His6-CidA (#AX1); 3, doubly tagged His6-CidA-His6 (#AW1); 4, C-terminally tagged CidA-His6 (#Y10); 5, a codon-optimized variant of N-terminally tagged His6-CidA (#AS1); 6, N-terminally tagged His6-CidA in BL21-AI cells, which have a deletion of the *lon* protease gene (#N15); and 7, N-terminally tagged His6-CidA coexpressed with His6-tagged *Wolbachia* Lon protease (#BN5). Switching expression of recombinant proteins from TOP10F' cells to BL21-AI cells eliminated the doublet (Lane 6). Because Lon protease often regulates toxin-antidote systems, we tested *Wolbachia*'s own Lon protease, but it did not cleave CidA (lane 7). Subsequent expression of CidA and other proteins was always performed in BL21-AI or Rosetta cells lacking Lon. **b.** Anti-His6 immunoblot corroborates the Coomassie staining patterns.



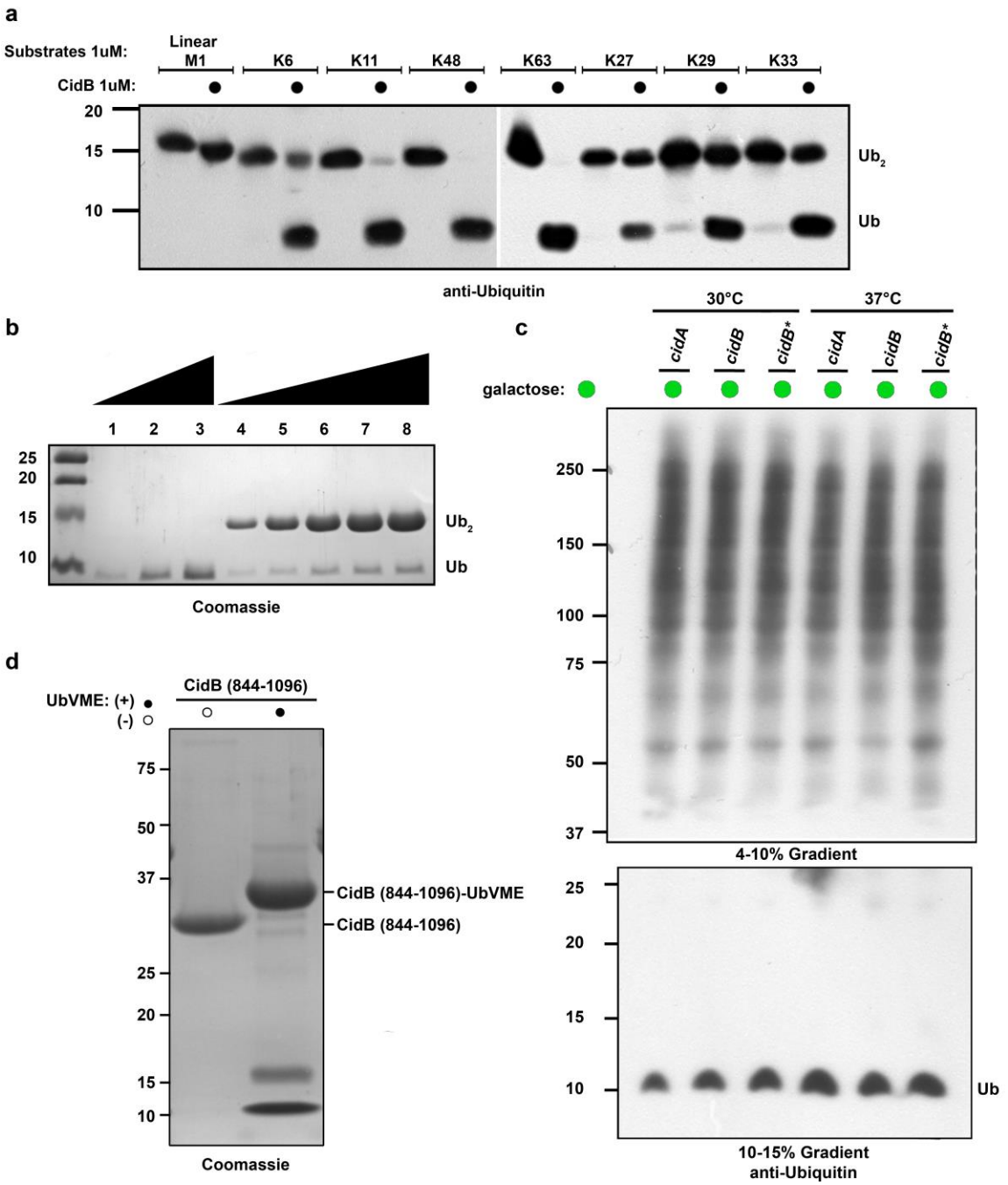
Supplementary Figure 3.

Supplementary Figure 3. Interaction of operon-encoded proteins. **a.** His6-mediated pull-downs reveal binding interactions of cognate operon partners (6 replicates). Lanes 1, 2 and 3 are His6-CidA, His6-CidB, and His6-CidA+CidB (full operon), respectively. **b.** Western blot analysis verifying that the co-pelleted species is CidB (2 replicates). CidB is C-terminally FLAG-tagged in lanes 4 and 6. **c.** His6-mediated pull-downs show interactions of CinA with CinB (3 replicates). Lanes 1, 2 and 3 are His6-CinA, His6-CinB, and His6-CinA+CinB (full operon), respectively. **d.** Western blot analysis verifying that the co-pelleted species is CinB (5 replicates). FLAG tags are analogous to panel **b**.



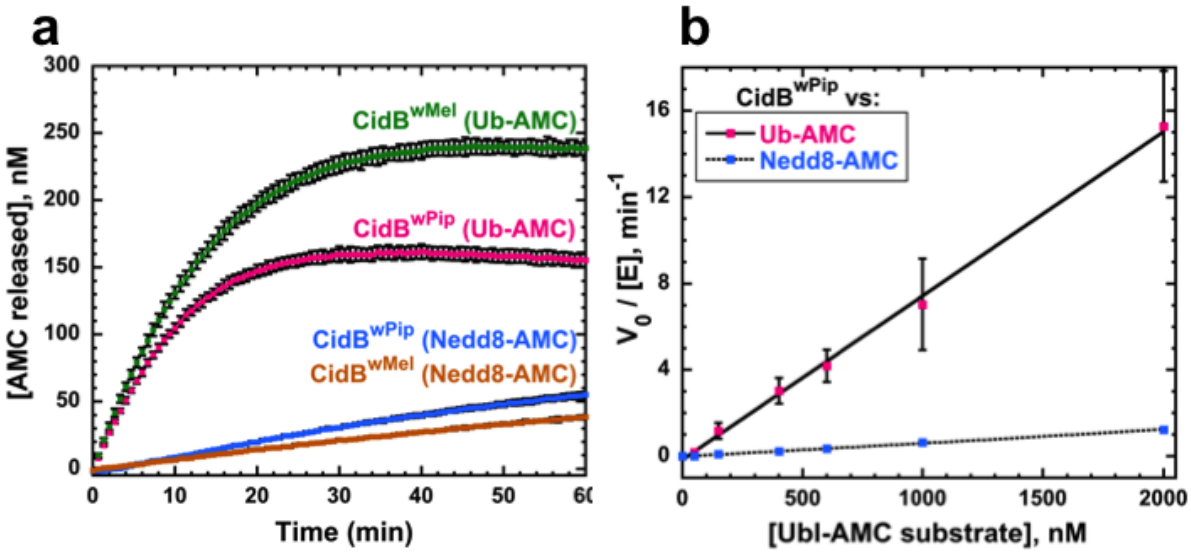
Supplementary Figure 4.

