Abstract

A Wolbachia Nuclease and Its Binding Partner Provide a Novel Mechanism for Cytoplasmic Incompatibility

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Wolbachia are maternally inherited obligate bacterial endosymbionts that infect nearly half of all arthropod species, a success largely due to their ability to selfishly manipulate host reproduction to favor infected females. Cytoplasmic incompatibility (CI), a phenomenon where Wolbachia infection renders male insects sterile when they mate with uninfected females, is the most common type of these manipulations. Since matings between infected females and infected (the “Rescue” cross) or uninfected males are both fully compatible, Wolbachia infection provides a selective advantage to infected females who in turn help the bacteria propagate in a population through the infected female germline.

For decades, Wolbachia and CI have been utilized as a strategy to control agricultural pests and reduce the spread of vector-borne human diseases. The most promising application involves using Wolbachia in mosquito population control through either sterilizing male mosquitoes or using a population replacement method that exploits Wolbachia’s ability to provide the host with resistance to mosquito-borne viruses. Release of Wolbachia-infected mosquitoes is EPA-approved in 20 states in the United States with the most recent large-scale release being conducted in Miami in an effort to reduce the population of Aedes aegypti, a mosquito vector for Zika virus.
CI embryos showed abnormal phenotypes during early developmental stages, generally resulting in embryonic death before hatching in many insect species. The incompatibility between Wolbachia-infected sperm and uninfected egg is due to the asynchronous development of male and female pronuclei at the early stage of mitosis after fertilization. The earliest observable embryonic defect from an incompatible CI cross between infected male and uninfected female is a delayed deposition of maternal H3.3 and H4 histone in the paternal pronuclei immediately after protamine removal. Paternal activation of cell cycle kinase Cdk1 and nuclear envelope breakdown are delayed. The consequence is improper condensation of male chromosomes that fail to segregate during anaphase, causing chromatin bridging and shearing of paternal DNA that generally lead to embryonic lethality.

Despite the success of Wolbachia in mosquito control, the molecular mechanism of CI had long eluded identification. The discovery of the Wolbachia two-gene cif (CI factor) operons as the main contributor of CI marks a major step in understanding its molecular mechanism. Within each cif operon, the downstream gene is annotated as the B gene while the upstream gene is annotated as the A gene. These operons were further divided into two groups based on the enzymatic activity of the B proteins; a cid (CI-inducing deubiquitylase) type which encodes deubiquitylases and a cin (CI-inducing nuclease) type which encodes nucleases. Significant progress has been made in recent years on understanding the genetic and molecular relevance of the cid genes on Wolbachia-induced CI. Expression of the cid operon in transgenic male Drosophila melanogaster induces CI-like postzygotic male sterility through interference with embryonic nuclear division. Transgenic expression of cidA gene in female flies can
rescue both transgenic CI and natural CI caused by male flies infected with *Wolbachia*. A large-scale population genomic screen of *Culex* mosquitoes linked crossing-type diversity in CI among mosquitoes infected with different wPip *Wolbachia* strains to genetic variations in the *cid* operon, further highlighting the important role of the *cid* genes in CI.

The facts that some CI-inducing *Wolbachia* strains, such as the wNo strain that infects *Drosophila simulans*, contain only *cin* but not *cid* type operons and that neither operon is present in wAu, a close relative of wMel that does not induce CI, suggested that the *cin* type operon should also be able to induce CI independent of the *cid* operon. Recent genetic analyses have uncovered natural variation in both *cid* and *cin* loci that correlates with CI in different *Wolbachia*-infected *Drosophila* species. While this supports previous speculations on the possible function of the *cin* operon in CI, the ability of these genes to cause CI had not been experimentally tested. Similarly, while there are distant sequence similarities between CinB and the PD-(D/E)xK superfamily of nucleases, no nuclease activity has been demonstrated. In this thesis work, I show that CinB has DNase activity. Mutation of putative active-site residues in either CinB PD-(D/E)xK domain abolishes activity *in vitro* and renders the resulting protein nontoxic to *Saccharomyces cerevisiae*. Most importantly, the *cin* operon induces a CI-like phenotype in transgenic flies, and expression of *cinA* alone in females is sufficient for rescue of transgenic CI. Therefore, the *cin* type nuclease operon provides a biochemically distinct mechanism for CI and its presence likely accounts for the ability of many *Wolbachia* strains to induce CI in their hosts despite not carrying an intact *cid* operon.
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Cytoplasmic Incompatibility

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Table of Contents

Abstract .................................................................................................................................................. 1

Table of Contents .............................................................................................................................. 6

List of Tables and Figures .................................................................................................................. 8

Acknowledgements ........................................................................................................................... 11

Chapter I: Introduction to Wolbachia and Cytoplasmic Incompatibility (CI)
  i.  Wolbachia and Its Reproductive Manipulations ................................................................. 12
  ii. Application of Wolbachia in Vector-Borne Disease Control ........................................ 18
  iii. Discovery of CI Factors and A Note on Nomenclature .................................................. 24
  iv.  Biochemistry and Genetics of CI Factors ........................................................................ 28

Chapter II: Materials and Methods
  i.  Sequence Alignment and Structure Prediction ................................................................. 31
  ii. Western Immunoblotting .................................................................................................... 31
  iii. Purification of Proteins for In Vitro Nuclease Assays and ITC ................................... 31
  iv.  Isothermal Titration Calorimetry (ITC) ....................................................................... 32
  v.  In Vitro Nuclease Assays ............................................................................................... 33
  vi.  Yeast Methods .................................................................................................................. 34
  vii. Yeast High-Copy Suppressor Screens ........................................................................ 35
  viii. Drosophila Hatch Rate and Cytology Analyses .......................................................... 35
  ix. Statistical Analyses ............................................................................................................ 38

Chapter III: In Vitro Biochemistry of CinA and CinB Proteins
  i.  Introduction ............................................................................................................................. 39
ii. Sequence Alignments and Structural Predictions ........................................ 40
iii. *In Vitro* Nuclease Activity of CinB Protein ............................................. 47
iv. Protein-Protein Interaction Between CinA and CinB ............................ 61
v. Discussion .................................................................................................. 63

Chapter IV: Analysis of the wPip *cin* Operon in *Saccharomyces cerevisiae*

i. Introduction .............................................................................................. 65
ii. *Cin* Operon Expression in Yeast .......................................................... 66
iii. CinB Suppressor Screen Utilizing a Yeast Genomic Tiling Library ..... 72
iv. Localization of CinA and CinB in Yeast .................................................. 77
v. Discussion .................................................................................................. 83

Chapter V: *Cin* Operon in Transgenic *Drosophila melanogaster*

i. Introduction .............................................................................................. 84
ii. Hatch Rate Analysis of Transgenic Fruit Flies ........................................ 85
iii. Cytology of Transgenic and Natural CI Induced by *cin* Operon .......... 91
iv. Discussion .................................................................................................. 96

Chapter VI: Summary and Discussion ............................................................... 98

Appendix I: Plasmids Used in This Study ....................................................... 102

Appendix II: Drosophila Lines Used in This Study ......................................... 105

Appendix III: Primers Used in This Study ...................................................... 108

References ..................................................................................................... 113
List of Tables and Figures

Chapter III

Figure 1: Illustration of cytoplasmic incompatibility ........................................... 14

Figure 2: A nomenclature proposal and schematic view of putative cytoplasmic incompatibility operon structures ................................................................. 25

Figure 3: Sequence of CinB<sup>w<Pip</sup> showing its two nuclease domains ................. 41

Figure 4: Protein sequence and secondary structure alignments of the NTND and CTND of CinB from various Wolbachia strains .............................................. 42

Figure 5: Predicted protein structure of CinB<sup>w<Pip</sup> by RaptorX ............................... 44

Figure 6: A model for a two-ion mechanism of phosphodiester bond cleavage by PD-(D/E)xK nucleases ....................................................................................... 46

Figure 7: Purification of recombinant CinB<sup>w<Pip</sup> protein .......................................... 48

Figure 8: CinB<sup>w<Pip</sup> cleaved both circular and linearized pBluescript SK+ plasmids ..... 49

Figure 9: Time-course experiment showing CinB<sup>w<Pip</sup> activity against linearized pBluescript SK+ plasmid ................................................................. 50

Figure 10: DNase activity of CinB<sup>w<Pip</sup> against purified pBluescript SK+ plasmid ...... 51

Figure 11: DNase activity of CinB<sup>w<Pip</sup> is ion-dependent ........................................ 52

Figure 12: Dnase activity of CinB<sup>w<Pip</sup> is weakly pH-dependent .............................. 53

Figure 13: CinB<sup>w<Pip</sup> cleaved both single- and double-stranded 70-mer DNAs .......... 54

Figure 14: Time-course experiment showing CinB<sup>w<Pip</sup> cleavage of Cy5-labeled single-stranded DNA ................................................................. 55

Figure 15: K636A or K279A mutations eliminated CinB<sup>w<Pip</sup> cleavage of linearized pBluescript SK+ ................................................................. 56

Figure 16: Catalytic K636A mutation eliminated DNase activity of CinB<sup>w<Pip</sup> against purified pBluescript SK+ plasmid .......................................... 57

Figure 17: Recombinant CidB<sup>w<Pip</sup> protein showed no DNase activity .................... 58
Figure 18: CinB<sup>W</sup>Pip showed no substrate preference against various DNA substrates .. 59

Figure 19: Recombinant CinB<sup>W</sup>Pip showed no RNase activity ............................................. 60

Figure 20: CinA-CinB<sup>W</sup>Pip protein binding affinity determined by ITC ............................ 62

Chapter IV

Figure 21: CinB<sup>W</sup>Pip toxicity in the <em>S. cerevisiae</em> BY4741 strain ............................................ 68

Figure 22: Relative expression levels of WT and mutant CinB<sup>W</sup>Pip proteins in yeast .......... 69

Figure 23: Effect of doubly tagged Flag-CinB<sup>W</sup>Pip-HA on its toxicity in yeast ............ 70

Figure 24: Western blot analysis of yeast expressing either Flag-CinB<sup>W</sup>Pip-HA or the catalytic K636A mutant derivative ............................................................... 71

Table 1: Yeast genomic suppressors for CinB-induced toxicity obtained by growing transformants first on glucose followed by replica-plating onto galactose .... 73

Table 2: Yeast genomic suppressors for CinB-induced toxicity obtained by growing transformants directly onto galactose media after transformation .......... 75

Figure 25: Effect of N-terminal fluorescent protein fusion of Cif<sup>W</sup>Pip on <em>S. cerevisiae</em> (BY4741) growth ................................................................. 78

Figure 26: Relative expression levels of eGFP-CifB<sup>W</sup>Pip and mCherry-CifA<sup>W</sup>Pip in yeast ................................................................. 79

Figure 27: Effect of C-terminal fluorescent protein fusion of Cin<sup>W</sup>Pip on <em>S. cerevisiae</em> (BY4741) growth ................................................................. 80

Figure 28: Effect of C-terminal fluorescent protein fusion of Cid<sup>W</sup>Pip on <em>S. cerevisiae</em> (BY4741) growth ................................................................. 81

Figure 29: Effect of 3xFlag-tagged CinB<sup>W</sup>Pip and HA-CinA<sup>W</sup>Pip on <em>S. cerevisiae</em> (BY4741) growth ................................................................. 82

Chapter V

Figure 30: Strategies used to generate transgenic flies ......................................................... 86
Figure 31: PCR verification of *Wolbachia* infection status in both *D. melanogaster* and *D. simulans* .................................................. 87

Figure 32: Expression of the *cinA-cinB*<sup>w<Psi>p</sup> genes in flies induces CI-like embryo killing and rescue phenotypes .................................................. 89

Figure 33: Hatch rate analysis with w<sup>No</sup> infected male *D. simulans* ............... 92

Figure 34: Embryos cytology from incompatible crosses between w<sup>No</sup>-infected males *D. simulans* and uninfected females ........................................ 93

Figure 35: Embryos cytology from crosses between transgenic *D. melanogaster* males expressing the *cinA-cinB*<sup>w<Psi>p</sup> operon and wild type females .......... 94

Figure 36: Quantification of embryo cytology .......................................................... 95
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Chapter I: Introduction to *Wolbachia* and Cytoplasmic Incompatibility (CI)

i. *Wolbachia* and Its Reproductive Manipulations

*Wolbachia* *pipiens* are Gram-negative obligate intracellular bacteria that infect insects, mites, crustaceans, and filarial nematodes. They are α–proteobacteria of the order *Rickettsiales*, a diverse group of mostly intracellular bacteria. Since first discovered in *Culex pipiens* mosquitoes nearly a century ago, *Wolbachia* has been found in every insect order and up to two-thirds of all insect species (Hilgenboecker *et al.*, 2008). The success of *Wolbachia* infection is in part due to their ability to manipulate host reproduction to selectively favor infected females. Since *Wolbachia* are transmitted by vertical transmission through the infected female germline, the reproductive advantage *Wolbachia* provides to an infected female hosts enhances its own ability to propagate in an insect population (Hoffmann, Turelli and Harshman, 1990; Bressac and Rousset, 1993; Turelli and Hoffmann, 1995; Clark, Jan and Jan, 1997; Hoffmann, Hercus and Dagher, 1998).

*Wolbachia*-induced host manipulations include parthenogenesis, feminization of genetic males, male killing, and cytoplasmic incompatibility (Serbus *et al.*, 2008). Among these reproductive manipulations, cytoplasmic incompatibility (CI) is the most frequently found phenotype in insects. CI is a phenomenon where mating between infected males and uninfected females causes severe developmental defects in the early stages of embryogenesis, which often results in embryonic lethality (Laven, 1953; Yen and Barr, 1973; Werren, Baldo and Clark, 2008). While uninfected females can only produce viable offspring with uninfected males, infected females are capable of
producing fully viable progeny with either infected or uninfected males (Figure 1) (Beckmann et al., 2019b).
**Figure 1.** Illustration of cytoplasmic incompatibility. Infected females can produce viable offspring with both infected and uninfected males, whereas uninfected females can produce viable progeny only if they mate with uninfected males. Abbreviation: w, *Wolbachia*-infected. Figure adapted from Beckmann *et al.*, 2019b.
CI embryos show abnormal phenotypes during early developmental stages, generally resulting in embryonic death before hatching in many insect species (Riparbelli et al., 2012). The incompatibility between Wolbachia-infected sperm and uninfected egg is due to the asynchronous development of male and female pronuclei at the early stage of mitosis after fertilization. In normal embryonic development, following the initial nuclear envelope breakdown of male and female pronuclei, protamine proteins used to package the paternal DNA are removed and nucleosomes are assembled using maternally supplied core histones and the histone variant H3.3 (Loppin et al., 2005; Balhorn, 2007; Tirmarche et al., 2014; Loppin, Dubruille and Horard, 2015). The male and female pronuclei migrate towards one another. Both sets of chromosomes are replicated, condensed and then separated at anaphase creating two diploid daughter cells.

In CI embryos, the earliest detectable developmental defect is the abnormal deposition of H3.3 and H4 histone in male pronuclei following normal protamine removal from paternal DNA (Landmann et al., 2009). Paternal activation of cell cycle kinase Cdk1 and nuclear envelope breakdown are delayed (Tram and Sullivan, 2002). Prolonged retention of DNA replication factor proliferating cell nuclear antigen (PCNA) was also observed in the male pronuclei, a possible indication of progression into mitosis with incompletely replicated DNA (Landmann et al., 2009). The consequence is improper condensation of male chromosomes that fail to segregate during anaphase, causing chromatin bridging and shearing of paternal DNA (Reed and Werren, 1995; Lassy and Karr, 1996; Callaini, Dallai and Riparbelli, 1997; Tram et al., 2006). Excess centrosomes unassociated with the maternal and paternal nuclei as well as mitotic spindles lacking centrosomes are also often observed in CI embryos, possibly a direct
outcome of delayed nuclei development and abnormal chromosome condensation (Lassy and Karr, 1996; Callaini, Dallai and Riparbelli, 1997; de Saint Phalle and Sullivan, 1998). In recent years, a group of Wolbachia genes called cif genes has been identified and shown to be the key genetic elements in Wolbachia-related CI. Detailed evidence supporting cif genes as the CI factors will be discussed in a later section of the Introduction.