Supplementary Figure 4. Yeast heterologous protein expression controls. **A.** Western immunoblotting of FLAG-tagged CidB and CinB proteins expressed from the yeast 2-micron plasmid pYES2 (*GAL1* promoter; 4 replicates). Closed green circles indicate 2% galactose (inducing conditions) in the growth medium, open green circles, 2% glucose (repressed). CidB and the catalytically inactivated CidB* (C1025A) are expressed at similar levels. The catalytically inactivated mutant (D614A; E634A; K636A), CinB*, does appear to be expressed at lower levels, and this could account at least in part for decreased toxicity. **b.** Western blotting of FLAG-tagged CidB and CinB proteins expressed from the low-copy pRS416 (*GAL1*) plasmid (3 replicates). Genes for the co-expressed putative antidotes were cloned into the high-copy 2-micron pRS425 (*GAL1*) vector. PGK is a loading control.



Supplementary Figure 5.

Supplementary Figure 5. Reactions with ubiquitin (Ub) substrates. **a.** Full length CidB cleaves all forms of lysine-linked (isopeptide-linked) diubiquitin, albeit with variable efficiency, but is inactive on linear Met1-linked diubiquitin (3 replicates). Digests of diubiquitin were performed overnight at 37°C with enzyme and substrate both at 1 μM concentration. Similar results were observed with shorter digests of 1 or 4 h. **b.** Representative gel from a single kinetic assay of diubiquitin cleavage. Three replicates for each diubiquitin substrate were used to generate the plot shown in **Figure 3.** Lanes 1-3 are ubiquitin standards of 6, 20, and 40 μM, respectively. In lanes 4-8, 400 nM CidB (762-1143) was incubated with Lys48-linked diubiquitin ranging in concentration from 20 μM (lane 4) to 120 μM (lane 8). All Lys48-linked diubiquitin reactions were carried out at room temperature for 15 min. The amount of ubiquitin produced from each reaction was quantified by densitometry using ImageJ software. **c.** Total cellular ubiquitylation as measured by anti-ubiquitin immunoblotting in yeast extracts (1 experiment). Induction of *GAL1*-driven CidB expression did not change the pattern of ubiquitin conjugates when compared to cells with induced CidA or CidB* (negative controls). These results suggest CidB activity is likely limited to a small number of cellular substrates rather than affecting gross protein ubiquitylation. The same sample was run on two separate gradient gels of 4-10% and 10-15% gels to create maximal separation of high and low molecular weight ubiquitylated species. Induction temperatures of 30°C and 37°C in the presence of galactose for 4 h were utilized; toxicity is most apparent at 37°C. **d.** Reaction of a truncated construct of CidB (844-1096) with UbVME leads to an observable mobility shift when compared to unreacted CidB. The reaction was carried out for 4 h at room temperature (1 experiment).



c

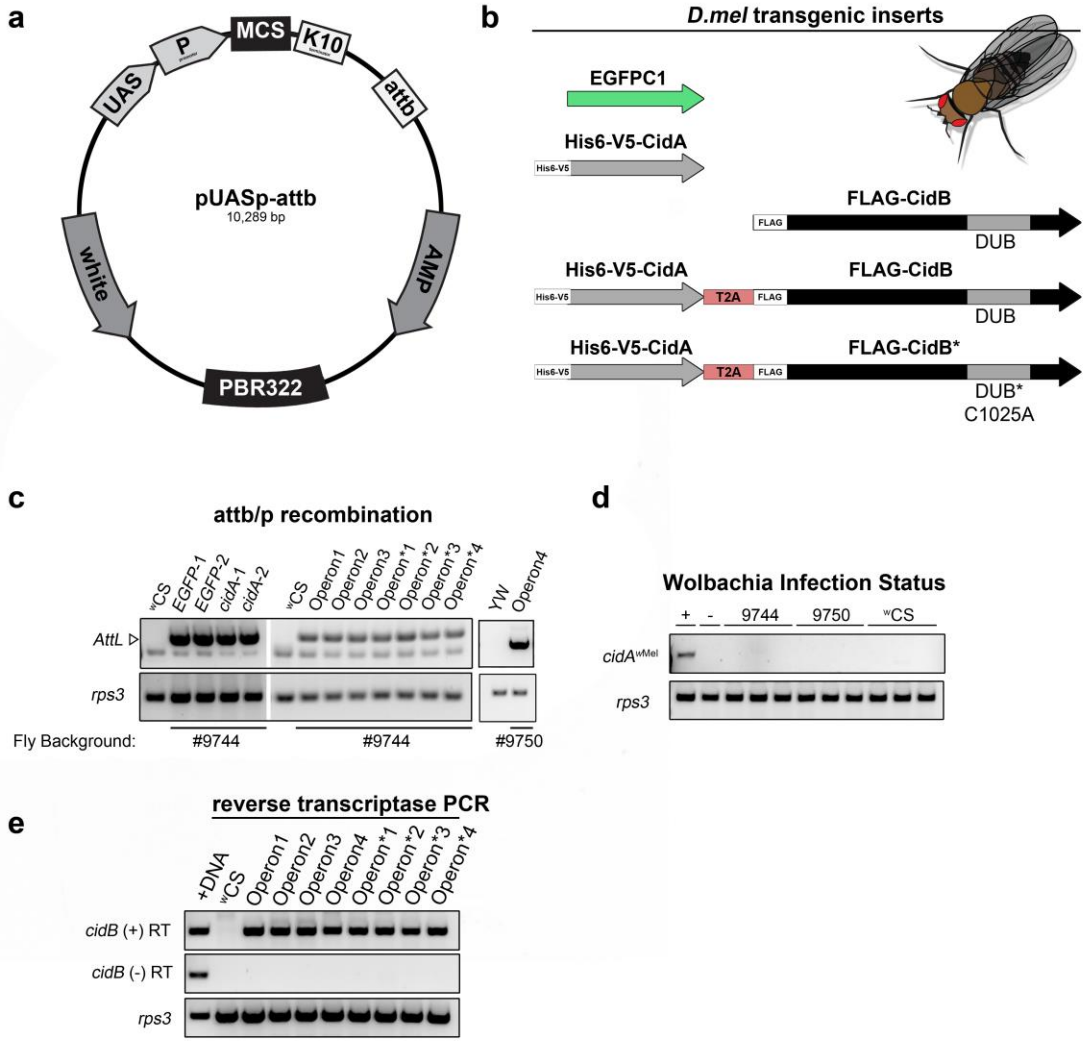
Substrate	k_{cat}/K_M ($\mu M^{-1}min^{-1}$)
Ub-AMC	7.59
Nedd8-AMC	0.624

d

DiUb Type	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M ($\mu M^{-1}s^{-1}$) x 10^{-3}
Lys63	55.6 ± 20.1	0.080 ± 0.01	1.44
Lys48	99.9 ± 28.1	0.035 ± 0.005	0.35

Supplementary Figure 6.