Other Wolbachia-induced reproductive phenotypes are less common than CI. Wolbachia-related parthenogenesis occurs in species such as mites, wasps and thrips (Stouthamer, Luck and Hamilton, 1990; Weeks and Breeuwer, 2001; Arakaki, Miyoshi and Noda, 2001). Most of these species have haplodiploid sex determination where unfertilized eggs develop into haploid males while fertilized eggs develop into diploid females. In Wolbachia-induced parthenogenesis, meiosis is followed by fusion of the two nuclei from the first mitotic division, resulting in restoration of diploidy and development of diploid females which unlike males are able to transmit Wolbachia to their offspring (Stouthamer and Kazmer, 1994; Stouthamer, Breeuwer and Hurst, 1999). Another Wolbachia-related host manipulation is feminization. The exact mechanism for Wolbachia-induced feminization of genetic males still remains unclear (Asgharian et al., 2014). Feminizing Wolbachia strains have been characterized in several arthropod species and most heavily studied in the common pill bug Armadillidium vulgare (Cordaux et al., 2004; Badawi, Greve and Cordaux, 2015). Only two insect species, Eurema hecabe and Zyginidia pullulan, are currently known to be feminized by Wolbachia (Hiroki et al., 2002; Negri et al., 2008).
*Wolbachia* has also been described to cause male killing in several arthropod orders (Fialho and Stevens, 2000; Jiggins *et al*., 2001; Dyer and Jaenike, 2004; Zeh, Zeh and Bonilla, 2005). Male-killing occurs mainly during early embryogenesis resulting in more resources for the surviving female offspring. In *Ostrinia scapulalis*, male killing occurs when genetic males become feminized and die during the larval stage in the presence of *Wolbachia* (Kageyama *et al*., 2002; Kageyama and Traut, 2004). In *Drosophila bifasciata*, *Wolbachia*-induced male killing is associated with defective chromatin condensation, chromosome segregation and mitotic spindle organization in the early development of male embryos, leading to embryonic death before hatching (Riparbelli *et al*., 2012). Recent evidence suggests the phage gene *wmk* from the *wMel* *Wolbachia* strain could be a candidate for *Wolbachia*-related male killing. Transgenic expression of *wMel wmk* gene in *D. melanogaster* caused a lower male-to-female sex ratio in the offspring and an increase in abnormal cytology in male embryos (Perlmutter *et al*., 2019; Perlmutter, Meyers and Bordenstein, 2020). However, since *wMel* is not known to naturally cause male killing, further genomic analyses and transgenic fly studies are needed to identify if similar *wmk* genes exist in male-killing *Wolbachia* strains that infect *Drosophila*, such as *wBif* and *wRec* (Hurst *et al*., 2000; Sasaki, Kubo and Ishikawa, 2002; Jaenike, 2007; Richardson *et al*., 2016).
ii. **Application of Wolbachia in Vector-Borne Disease Control**

A primary target for mosquito-borne disease control is *Aedes aegypti*. This type of mosquito prefers tropical and subtropical regions of the world where they live and breed near or within human habitats (Flores and O'Neill, 2018). With rapid urban growth and the convenience of global air travel, the pandemics of mosquito-transmitted viral diseases have increased significantly over the past few decades (Gubler, 2002). For example, the incidence of dengue fever has grown over 30-fold during this period, now becoming the world’s most common mosquito-borne virus (Pang, Mak and Gubler, 2017). Dengue virus is estimated to infect 390 million people per year, of which about 100 million would manifest significant clinical severity (Bhatt *et al.*, 2013).

Chikungunya, an arbovirus originated in sub-Saharan Africa, has also recently expanded its geographic range and spread into new areas around the world (Powers *et al.*, 2000). Since a reported emergence of chikungunya in the coastal area of Kenya in 2004, the virus has spread to different regions of Africa (Chretien *et al.*, 2007), Asia, several islands in the Indian Ocean (Hochedez *et al.*, 2006; Lanciotti *et al.*, 2007; Taubitz *et al.*, 2007) and temperate areas in Europe (Rezza *et al.*, 2007; Grandadam *et al.*, 2011) and reached the Americas in 2013 (Leparc-Goffart *et al.*, 2014; Mayer, Tesh and Vasilakis, 2017). Zika virus is another mosquito-borne arbovirus that has been rapidly introduced to areas where it was not previously reported. After its first outbreak on the Island of Yap in Micronesia in 2007, the Zika virus reached French Polynesia in 2013 causing another major epidemic (Ioos *et al.*, 2014; Mayer, Tesh and Vasilakis, 2017). In the following years, the virus spread to Thailand (Buathong *et al.*, 2015), other South Pacific regions
such as Easter Island (Musso, Nilles and Cao-Lormeau, 2014) and eventually reached the Americas with a major outbreak in Brazil in 2015 (Musso, 2015; Zanluca et al., 2015).

A subset of vector control focuses on reducing the spread of mosquito-borne viruses by direct suppression of the mosquito population or modification of the mosquitoes to make them resistant to the pathogens. The approaches used to suppress mosquito population work by reducing mosquito bite rates, thus lowering virus transmission and risk of disease. Although the assumption certainly is true if a mosquito population is completely eliminated, more evidence is required to evaluate the effect when mosquito population is only partially suppressed (Wilson et al., 2015; Bowman, Donegan and McCall, 2016; Flores and O'Neill, 2018). Currently, one promising approach in reducing mosquito population involve rearing and releasing large numbers of male mosquitoes that cannot produce viable offspring when mating with wild females. These methods include the sterile insect technique (SIT) and incompatible insect technique (IIT).

Traditionally, SIT involves irradiating or chemically treating male insects to sterilize them (Bushland, Lindquist and Knipling, 1955). When these males are released and mate with wild females, they cannot produce viable offspring, leading to a decrease in the wild mosquito population. A major limitation of SIT is that irradiation and chemical treatment can reduce the fitness of the treated males, rendering them less reproductively competitive comparing to the wild males. IIT, a version of SIT that utilizes Wolbachia to effectively sterilize males, can overcome the fitness costs associated with the traditional SIT methods. In IIT, Wolbachia-infected males are released into a mosquito population to mate with uninfected wild females. Due to CI,
such matings between *Wolbachia*-infected males and uninfected females would produce few offspring, leading to a decrease in mosquito population size. The main concern about IIT is that accidental release of infected females during sex sorting might cause the *Wolbachia*-infected mosquitoes to replace the field mosquito population, due to CI, and thus prevent future population suppression through IIT.

IIT also shares many limitations with SIT. Since both methods require continual release of large numbers of males into the wild mosquito population, effective rearing/sex-sorting facilities are required to constantly produce large amounts of male mosquitoes. Migration of mosquitoes from untreated neighboring areas can also limit the long-term effectiveness of these methods. Other major concerns of IIT include introgression of unfavorable genetic alleles into the mosquito population and development of population resistance against the sterilization or embryo-killing mechanisms.