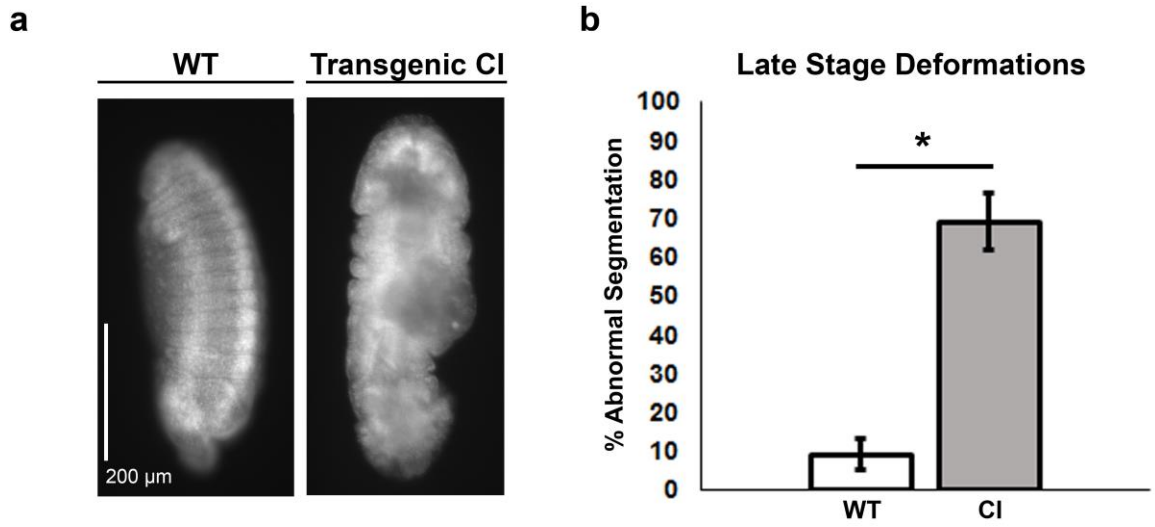
Supplementary Figure 6. Cleavage of Ub-AMC and Nedd8-AMC by *w*Pip and *w*Mel CidB enzymes. **a.** Progress curves of AMC release from Ub-AMC and Nedd8-AMC catalyzed by CidB^{wPip} and CidB^{wMel} are shown. Enzyme (5 nM) was mixed with 400 nM substrate, and the reactions proceeded at 30°C; the enzymes share a similar preference for ubiquitin over the UBL Nedd8. The activity of CidB^{wMel} is comparable to that of CidB^{wPip}. **b**. CidB^{wPip} catalytic efficiency for hydrolyzing Ub-AMC is 11-fold greater than for Nedd8-AMC. In Fig. 6a and Fig. 6b, error bars are standard deviations from three independent experiments. **c.** As the kinetics from **Fig. S6b** exhibited a linear response over the substrate concentration range tested, which is typical of other DUBs toward the Ub-AMC substrate, k_{cat}/K_M values were determined by fitting the data to the equation: $v/[E] = k_{cat}/K_M[S]$. **d.** Steady-state kinetic parameters for CidB^{wPip} cleavage of Lys63- and Lys48-linked ubiquitin dimers indicate a modest preference for Lys63-linked diubiquitin. This suggests that the physiological targets of CidB^{wPip} might bear Lys63-polyubiquitin linkages and are less likely to be targets of Lys48-polyubiquitin-based proteasomal degradation.



Supplementary Figure 7.

Supplementary Figure 7. Creation of transgenic *D. melanogaster* strains. **a.** pUASp-attB vector.[36,37] UAS is the *GAL4* upstream activating sequence; P is the P-element basal germline promoter; and MCS is the multi-cloning site. K10 has 3'UTR sequences from the K10 terminator, and attB is the ΦC31 integrase recombination site. **b.** Five transgene injection constructs were created by heterologous gene insertion into pUASp-attB: four *cidA*-*cidB*-derived constructs and an EGFPC1 negative control. T2A is a viral peptide sequence that causes translation of two separate polypeptides from the fused ORFs by ribosome skipping, mimicking the bicistronic bacterial operon; no efficient IRES system has been described for *D. melanogaster*. No transgenic lines expressing CidB^{wPip} alone could be established after 3 trials totaling 600 embryo microinjections, whereas all other constructs readily recombined into the *Drosophila* chromosome-3 attP site. **c.** Transgenic fly lines were created and screened for proper attB/attP recombination by PCR in triplicate. "AttL" is a PCR product indicating correct recombination; *rps3* is a PCR positive control. The ^{w}CS and YW lanes are negative controls using genomic DNA from these two untransformed fly strains. We created multiple transgenic fly lines for each construct. A total of four sterile "Operon" (*cidA*-*cidB*) lines were created in two different fly backgrounds bearing independent attP insertion sites (#9744 and #9750). Four independent lines with the catalytically inactive DUB (Operon*) were isolated in the #9744 background. All replicate lines showed the same phenotypes. **d.** Verification that lines used in transgenic crosses (#9744, 9750, and ^{w}CS) were uninfected with native *Wolbachia* strains (triplicate). CidA^{wMel} (WD_0631) is from the *w*Mel *Wolbachia* strain. **e.** Reverse transcriptase-PCR analysis confirming transcription of the transgenic operons from the basal P-element promoter despite the absence of a Gal4 driver (triplicate). DNA is a positive PCR control

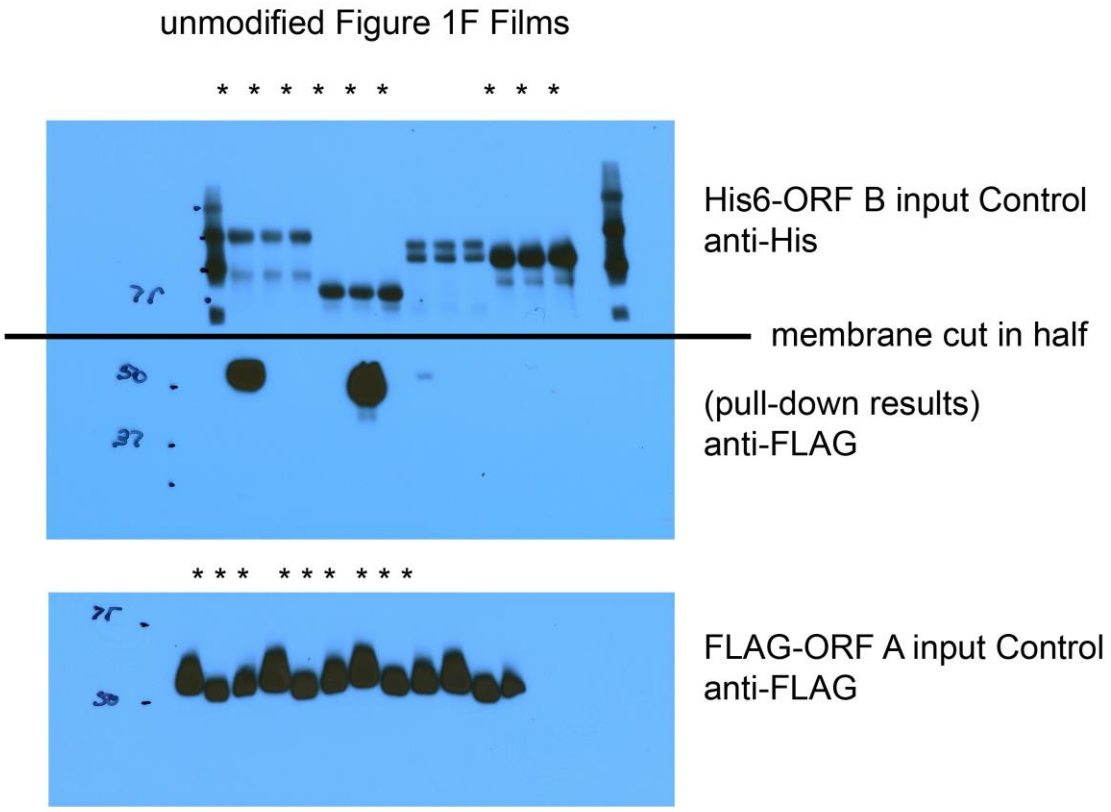
to show correct band size. RNA samples from pooled adult males were assayed with reverse transcriptase "(+) RT"-PCR to verify the presence of transcript; omission of reverse transcriptase, "(-) RT," served as a negative control for DNA contamination. The cDNAs were amplified with primers specific for *cidB*^{wPip}. Analysis of the fly *rps3* transcript was a positive control for RNA quality. The Operon and Operon* (*cidA-cidB w*Pip) fly lines express active CidB and catalytically inactive CidB* (C1025A), respectively.

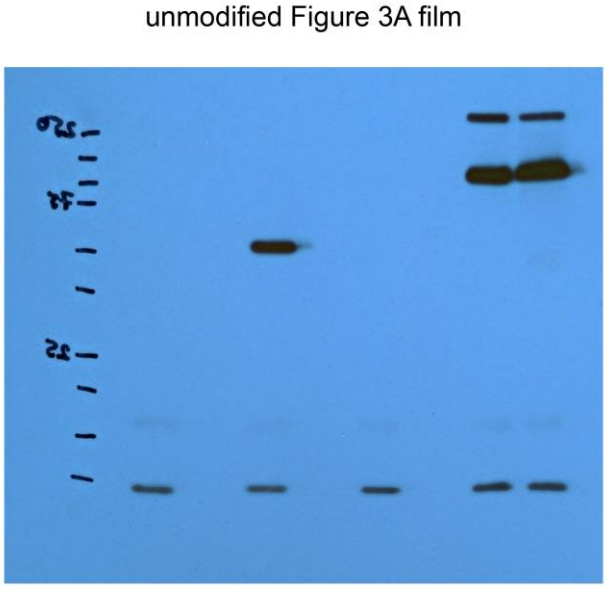


Supplementary Figure 8.

Supplementary Figure 8. Additional analysis of transgenic *cidA-cidB* embryo cytology. **a.** Examples of late-stage embryos; transgenic embryos that develop to the late stage show significant deformations of segmentation patterns including pinching, gaps in segmentation, and asymmetry. This was observed in 148 CI embryos. **b.** Of the 20% of transgenic CI embryos that develop to the late stage, 69% showed deformations and abnormal segmentation. Error bars are standard deviation.

Supplementary Figure 9.





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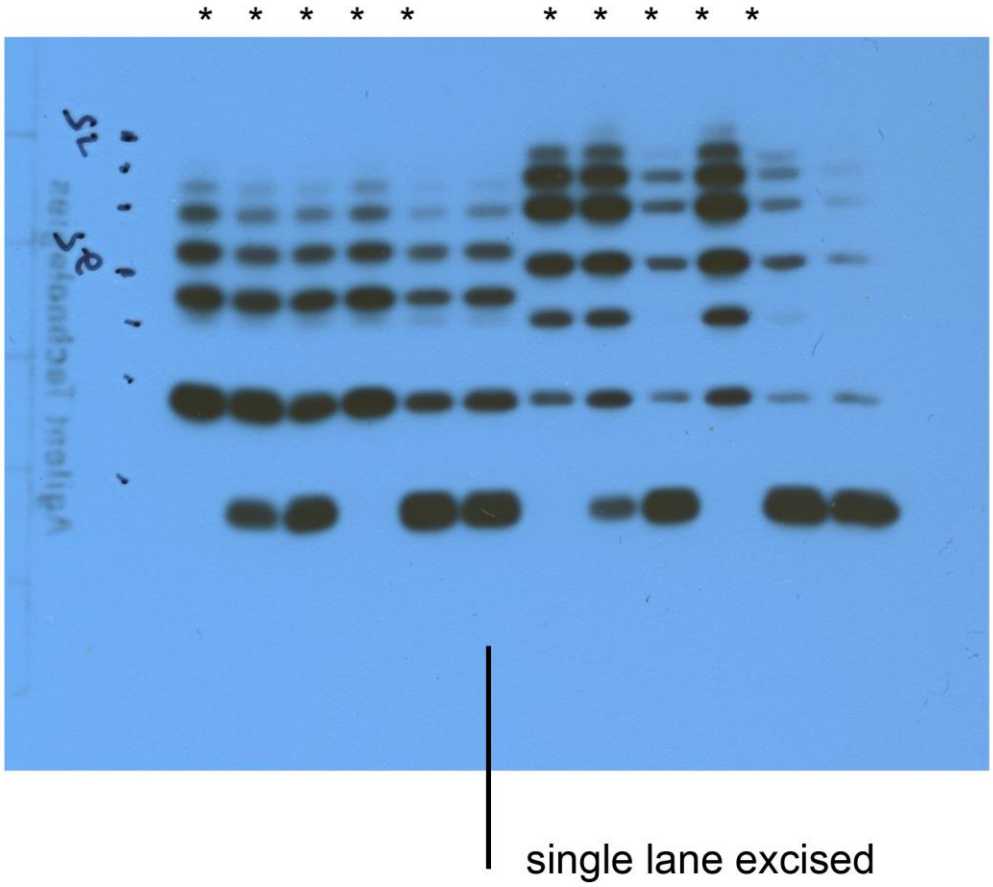