The first field trial of IIT utilizing *Wolbachia*, which achieved complete elimination of a wild *Culex quinquefasciatus* population, was conducted in Burma in 1967 (Laven, 1967). More recently, *Wolbachia*-infected male *Aedes polynesiensis* mosquitoes were released in French Polynesia, resulting in a significant decrease in mosquito egg hatch rate in the treated area (O'Connor et al., 2012). In the United States, release of *Wolbachia*-infected mosquitoes is EPA-approved in 20 states and Washington D.C. In 2016, release of male *Aedes albopictus* mosquitoes infected by *Wolbachia* in Lexington, Kentucky over a 17-week period caused significant reduction in both the number of adult females and egg hatch rates (Mains et al., 2016).
The latest release of Wolbachia-infected mosquitoes in the United States was conducted in Miami, Florida in 2019 where researchers released male Aedes aegypti mosquitoes infected by Wolbachia over the course of six months in response to heightened concern of Zika virus transmission, leading to a significant decrease in both the egg hatch rate and number of adult mosquitoes (Mains et al., 2019). Organizations such as the World Mosquito Program (formerly known as the Eliminate Dengue Program) for years have also been using Wolbachia-infected mosquitoes to control mosquito population in order to reduce the spread of mosquito-borne diseases in Australia, Indonesia, Brazil, and many other places around the world (O'Neill, 2018; O'Neill et al., 2018).

The combination of SIT and IIT has also proven to be effective in mosquito population suppression. Treating Wolbachia-infected mosquitoes with low-level irradiation will sterilize females while leaving males generally unaffected (Arunachalam and Curtis, 1985; Shahid and Curtis, 1987; Zhang et al., 2015a; Zhang et al., 2015b). Therefore, females that might escape during sex sorting and be released into the field would not be able to produce viable offspring, eliminating the risk of population replacement. A recent field study conducted in Guangzhou, China using the combined SIT-IIT approach; millions of factory-reared male mosquitoes were released and achieved near elimination of field A. albopictus populations (Zheng et al., 2019).

In contrast to the population reduction approach using Wolbachia-induced CI, the population modification method exploits the ability of Wolbachia to protect host insects from pathogenic viruses. Since Wolbachia was first discovered to protect host Drosophila melanogaster from Drosophila C virus, many laboratory studies have shown that
Wolbachia infection can prevent the transmission of various viruses (Teixeira, Ferreira and Ashburner, 2008), particularly RNA viruses such as dengue (Walker et al., 2011; Blagrove et al., 2012), chikungunya (Moreira et al., 2009), Zika and West Nile virus (Joubert and O'Neill, 2017), by inhibiting pathogen replication in the host insect (Hedges et al., 2008; Kambris et al., 2009; Walker et al., 2011). This ability of Wolbachia to provide pathogen resistance coupled with CI allows an alternative strategy for controlling mosquito-borne viruses. Due to the reproductive advantage in Wolbachia-infected females and Wolbachia’s ability to transmit vertically through female germlines, releasing both male and female mosquitoes infected by Wolbachia would allow Wolbachia infection to spread throughout a wild population, thus reducing the probability of the mosquitoes transmitting viruses to humans.

A major advantage of this approach over SIT or IIT is that the method requires the release of far fewer mosquitoes and, once Wolbachia infection is established in the wild population, it is expected to be maintained at a high frequency indefinitely (Turelli, 2010; Hoffmann et al., 2011). As a result, the Wolbachia-based population replacement strategy has much lower cost than the population suppression approach. Many field studies have proven the effectiveness in spreading Wolbachia infection throughout wild mosquito populations. In Australia, since the initial release of wMel-infected A. aegypti in 2011 by the World Mosquito Program, the frequency of the wMel Wolbachia strain has been maintained at rate of 90% or greater (Hoffmann et al., 2011; Hoffmann et al., 2014). However, the effectiveness of this approach in suppressing viral transmissions remains unclear. An ongoing field trial in Yogyakarta, Indonesia is expected to provide
evidence on the degree of disease reduction through the use of the *Wolbachia* population replacement method (Flores and O'Neill, 2018).

Lastly, another potential strategy of using *Wolbachia* in mosquito control is by creating a gene-drive system utilizing genomic editing to increase the odds of the drive system to be passed onto offspring. Recent study showed that in laboratory settings, CRISPR-Cas9 could be used to spread anti-*Plasmodium falciparum* effector genes m2A10-m1C3 into an *Anopheles stephensi* population (Gantz and Bier, 2015; Gantz et al., 2015). Subsequent work also exploited CRISPR-Cas9 gene-drive system for population suppression in *Anopheles gambiae* by either targeting female reproduction or creating sex-ratio distortion (Hammond et al., 2016; Galizi et al., 2016). The effectiveness of gene-drive systems in mosquito-borne disease control has yet to be tested in field studies. Several concerns about the gene-drive approach include the difficulty in receiving public support in releasing transgenic organisms into the wild, potential risk of spreading the gene-drive system into neighboring populations, and the possibility for viruses to develop mutations over time that can render them resistant to the transgenes.
iii. Discovery of CI Factors and A Note on Nomenclature

Despite the success of *Wolbachia* in reducing mosquito-borne viruses and agricultural pest control, the molecular mechanisms of *Wolbachia*-induced CI have long eluded identification. Since CI is caused by improper condensation and segregation of chromosomes from *Wolbachia*-infected male sperm, it was thought that *Wolbachia* must somehow modify the sperm and such modification has to take place during spermatogenesis because the bacteria are removed from mature sperm (Bressac and Rousset, 1993). The fact that sperm from *Wolbachia*-infected males induce CI and embryonic lethality only in uninfected but not infected embryos could be explained by a toxin-antidote model wherein the sperm from the infected male carries the toxin and the egg from the infected female carries the *Wolbachia*-derived antidote. Driven by these hypotheses, a proteomic analysis was conducted on sperm and ovaries collected from wPip *Wolbachia*-infected *Culex pipiens*. Peptides of a *Wolbachia* protein, now called CidA, were identified by mass spectrometry in both sperm and ovaries of wPip-infected mosquitoes but were absent from mosquitoes not infected by *Wolbachia* (Beckmann and Fallon, 2013). Further genetic analysis revealed that *cidA* is part of a two-gene operon, which we now call *cidA-cidB*, and there coexists a paralogous operon in the same wPip *Wolbachia* genome termed *cinA-cinB* (Figure 2).

The first evidence demonstrating that genes in either the *cid* or *cin* operons can behave as toxin-antidote pairs came from transgenic studies in yeast. Using *Saccharomyces cerevisiae* as a model system, expression of *cidB* or *cinB* caused a temperature-dependent growth defect at 37°C that can be rescued when the cognate *A* genes (*cidA* or *cinA*, respectively) were co-expressed, suggesting that the *A* genes in each
**Figure 2.** A nomenclature proposal and schematic view of putative cytoplasmic incompatibility operon structures. In this naming system, the *cif* (and Cif) terms designate CI genes (and proteins) in general, while genes from specific operon categories are named according to the enzymatic activity of the putative toxin. The first and second genes within each operon are denoted *A* and *B*, respectively, and the *Wolbachia* strain is indicated as a superscript when relevant. The structure of several CI operons is shown to illustrate this system; active-site residues are labeled. Abbreviation: ORF, open reading frame. Figure adapted from Beckmann *et al.*, 2019b.
operon are likely the antidote genes while the $B$ genes are toxin-encoding genes
(Beckmann, Ronau and Hochstrasser, 2017). *In vitro* binding experiments also showed
that the two proteins within each operon bind tightly to one another in a cognate-specific
manner, a common feature among type II toxin-antitoxin systems in free-living bacteria
(Beckmann, Ronau and Hochstrasser, 2017; Yamaguchi, Park and Inouye, 2011).

Subsequent studies in transgenic *D. melanogaster* confirmed the role of the *cid*
operon in *Wolbachia*-induced CI and rescue. Transgenic expression of *cid*\textsuperscript{wPip} or *cid*\textsuperscript{wMel} in uninfected male fruit flies recapitulate CI embryonic lethality and cytological defects
when mated with wild-type uninfected females (Beckmann, Ronau and Hochstrasser,
2017; LePage *et al.*, 2017). Importantly, female flies transgenic for *cidA*\textsuperscript{wMel} can rescue
CI when crossed with either wild-type *wMel*-infected males or uninfected males
transgenic for *cidA-cidB*\textsuperscript{wMel}, indicating that the *cid* operon is capable of inducing both CI
and rescue (LePage *et al.*, 2017; Shropshire *et al.*, 2018; Shropshire and Bordenstein,
2019; Beckmann *et al.*, 2019c). However, the notion of *cidB* as the sole gene remains
uncertain as transgenic expression of *cidB*\textsuperscript{wMel} alone in male flies was unable to induce CI
when crossed with wild-type uninfected females.