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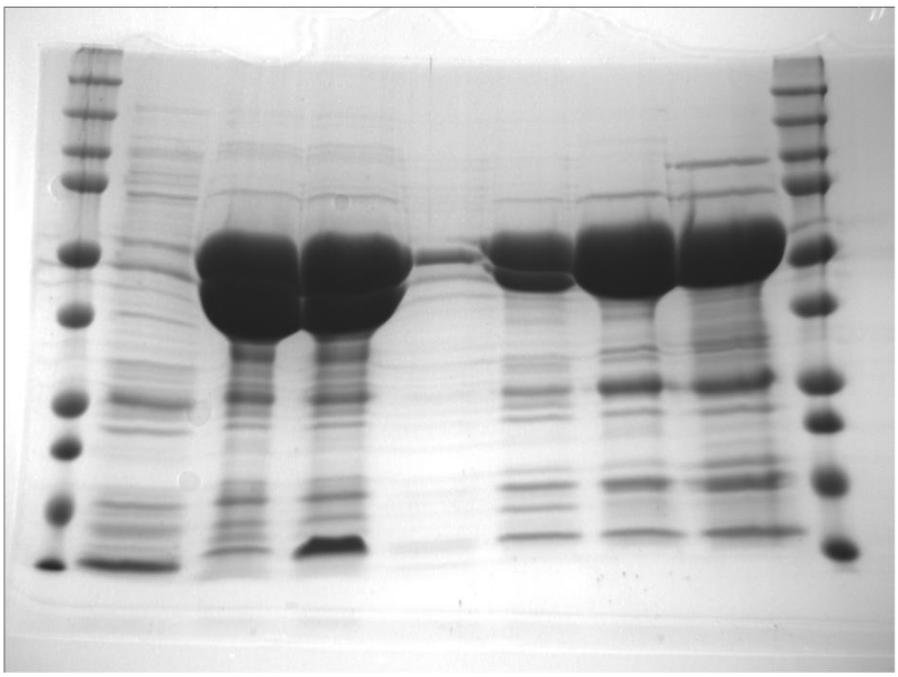

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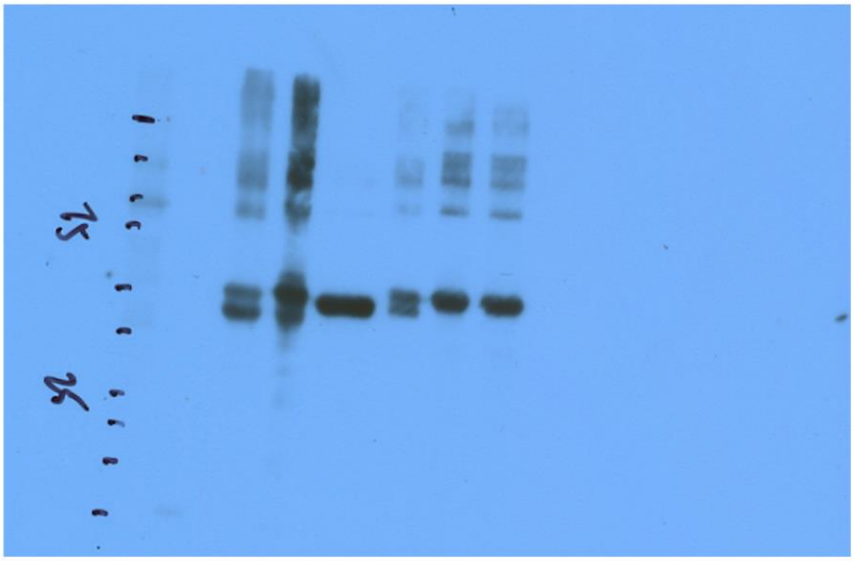

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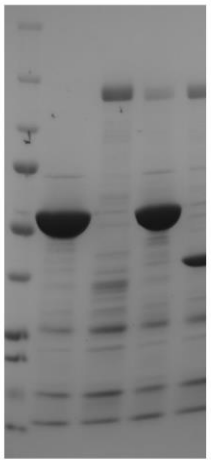

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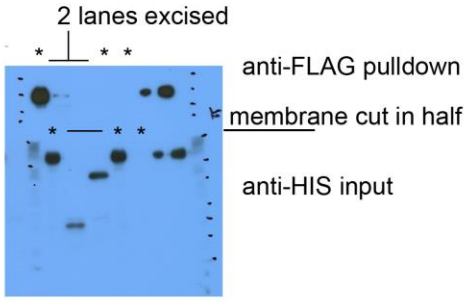

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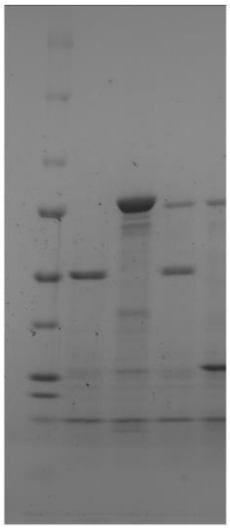

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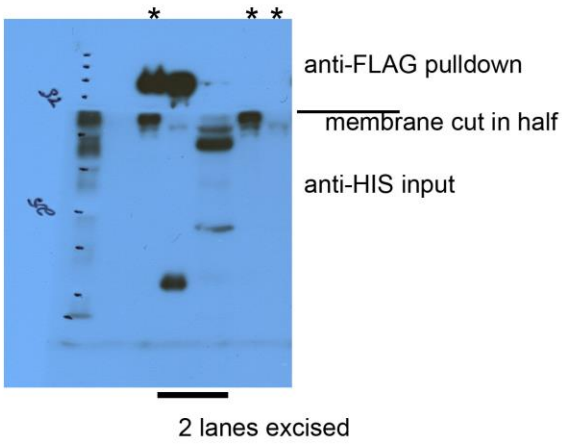

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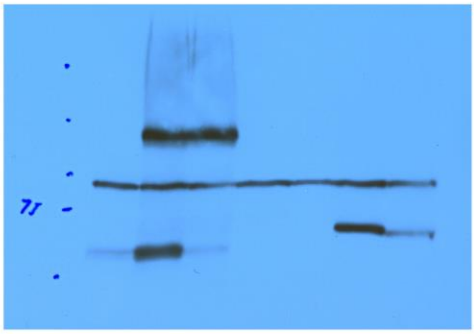

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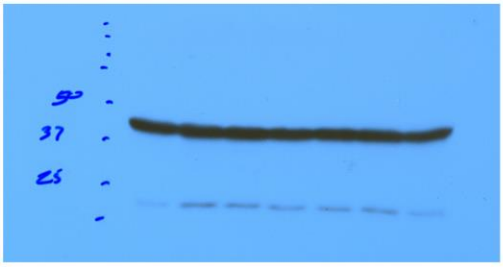

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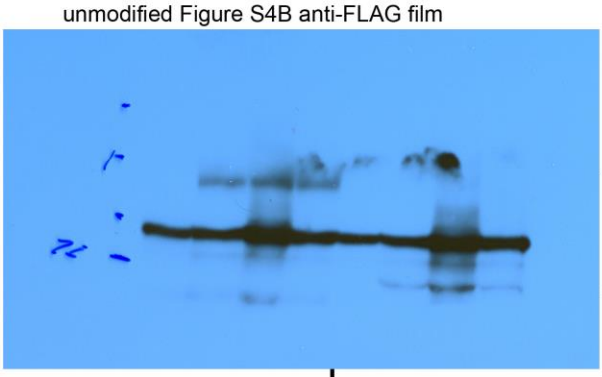

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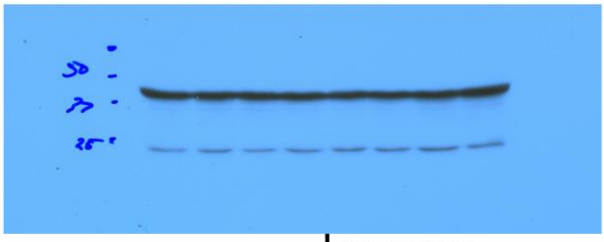

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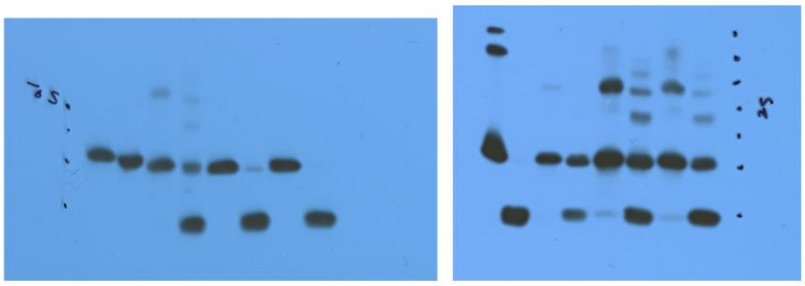

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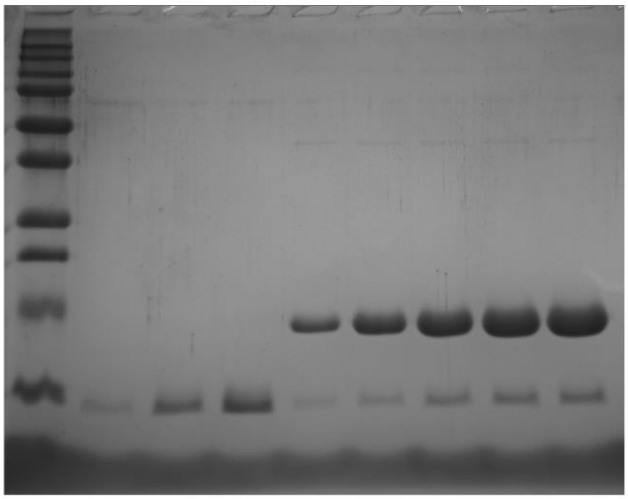

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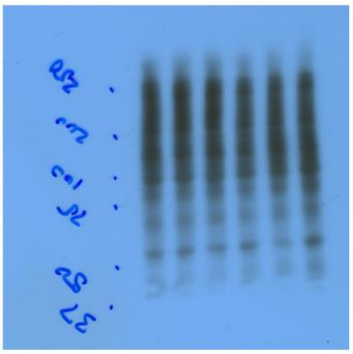

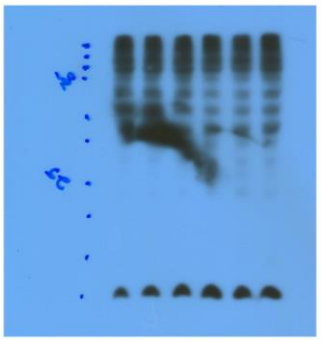

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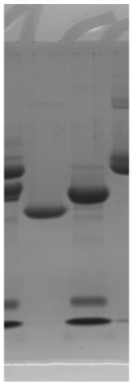

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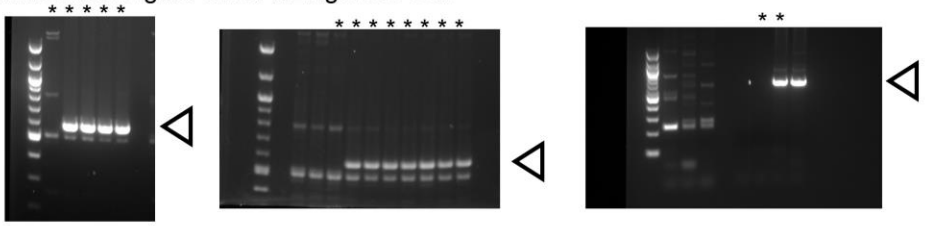

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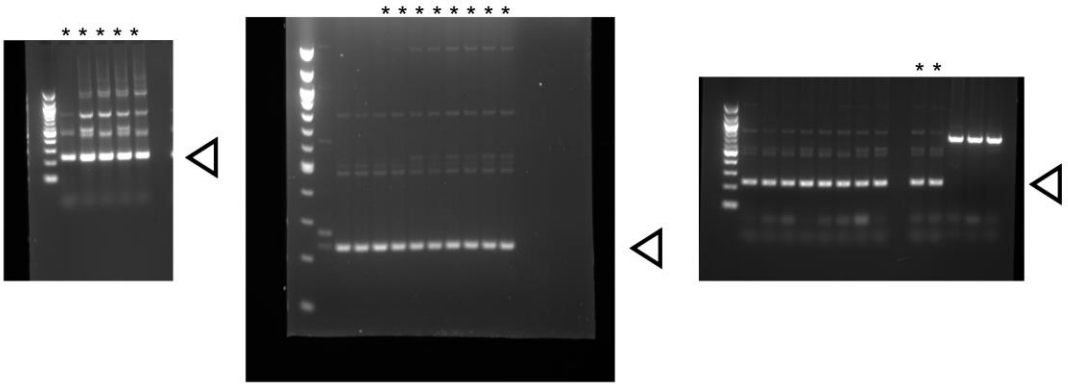

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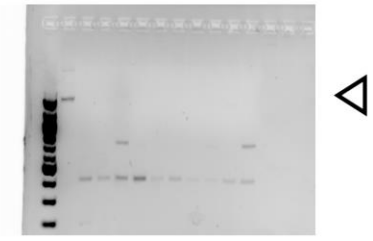

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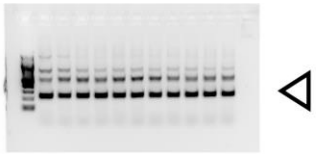

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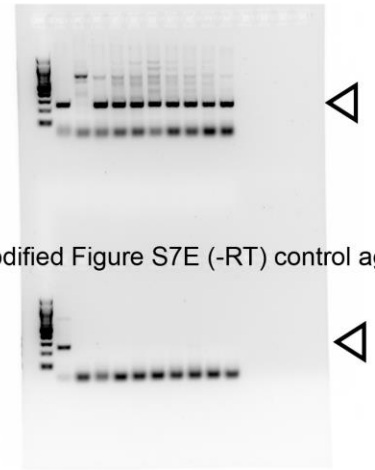

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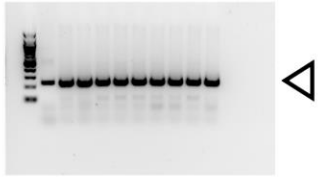