Before further discussion on the biochemistry and genetics of these CI-inducing
operons, it is important to note that there are currently two different CI gene
nomenclatures coexisting in the literature. One nomenclature system proposed naming
the CI operons based on the enzymatic function of the proteins encoded by the $B$ genes
and using *cif*, short for CI factors, only to designate CI genes in general (Beckmann,
Ronau and Hochstrasser, 2017; Beckmann *et al.*, 2019b; Beckmann *et al.*, 2019a). The B
protein in *cid* type operon, short for CI-inducing deubiquitylase (DUB), has been
confirmed to have DUB activity \textit{in vitro} when it is expressed recombinantly; moreover, mutating the DUB active site abrogates toxicity in yeast and flies (Beckmann, Ronau and Hochstrasser, 2017). Prior to the work presented in this thesis, the nuclease function of the B protein in \textit{cin}-type operon, short for \textit{CI}-inducing nuclease, was only proposed based on its weak protein sequence homology to other known nucleases.

The second nomenclature system names all CI-related genes as \textit{cif} regardless of the biochemical functions of the proteins encoded by the CI operons (LePage \textit{et al.}, 2017; Shropshire \textit{et al.}, 2019). Bordenstein and colleagues argued that it was premature to name the nuclease-type operons \textit{cin} since it had not been shown that these operons caused CI or that CinB had nuclease activity. They further argued that the B proteins are potentially polyvalent, suggesting that other putative protein domains aside from the DUB or the nuclease domains could contribute to their CI-related functions. However, in both nomenclature systems, the upstream gene in each operon is denoted as the \textit{A} gene (\textit{cifA}, \textit{cidA} or \textit{cinA}), while the downstream gene is denoted as the \textit{B} gene (\textit{cifB}, \textit{cidB}, or \textit{cinB}). In this thesis work, we use the first nomenclature system in which “\textit{cif}” is used only when discussing CI genes generally and the more specific “\textit{cid}” or “\textit{cin}” names when the enzymatic function of particular toxins is known or strongly predicted. The relevant \textit{Wolbachia} strain is denoted by a superscript (\textbf{Figure 2}) (Beckmann \textit{et al.}, 2019b).
iv. Biochemistry and Genetics of CI Factors

The discovery of the Wolbachia cif genes marked a major step in understanding the molecular mechanisms of CI. Prior to the work presented in this thesis, most research had focused on understanding the biochemistry and relevance of cid-type operons in inducing CI and its underlying mechanism. Little was known about the cin operon.

Protein sequence analysis suggested that CidB protein contains a C-terminal Ulp1-like (ubiquitin-like protein-specific protease 1) domain (Beckmann, Ronau and Hochstrasser, 2017). Ulp1 is a cysteine protease that catalyzes the deconjugation of the small ubiquitin-related modifier (SUMO) from proteins (Li and Hochstrasser, 1999; Hickey, Wilson and Hochstrasser, 2012).

Interestingly, recombinant CidB protein showed no SUMO protease activity but instead, reacted with the ubiquitin-based suicide inhibitor ubiquitin vinyl methyl ester (UbVME) and exhibited activity towards ubiquitin-AMC and polyubiquitin chains with isopeptide linkages. Active site cysteine-to-alanine mutation renders CidB unreactive toward UbVME. These results suggested that CidB is a DUB, a group of enzymes that specifically remove ubiquitin from ubiquitin-modified proteins (Ronau, Beckmann and Hochstrasser, 2016; Beckmann, Ronau and Hochstrasser, 2017).

A follow-up study aimed to uncover the molecular mechanism of cid-induced CI using an in vitro affinity purification approach found that the catalytic inactive CidB protein interacts with both karyopherin-α (Kap-α), a nuclear import receptor, and the P32 histone chaperone from Drosophila melanogaster protein extracts (Beckmann et al., 2019c). Transgenic expression of these two proteins in uninfected female D. melanogaster partially suppressed CI when crossed with wild type males infected by
wMel Wolbachia. Overexpression of yeast Kap-α (Srp1) also suppressed CidB toxicity in yeast. However, it is still unclear how exactly Kap-α and P32 are involved in the molecular mechanism of CI. Ubiquitylation of Kap-α might be important for promoting nuclear import of key proteins involved in protamine-histone exchange. It is also possible that protein interaction between CidB and P32 could allow the DUB to deubiquitylate certain histone components, resulting in impaired histone deposition.

A large-scale population genomic screen of Culex mosquitoes correlated crossing-type diversity in CI among mosquitoes infected with different wPip strains to genetic variation in the cidA-cidB\textsuperscript{wPip} operon (Bonneau et al., 2018a). The intensity of CI embryonic defects was also correlated specifically with variations in the cidB gene in the genome of the wPip strain hosted by the male mosquitoes (Bonneau et al., 2018b). A follow-up genetic analysis using wPip strains from North Italy also linked cidB variants to CI phenotypes but found no association between cidA variants and CI diversity (Bonneau et al., 2019). These results together provide genetic evidence highlighting the important role of the cid genes in CI and support the model that cidB is the CI-inducing toxin in cid.

Another set of Wolbachia cif factors hypothesized to contribute to CI is the two-gene cin operon, named after the putative nuclease domains in the CinB protein suggested by protein sequence analysis (Beckmann, Ronau and Hochstrasser, 2017). The same genomic screen found that the cin operon genes were monomorphic among the incompatible wPip strains, suggesting that CI in C. pipiens are only related to cid but not cin operons (Bonneau et al., 2018a). However, the fact that some CI-inducing Wolbachia strains, such as the wNo strain that infects Drosophila simulans, contain only cin but not
cid operons and that neither operon is present in \textit{w}Au, a close relative of \textit{w}Mel that does not induce CI, suggested that the cin operon might also be able to induce CI independently of the cid operon (Sutton \textit{et al}., 2014; Lindsey \textit{et al}., 2018). As was true for CidB, CinB was shown to inhibit growth when expressed in yeast (Beckmann, Ronau and Hochstrasser, 2017).

Recent genomic analyses have also uncovered natural variation in both cid and cin loci that correlates with CI in different \textit{Wolbachia}-infected \textit{Drosophila} species (Cooper \textit{et al}., 2019; Meany \textit{et al}., 2019). While this supports previous speculations on the possible function of the \textit{cinA-cinB} operon in CI, the ability of these genes to cause CI has not been experimentally tested. Similarly, while there are distant sequence similarities between CinB and the PD-(D/E)xK superfamily of nucleases, no nuclease activity has been demonstrated. In this thesis work, I show that CinB has DNase activity. Mutation of putative active-site residues in either of two CinB PD-(D/E)xK domains present in CinB abolishes activity \textit{in vitro} and renders the resulting protein nontoxic to yeast. Most importantly, the \textit{cinA-cinB}^\textit{wPip} operon induces a CI-like phenotype in transgenic flies, and \textit{cinA}^\textit{wPip} is sufficient for rescue of transgenic CI. Therefore, the \textit{cinA-cinB} nuclease operon provides a biochemically distinct mechanism for CI and its presence likely accounts for the ability of many \textit{Wolbachia} strains to induce CI in their hosts despite not carrying an intact \textit{cidA-cidB} gene pair. A majority of the work presented in this thesis has been previous published in four peer-reviewed journals (Beckmann \textit{et al}., 2019b; Beckmann \textit{et al}., 2019a; Chen \textit{et al}., 2019; Beckmann \textit{et al}., 2019c).
Chapter II: Materials and Methods

**Sequence Alignment and Structure Prediction.** Multiple sequence alignments of CinB orthologs from several *Wolbachia* strains and known PD-(D/E)xK nucleases were generated using the Clustal Omega Multiple Sequence Alignment program from EMBL-EBI followed by manual adjustment (Kanz et al., 2005). Secondary structure predictions and alignments of the protein sequences were performed using the PSIPRED Protein Sequence Analysis Workbench program (Jones, 1999). Structure prediction of CinB\textsuperscript{wPip} was done using the RaptorX Structure Prediction server with a few unstructured regions removed in the displayed figure (Wang et al., 2016).