Supplementary Discussion:

Wolbachia Genomics Supports a Role for the cidA-cidB Operon in CI

The *lock-and-key* model, originally proposed as the *toxin-antidote* model by Hurst 1991,[1] has gained traction as the model that best describes the phenomenology of CI in insects.[2] According to the lock-and-key model, i) lock and key functions are genetically distinct (**Fig. 1c-e**); ii) independent sets of locks and keys exist;[3] iii) pairs of locks and keys interact in a specific or preferential manner (**Figs. 1f, 2b**); and iv) locks and keys are co-evolving/diverging from a common ancestor (**Supplementary Figure 1**).[3]

Although the proposed rescue function for CidA has not been demonstrated in an insect host, genomic evidence supports our biochemical and genetic specification of the lock and key components. For instance, *Wolbachia* that infect *Drosophila simulans* show different CI phenotypes. Three CI-inducing strains exhibit mutual bi-directional incompatibilities (mismatched locks and keys) – *w*Ha, *w*No, and *w*Ri;[4] each strain has a unique lock/toxin variant: one with a Ulp1-like cysteine protease domain (WHA_RS01430), one with the DUF1703/PD-(D/E)XK putative nuclease domain (WNO_01980), and one that is unannotated but evolutionarily diverged PD-(D/E)XK-related motif (WRI_RS03365), respectively. Co-divergence of such lock domains and their corresponding keys can rationalize these incompatibilities. A fourth strain, *w*Au, which is unable to induce or rescue CI, lacks the operon altogether.[5] Finally, all sequenced genomes from so-called A and B supergroups that induce CI have orthologs of the putative *w*Pip CI operons, and strains of *Wolbachia* not observed to induce CI (*w*Au, *w*Oo, and *w*Bm) lack an orthologous

operon.[3] Therefore, all the assembled genomes of *Wolbachia* show a strong correlation between their CI phenotypes and *cid*/*cin* operon structures.

CidB sequences beyond the DUB domain are likely necessary for CI. BLAST analysis of the Ulp1-like CidB domain shows that small truncated orthologs of the enzymatic Ulp1-like domain are present in non-inducing CI strains as well as *Rickettsia* relatives. We make a distinction between these truncated versions and full-length genes. The truncated versions are exemplified by the paralogous *w*Pa_1291 of *w*Pip, which encodes just the Ulp1-like domain and lacks potentially important N-terminal residues, an operon structure, or an associated cognate partner gene. Notably, when we tested *w*Pa_1291 (which encodes residues equivalent to 894-1177 of CidB), we found that it would not induce toxicity in yeast. This suggests that N-terminal residues and possibly even the hypothetical rescue factor (CidA) are important for toxin functionality and CI induction.

In Beckmann and Fallon (2013), a reciprocal toxin-antidote hypothesis was originally postulated in which CidA acted as toxin and CidB as antidote. This was because we had detected CidA in mature mosquito sperm purified from spermathecae.[3] Our analyses in yeast and *Drosophila* (**Figs. 2 and 4**) now suggest the opposite, namely, that CidB acts as toxin and CidA more likely as antidote. Although CidB had not yet been detected within sperm, this does not invalidate the revised model. Because CidA binds to CidB, it is possible that CidA might even play a positive role in localizing CidB within the mature sperm or in the zygote. We were unable to generate a transgenic line expressing just the CidB enzyme. In contrast, all other constructs including the full operon, could be readily

inserted into the fly genome. We suspect that CidB, when expressed by itself, is lethal to the injected flies. The CidA protein might mitigate toxicity of the CidB DUB. Further investigation of molecular interactions and localization of the two proteins will be needed to test these ideas.

Failure to Rescue Embryonic Lethality with cidA or cidA-cidB in Female Flies

In yeast, CidA coexpression with CidB suppressed the toxicity caused by the latter enzyme. In flies, however, we were not able to recapitulate the "molecular rescue" of *cidB*-derived toxicity. Although this might mean that CidA is not in fact a CI rescue factor, there are a number of reasons to suspect that these negative results are due to experimental complexities in achieving proper transgenic gene expression in fly embryos. Expression of transgenes before blastoderm formation is notoriously difficult[6] because the early embryo relies entirely upon maternal factors deposited by the nurse cells.[7] Rescue must occur prior to the first zygotic division. If CidA were targeted for rapid degradation, it might be difficult to achieve levels sufficient to rescue CI. In contrast to a chromosomal transgene, *Wolbachia* themselves are not subject to the transcriptional/translational shutdown of early embryos. An embryo infected by many *Wolbachia* bacteria may receive large quantities of secreted CidA, something that may be difficult to replicate by transgenesis. Indeed, a previous proteomic dataset suggested that the CidA protein is one of the most abundant *Wolbachia* proteins in ovarian tissues, on par with the highly abundant *Wolbachia* surface protein WSP.[8] Conversely, it is also possible that too much active CidB is being introduced by the transgene to be countered by CidA in the egg. Other technical issues might arise from interference of the protein tag, incorrect protein folding, or limiting concentrations of

CidA in the regions of the embryo where CidB activity is most relevant (most likely the male pronucleus).

In our view, the ability of CidA and CinA to specifically rescue toxicity in yeast caused by CidB and CinB, respectively, suggests that both components of CI (modification and rescue) are built into these two-gene operons. However, this conclusion requires either a "molecular rescue" of toxicity in an insect or a complementation analysis of transgenic insects crossed to insects infected with *Wolbachia* bearing alterations of these operons. On the other hand, the induction of a fully penetrant and highly specific CI phenocopy in transgenic flies indicates that the *cidA*-*cidB* operon encodes a critical "modification" component of CI.

Bacterial DUBs are Secretion System Effectors That Modulate Host Ubiquitin Systems

Prokaryotic ubiquitin-like protein (UBL) proteases (ULPs) and DUBs are frequently encoded by pathogenic gram-negative and obligate intracellular bacteria.[9] This is intriguing because prokaryotes do not have their own full ubiquitin-proteasome system.[10] All identified bacterial DUBs specifically tested for secretion have been shown to be secreted as effector proteins. Type III secretion system (T3SS) substrates include ChlaOTU (*Chlamydia*), a DUB which is thought to interact with intrinsic cellular immunity/autophagy systems regulated by ubiquitin;[11] XopD (*Xanthomonas*) a SUMO protease which affects modification of important plant transcription factors by the UBL SUMO;[12-14] and SseL (*Salmonella*), a DUB that was shown to be a virulence factor important for regulation of cytotoxicity in macrophages.[15] A Type IV secretion system

(T4SS) substrate is SdeA (*Legionella*), a DUB which is essential for virulence in protozoan hosts.[16] No reports describe an intrabacterial function for any prokaryotic ULP or DUB. Because the CidA protein was detected in spermathecal tissues lacking endogenous *Wolbachia* infections, secretion of at least this protein is suggested.[3] Interestingly, the *cidA-cidB* operon was shown to be incorporated into WO prophage genomes,[17] making the translated proteins' escape from bacterial cells by phage-induced cell lysis, or incorporation into transmissible viral particles, another possibility. Overall, these data strongly suggest that the CidA and CidB proteins are secreted or released from intracellular *Wolbachia*, although this remains to be explicitly tested.