**Western Immunoblotting.** The following antibodies were used: mouse anti-FLAG M2 (Sigma, 1:10,000); mouse 16B12 anti-HA (Covance, 1:1000), mouse anti-PGK (Molecular Probes, 1:20,000), and HRP-conjugated sheep anti-mouse NA931V (GE Healthcare, 1:10,000). Protein samples were resolved by SDS-PAGE and transferred to PVDF membranes (Millipore) for immunoblotting. Proteins were visualized by HRP-based chemiluminescence (Mruk and Cheng, 2011).

**Purification of Proteins for In Vitro Nuclease Assays and ITC.** Full-length CinB, catalytically inactive CinB mutants (K279A, K636A, and KK279/636AA), CidB\textsubscript{1-761}(V686E/R688K), and CinA were expressed as glutathione-S-transferase (GST) fusions from the pGEX6P1 vector in Rosetta DE3 (Novagen) *E. coli* as previously described (Beckmann, Ronau and Hochstrasser, 2017). To reduce the likelihood of CinB
copurification with DNA, we used a protocol to isolate DNA-free protein described by Epling et al (Epling et al., 2015). 2 L of back-diluted bacterial cultures were grown to \( \text{OD}_{600} = 0.5 \) in LB medium containing ampicillin at 37 °C followed by induction of protein expression with 0.3 mM isopropyl-β-D-thiogalactoside (IPTG) at 18 °C overnight. Cells were harvested by centrifugation, resuspended in a buffer containing 50 mM Tris pH 8, 250 mM NaCl, 10% glycerol, 2 mM β-mercaptoethanol, and lysed on ice via French press. The lysate was clarified by centrifugation at 50,000 x g for 1 hr at 4°C (Thermo Sorvall Lynx 600 F20-12x50 LEX). Nucleic acids were separated from protein upon the addition of 1M (final concentration) sodium chloride to the clarified lysate, followed by precipitation of nucleic acids by addition of 0.3% (final concentration) of a solution composed of 10% polyethylenemine in 10% hydrochloric acid. After centrifugation (5,000 rpm, 15 min), the supernatant was treated with 70% ammonium sulfate to remove excess PEI by precipitation of the protein. The protein was centrifuged at 50,000 x g for 1 hr at 4°C and the pellet was resuspended in PBS supplemented with 400 mM KCl. The protein was further purified via GST affinity chromatography and size-exclusion chromatography as described previously (Beckmann, Ronau and Hochstrasser, 2017). All proteins were concentrated using Amicon Ultra centrifuge filter units and stored at -80°C in 25 mM Tris-HCl, pH7.5, 150 mM NaCl, and 4 mM dithiothreitol (DTT).

**Isothermal Titration Calorimetry.** Isothermal titration calorimetry (ITC) experiments were carried out at 25°C using a NanoITC (TA Instruments). First, CinA\(^{wPip}\) and CinB\(^{wPip}\) were dialyzed extensively against a buffer of 50 mM HEPES pH 7.4 over the course of two days with 3-4 buffer exchanges. To determine the binding affinity of CinB
for CinA, 500 μM CinA was loaded into the syringe and titrated into a 50 μM solution of CinB. A total of 22 injections (2 μl/injection) were performed over the course of the experiment with a spacing of 300 seconds between injections to ensure a return to baseline prior to the next injection. The data were baseline corrected using NITPIC and analyzed in SEDPHAT using the one-site (A+B → AB) binding model. The figure was prepared in GUSSI, which was downloaded from the MBR Software Page (http://biophysics.swmed.edu/MBR/software.html) (Roe and Cheatham, 2013; Keller et al., 2012; Houtman et al., 2007).

**In Vitro Nuclease Assay.** All *in vitro* nuclease activity assays were performed with full-length CinB$^{wPip}$ (1-733) with or without CinA$^{wPip}$ (1-446). For DNase and RNase activity assays, 1 μM CinB or CinB mutant proteins was incubated in a reaction buffer containing 20 mM HEPES (pH 8.0), 5 mM MgCl$_2$, 2.5% sucrose, 150 mM NaCl, 0.001% Triton X-100, and 2 mM DTT with 15 nM of either linearized or supercoiled pBluescript SK+, 500 nM single-stranded or double-stranded Cy5-labeled DNA (70-mer: Cy5-GCAATTTCGATCGTTGACATCTCGTGCGCTGTCGTCGATCAATCGGCAGATGCGGAGTGAAGTTCCAACGTTCGGC, previously used for ssDNA cleavage analysis (Komori et al., 2000); 45-mer: Cy5-GGGTCAACGTGGGCAAAAGATGTCCTAGCAAGCAGCCAGAATTCGTCGGCAG, which was tested with the online calculator OligoCalc to confirm that the sequence should not form any strong hairpins; and the respective complements to generate dsDNA) or 500 ng of either yeast tRNA (Thermo Fischer) or *D. melanogaster* total RNA (Bogart and Andrews, 2006). In reactions where CinA was present, 10 μM CinA was used.
All reactions were carried out at 25 °C for 90 min and quenched by adding EDTA to a final concentration of 10 mM unless otherwise noted.

For reactions using linear or circular pBluescript SK+ vector or RNA, samples were run in agarose gels with a range of concentrations between 0.8 and 2.0% containing 0.4 µg/mL ethidium bromide in 1x TAE buffer at 100 V for 40-60 min and imaged on a Syngene G:box with GeneTools software. For reactions using Cy5-labeled oligodeoxynucleotides, samples were run in 9% TBE polyacrylamide native gels at 100 V for 45-60 min and imaged on Typhoon FLA 7000 with Typhoon FLA 7000 control software.

**Yeast Methods.** The yeast growth assays shown were done in the BY4741 strain (Brachmann et al., 1998). The 2-micron plasmid pYES2 (URA3) utilizes a GAL1 promoter and a CYC1 terminator and was used for galactose-inducible cinB expression in yeast. Standard site-directed mutagenesis was used to create point mutations in CinB. For yeast growth assays, cultures were grown overnight at 30 °C in glucose minimal medium (SD) lacking uracil. Cultures were spotted in six-fold serial dilution from an initial concentration of 0.2 OD₆₀₀ on solid minimal SD media lacking uracil and containing either 2% glucose or galactose. Plates were then left at 30, 34, and 36 °C for 2-3 days.

For immunoblotting, cells were grown overnight in either glucose (non-inducing) or galactose (inducing) media lacking uracil, diluted to 0.2 OD₆₀₀ the next morning and left to grow at 30 °C until reaching 0.8-1.0 OD₆₀₀ at which point the equivalent of 2-3 OD₆₀₀ units of cells were harvested, washed and resuspended in 1 mL cold water followed by the addition of 150 µL of a 2 N NaOH/1 M β-mercaptoethanol solution (Kushnirov, 2000).
Cells were vortexed vigorously for 20 s, incubated on ice for 15 min, and pelleted. Pellets were resuspended in 1x SDS-PAGE sample buffer and heated at 100 °C for 3 min before immunoblot analyses.

**Yeast High-Copy Suppressor Screens.** The high-copy suppressor screen was performed in the BY4741 yeast strain. Flag-CinB<sup>uPip</sup> was cloned into the pRS416GAL1 (CEN) plasmid with a galactose-inducible promoter and a *URA3* selection cassette. Plasmids from a yeast high-copy genomic tiling library utilizing a *LEU2* selection cassette were transformed into yeast expressing Flag-CinB<sup>uPip</sup> using a standard lithium acetate transformation method (Jones *et al.*, 2008; Gietz and Schiestl, 2007). Transformed cells were plated directly on selective media containing either galactose or glucose and incubated at 37°C. If first plated on glucose media, cells were then allowed to grow at 37°C for 3 days followed by replica-plating onto galactose-containing selective media. After plating on selective media containing galactose, colonies were allowed to grow for 3-5 days before they were picked and re-streaked onto a fresh selective media. Plasmids were extracted from yeast by standard phenol/chloroform extraction followed by ethanol precipitation and electroporated into electrocompetent Top10F<sup>+</sup> *E. coli* cells for better plasmid recovery (Hoffman and Winston, 1987). Each plasmid was then sequenced to identify its yeast genomic insert and also retransformed back into yeast expressing Flag-CinB<sup>uPip</sup> to confirm suppression.

**Drosophila Hatch Rate and Cytology Analyses.** Each CI candidate gene (*cinA*, *cinB*, *cinB-K636A*, *cinA-T2A-cinB*) was inserted into the pUASp-attB vector by standard cloning
techniques without codon optimization. In brief, the open reading frames of the genes were amplified by PCR and cloned into the pBluescript SK+ vector followed by restriction digestion and re-ligation of the genes into the pUASp-attB vector. All plasmids were verified by fully sequencing the inserted genes before they were sent to BestGene, Inc. for microinjection of *D. melanogaster* embryos (Beckmann, Ronau and Hochstrasser, 2017). Fly background #9744 was chosen for all gene constructs for site-directed attP/B integration on the 3rd chromosome by PhiC31 integrase with the exception that background #9723 was chosen for site-specific integration of *cinA* on the 2nd chromosome (Groth *et al.*, 2004). Drosophila DNA was purified by homogenizing 30 flies (infected or uninfected by *Wolbachia*) and recovering DNA with phenol/chloroform extraction (Livak, 1984; Beckmann and Fallon, 2012; Beckmann, Ronau and Hochstrasser, 2017). Integrations were confirmed independently by PCR amplifying the candidate genes.

* D. melanogaster* stocks were verified to be uninfected with native *Wolbachia* isolates by PCR amplification of *cidA*<sup>wMel</sup> gene. The MTD-Gal4 line from the Bloomington stock center was found to be infected by wMel and was treated by addition of 20 µg/ml tetracycline to the growth medium for three generations. Once the infection was confirmed to be cleared by PCR amplification of the *cidA*<sup>wMel</sup> gene, flies were reared on untreated media for at least three additional generations to allow for mitochondrial recovery (Chatzispyrou *et al.*, 2015). Both uninfected and wNo-infected *D. simulans* were also verified by PCR amplification of *cinB*<sup>wNo</sup> gene. Flies were reared on standard cornmeal-based solid media and maintained at room temperature. During virgin female collection, stocks were maintained at 18 °C overnight and room temperature the following day. All transgenic flies were maintained as homozygous lines.
Parental flies were generated by crossing either NGT-Gal4 or MTD-Gal4 virgin females with cin transgenic males (Rorth, 1998; Petrella, Smith-Leiker and Cooley, 2007). Only the males emerging between 0 and 30 h from these crosses were collected and used in CI analyses (Yamada et al., 2007). All flies used in both hatch rate and cytological analyses were aged for 2-4 days. Hatch-rate analysis was performed and embryos for cytological analyses were prepared as previously described (Beckmann, Ronau and Hochstrasser, 2017; LePage et al., 2017). In the hatch-rate analysis, one male and one virgin female were placed in a 100-mL polypropylene bottle with the bottom punctured for aeration. An apple juice agar plate (made by adding 26.1 g of dextrose, 13.03 g of sucrose, 9.9 g of agar, 12 mL of 1.25 N NaOH and 203 mL of apple juice to 242 mL of de-ionized water followed by autoclaving) with a small amount of yeast paste smeared in the center of the plate was taped at the bottle opening. Bottles were placed in an incubator at 25 °C overnight. The next morning the agar plates were replaced with freshly yeasted plates which were collected after another 24 h incubation at 25 °C. Both sets of plates were incubated at 25 °C for a total of 48 h before embryo counting. The numbers from the two sets of plates were pooled, and the counting was not blinded. Any crosses with fewer than ten total embryos laid were removed from hatch rate analysis.

To prepare embryos for cytological analyses, ~100 males and ~300 virgin females were placed in a 100 mL plastic cup and allowed to mate for two days at 25 °C with a freshly yeasted apple juice agar plate replaced every day. After two days, a freshly yeasted apple juice agar plate was provided and removed after 1 h. Embryos were then incubated at 25 °C for another hour to ensure each embryo has undergone 1-2 h development. These were then collected, dechorionated, washed in embryo wash buffer (0.6% NaCl, 0.04%
Triton X-100) and fixed immediately in a small scintillation vial containing 5 mL heptane and 5 mL methanol followed by shaking vigorously for 30 s (Sullivan, Ashburner and Hawley, 2000). De-vitellinated embryos were collected, washed three times with methanol, and stored overnight at 4 °C. The old methanol was then removed and replaced with 250 µL of fresh methanol and 750 µL PBTA (1x PBS, 1% BSA, 0.05% Triton X-100, 0.02% sodium azide). After inverting the tube a few times, the solution was replaced with 500 µL PBTA to rehydrate the embryos. PBTA was then replaced with 200 µL of 10 mg/ml RNase A and incubated at 37 °C for 2 h. The RNase was then removed, and the embryos were washed several times with PBS followed by a final wash of PBS-azide. The samples were stained with either Hoechst 33342 at 1:1000 in PBTA or 1 µg/ml propidium iodide. Stained embryos were mounted on glass slides and sealed under cover slips by nail polish. Imaging was done either on a Zeiss Axioskop microscope with AxioCam MRm camera using 10X and 40X objective lenses or a Zeiss LSM 880 Airyscan/NLO confocal microscope with internal PMT using a 20X objective lens. Software used to capture and analyze the images were AxioVision Rel. 4.8 or Zen (blue edition), respectively.

**Statistical Analyses.** All statistical analyses were done in GraphPad Prism 7. Hatch rate analyses were performed by either using one-way ANOVA with pairwise comparison after removal of outliers identified by ROUT method with Q = 1%, or unpaired two-tailed Mann-Whitney U test. Pairwise $\chi^2$ test was used in cytological analyses to compare normal and defect cytological phenotypes.
Chapter III: *In Vitro* Biochemistry of CinA and CinB Proteins


i. **Introduction**

The *cinA-cinB*<sup>wPip</sup> operon was first hypothesized to be another potential CI-inducing genes pair due to its paralogous relationship to the *cidA-cidB* operon identified in the initial proteomic analysis of *C. pipiens* spermathecae isolated following insemination by *wPip*-infected males (Beckmann and Fallon, 2013). That study found that the *Wolbachia* CidA protein was present in the sperm of *wPip*-infected *C. pipiens* and absent from the uninfected mosquitoes. Protein sequence analysis suggested that CinB encodes a putative DUF1703 putative nuclease domain within the PD-(D/E)xK nuclease superfamily (Knizewski *et al.*, 2007; Beckmann and Fallon, 2013). Interestingly, the DUF1703 domain is also found in another selfish genetic element named Medea (Maternal-Effect Dominant Embryonic Arrest) that is involved in a different reproductive manipulation phenomenon in *Tribolium castaneum* (Lorenzen *et al.*, 2008). Here we report additional sequence analysis suggesting that CinB has a second PD-(D/E)xK nuclease domain located at the N-terminus of the protein in addition to the previously reported C-terminal nuclease fold. Furthermore, using recombinant CinB<sup>wPip</sup>
protein, we showed that, for the first time, CinB is indeed a nuclease whose nuclease activity requires both of its PD-(D/E)xK nuclease domains to be active.

ii. Sequence Alignments and Structural Predictions

PD-(D/E)xK nucleases constitute a large and diverse group of enzymes that share little sequence similarity despite retaining a common core structural fold and conserved catalytic residues. CinB has putative PD-(D/E)xK domains located near both the N- and C-termini of the protein (Figure 3). Sequence and secondary structure alignments revealed that these domains have homology to other PD-(D/E)xK nucleases such as archaeal Holliday junction resolvase (Nishino et al., 2001; Knizewski et al., 2007) and are conserved across the so-called type II and type III Wolbachia cif operons, which lack the DUB domain (Figure 4) (Gillespie et al., 2018; Lindsey et al., 2018). Secondary structure predictions by PSIPRED (Jones, 1999) and protein structure prediction by RaptorX (Wang et al., 2016) suggest both the N-terminal and C-terminal nuclease domains (NTND and CTND) share a conserved \( \alpha\beta\beta\alpha\beta \) fold where the two \( \alpha \)-helices are predicted to sandwich a four-stranded \( \beta \)-sheet, a conserved feature in almost all PD-(D/E)xK nucleases (Figure 4 and Figure 5) (Steczkiewicz et al., 2012).