The CidB enzyme showed no activity toward mammalian SUMO1-AMC or SUMO2-AMC substrates or toward yeast SUMO (Smt3) fusions. Because XopD from *Xanthomonas* specifically targeted plant SUMO isoforms and would not cleave SUMO from other species,[18] we thought it possible that the $CidB^{wPip}$ enzyme might specifically cleave *Culex* mosquito SUMO and not other isoforms. We cloned the mosquito SUMO as a fusion substrate with ubiquitin and tested this protein for cleavage by CidB; it did not cleave. CidB also was inactive toward ISG15-AMC, but it showed weak activity toward Nedd8-AMC (**Supplementary Figure 6**). These data imply that CidB is a highly specialized protease that specifically cleaves ubiquitin conjugates. Identification of its *in vivo* substrates will be needed to understand its precise function in CI.

We investigated the ability of CidB to cleave all seven possible ubiquitin-C-terminus-lysine linkages in ubiquitin dimers as well as the linear Met1-ubiquitin linkage because

ubiquitin chains of different linkages are associated with distinct cellular functions.[19] CidB displayed activity towards all of the lysine-linked diubiquitins but was unable to cleave linear diubiquitin in 1 h or overnight at 37°C. Other DUBs, mainly from the USP family, such as USP7 and USP28, are similarly active against multiple chain linkages but not linear diubiquitin.[20] Of all the possible linkages explored in our diubiquitin panel digest (**Supplementary Figure 5a**), CidB appeared to have the highest activity toward Lys48 and Lys63. The Lys48 polyubiquitin linkage often signals for substrate degradation by the proteasome, whereas Lys63 linkages are typically involved in certain DNA repair pathways and endocytosis.[9] The preference of CidB for Lys63 ubiquitin dimers over Lys48 dimers is relatively modest (~4-fold), so we cannot conclude which chain types might be most relevant to CI induction, although Lys63 chain-modified (or monoubiquitylated) substrates appear most likely.

Possible Molecular Mechanisms of CI

Following our original proposal that the *cidA-cidB* operon might be the executer of CI (Beckmann and Fallon 2013), we propose here that CidB (along with CidA) is an effector protein secreted by *Wolbachia* through its Type IV secretion system into sperm precursors during spermatogenesis. The proteins might not be secreted in precise 1:1 stoichiometry, and given the original detection of CidA, but not that of CidB, in fertilized female spermathecae, we suspect CidA might be in greater abundance. A unique event in early fertilization (or late spermatogenesis), such as protamine deposition/replacement, might stimulate the targeted degradation or inactivation of the CidA antidote, activating the CidB toxin. We suspect that in the zygote, CidB is toxic to the developing embryo unless

neutralized by CidA, which must be replenished by secretion or release from a maternal *Wolbachia* germline infection. CidA is proposed to be more rapidly degraded than CidB, as is typical of bacterial toxin-antidote systems.[21] Therefore, toxin inhibition would only occur while present in cytoplasm with concentrated CidA secreted from a maternal infection.

Because the toxin is an enzyme, only a small amount may be sufficient to induce incompatibility. The full-length epitope-tagged CidB was very difficult to detect during heterologous expression in yeast under conditions where it was either minimally or highly toxic. Moreover, Gal4/UAS driver induction of the *cidA-cidB* transgene was unnecessary to induce CI in flies, and fully sterile crosses were observed with male individuals heterozygous for the transgenic insert. Because developing sperm are connected by a shared cytoplasm,[22] the CidB toxin presumably distributes with sufficient abundance in all the haploid sperm to induce complete sterility. A similar phenomenon characterizes native *Wolbachia* infections insofar as CI induction occurs without bacterial residence uniformly in all spermatocytes.[22]

With respect to rescue of CI, it might be more complex than the presence of CidA binding alone. Our biochemical data suggest that the CidA rescue effect (observed in yeast) is not achieved by catalytic inhibition of the DUB enzyme but by another mechanism. Additionally, CidA might act with other factors to counteract the DUB in the egg. An antidote from a mosquito-derived *Wolbachia* strain may also not be adapted to function as effectively in fruit flies. Other hypotheses are that rescue is achieved by altering the

localization of the toxin or by blocking activity toward specific substrates. Modulating localization of the toxin might better explain how structurally similar antidotes can rescue toxins with distinct enzymatic "warheads." For example, if both CidB and CinB toxins are toxic due to activities in the nucleus, then antidotes might mediate their export from the nucleus.

It has been speculated that CI targets a core conserved biochemical machinery involved in mitosis because delays in chromosome condensation and bridging are, without exception, observed in a wide range of insects, including mosquitoes (*Culex* and *Aedes*), fruitflies (*Drosophila*), and wasps (*Nasonia*).[22,23] Furthermore, artificial injection of heterologous *Wolbachia* strains into diverse hosts still results in induction of CI (*w*AlbB into *Anopheles stephensi*:[24] *w*Ri into *Drosophila melanogaster*;[25] *w*Mel into *Aedes aegypti*).[26] Our data with heterologous expression of the *Wolbachia cid* and *cin* genes in yeast fully support this idea of broad host range. Similarly, we could induce robust transgenic CI in *Drosophila* flies with an operon from a *Wolbachia* strain that normally infects *Culex* mosquitoes. This CI-like effect over a broad host range also means that the transgenic operon might be utilized in many different insect pests or disease vectors to limit their populations.

Finally, the *cidA-cidB* operon is unlikely to be the only means of inducing CI. In fact, we hypothesize that there are redundant paralogous operons simultaneously capable of inducing CI, such as in *w*Pip. In the case of *w*No, for example, which lacks a functional *cidA-cidB* operon, we hypothesize that it induces CI by virtue of the orthologous DUF1703 nuclease-type operon. The DUF1703 domain has previously been implicated in insect

sterility.[27] We suggest that both paralogous operons in *w*Pip induce CI simultaneously, creating multi-directional incompatibility dynamics. Notably, we detected peptides from both operon systems in an ovarian proteome.[8]

Interestingly, a divergent version of the apparent CI toxin from *Rickettsia gravesii* has both a DUF1703 nuclease and a DUB domain (WP_024547315.1). This ortholog may be an evolutionary "missing link" between the paralogous forms diverging in *Wolbachia*. It will be interesting to determine if the *cid* and *cin* operons are also capable of inducing the other reproductive phenotypes associated with *Wolbachia*, such as parthenogenesis, male killing, or feminization. These different phenomena might reflect differences in enzyme targets or the same substrates being altered in hosts of distinct genetic composition (such as haploid versus diploid). Furthermore, the only other known CI-inducing bacterium, the phylogenetically distant *Cardinium hertigii*, was shown to possess a USP-type DUB in its genome, making it a possibility that *Cardinium* uses this effector to induce CI by a related pathway.[28,29]

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