Similar to other PD-(D/E)xK nucleases, both CinB nuclease domains also contain a highly conserved set of catalytic residues (highlighted in Figure 4 and Figure 5) (Pingoud et al., 2005; Knizewski et al., 2007; Steczkiewicz et al., 2012). In most PD-(D/E)xK nucleases, the negatively charged aspartate and glutamate residues help coordinate up to three metal ions that serve as Lewis acids to stabilize the transition state, while lysine functions as a general base for deprotonation of the nucleophilic water
Figure 3. The NTND and CTND of CinB<sup>wpip</sup> with their predicted catalytic aspartate, glutamate and lysine residues labeled. CidB<sup>wpip</sup> also has two predicted PD-(D/E)xK nuclease folds upstream of its deubiquitylase domain; these are related to the dual nuclease domains in CinB<sup>wpip</sup> but lack two or three of the three predicted core catalytic residues.
**Figure 4.** Protein sequence and secondary structure alignments of the NTND and CTND of CinB from various *Wolbachia* strains as well as several known PD-(D/E)xK nucleases. Predicted α-helical residues are labeled H and residues predicted to be part of β-sheets are labeled E. The numbers of excluded residues are shown in parentheses. The last residues numbers are shown at the end of each sequence. Catalytic D-E-K residues are highlighted in black. Residues in red are conserved among all three groups. Residues in gray are conserved within the respective groups.
**Figure 5.** Predicted protein structure of CinB<sup>W<sub>Pip</sub></sup> by RaptorX (A) with zoomed in views to highlight the catalytic triads of the CinB<sup>W<sub>Pip</sub></sup> NTND (B) and CTND (C). Structure 2EWF was determined to be the best template with p-value of 2.16e-03 and an overall uGDT (GDT) score of 138 (18).
molecule (Figure 6) (Pingoud et al., 2005; Knizewski et al., 2007). Interestingly, CidB also contains two potential PD-(D/E)xK folds upstream of its catalytic deubiquitylase (DUB) domain with significant sequence similarities to the NTND and CTND of CinB, respectively (Figure 3). However, these domains in CidB lack residues predicted to be essential for catalytic activity (Gillespie et al., 2018).
**Figure 6.** A model for a two-ion mechanism of phosphodiester bond cleavage by PD-(D/E)xK nucleases. The catalytic aspartate and glutamate help in coordinating two Mg\(^{2+}\) ions that stabilize two neighboring water molecules and also interact with the oxygen atoms in the phosphodiester bond during the transition state of catalysis. The lysine deprotonates one of the water molecules to create a hydroxide ion that serves as the nucleophile to attack the phosphate group. Lastly, the 3’ hydroxyl becomes the leaving group and picks up a hydrogen atom from the adjacent water molecule to complete the reaction. The color of the scheme is as follows: catalytic site of the nuclease in burgundy, DNA in amber with phosphorus in yellow, water molecules in blue, and magnesium ions in green.
iii. **In Vitro Nuclease Activity of CinB Protein**

To test if CinB has nuclease activity, we purified recombinant CinB\textsuperscript{wPip} using polyethylenemine to separate DNA from the protein (Figure 7) (Epling et al., 2015). CinB\textsuperscript{wPip} degraded both linearized and circular dsDNA plasmids (Figure 8-10). The DNase was activated by magnesium or manganese ions but not calcium and was active over a broad range of pHs (Figures 11 and 12). We further examined the nuclease activity of CinB against shorter ssDNA and dsDNA substrates and found it cleaves both forms of DNA (either 45 or 70 residues in length; Figures 13 and 14). Importantly, the catalytic CTND (K636A) or NTND (K279A) mutations each abolished DNase activity (Figures 8, 13, 15 and 16). By contrast, the purified CidB\textsuperscript{wPip} DUB protein did not exhibit DNase activity even after reinstating key catalytic residues in the CTND of CidB\textsuperscript{wPip} (Figure 17). We also tested the nuclease activity of CinB\textsuperscript{wPip} against several different DNA structures, which included both four-way and three-way junctions, but found no substrate preference towards any of the structures tested (Figure 18) (Komori et al., 2000).

We further tested whether CinB\textsuperscript{wPip} could cleave RNA substrates but did not detect activity against either yeast tRNA or a *Drosophila* total RNA extract under our conditions (Figure 19). Thus, CinB is a DNase capable of cleaving both ssDNA and dsDNA. Its activity might be higher against specific DNA sequences or structures, but we have not yet been able to identify such substrates. These *in vitro* experiments support the idea of CinB as a nuclease that requires both PD-(D/E)xK domains for its activity.
Figure 7. An example of purification of full-length recombinant CinB\textsuperscript{wPip}. (A) Chromatogram of CinB\textsuperscript{wPip} purification by size-exclusion chromatography using HiLoad 16/600 Superdex 200 PG column. The first peak between 40 ml and 50 ml elution volume represents the void peak and the second peak between 70 ml and 80 ml elution volume represents CinB\textsuperscript{wPip}. (B) SDS-PAGE gel of the samples collected in the second peak from size-exclusion chromatography. Lanes highlighted represents the fractions that were pooled, concentrated and used as the purified CinB\textsuperscript{wPip} in \textit{in vitro} nuclease assays. Protein purification was done with Judith Ronau.
**Figure 8.** CinB<sub>W</sub>Pip cleaved both circular and linearized pBlueScript SK<sup>+</sup> plasmids. In all reactions, 1 µM CinB<sub>W</sub>Pip was incubated with 15 nM DNA for 90 min. In reactions where CinA<sub>W</sub>Pip was present, 10 µM CinA<sub>W</sub>Pip and 1 µM CinB<sub>W</sub>Pip were incubated on ice for 30 min to allow complex formation before adding to substrate. To stop the reactions, EDTA was added to a 2x molar excess over Mg<sup>2+</sup>. Samples were run in a 0.8% agarose gel, and the gel was stained with ethidium bromide.
**Figure 9.** Time-course experiment showing CinB<sup>Pip</sup> activity against linearized pBluescript SK+ plasmid. In all reactions, 1 μM CinB<sup>Pip</sup> was incubated with 15 nM DNA. To stop the reactions, EDTA was added to a 2x molar excess over Mg<sup>2+</sup>. Samples were run in a 0.8% agarose gel, and the gel was stained with ethidium bromide.
Figure 10. DNase activity of CinB<sup>W<pip></sup></sup> against purified pBluescript SK<sup>+</sup> plasmid, which includes both linear, relaxed circular, and supercoiled forms, with various concentrations of the protein. All forms appear to be susceptible to cleavage, as also seen in Figure 8. Lanes starting from the left: 0, 20, 50, 100, 200, 500 nM, 1, 2, 5, 10, 25, 50 µM. In all reactions, 1 µM CinB<sup>W<pip></sup></sup> was incubated with DNA for 90 min. To stop the reactions, EDTA was added to a 2x molar excess over Mg<sup>2+</sup>. Samples were run in a 0.8% agarose gel, and the gel was stained with ethidium bromide.
Figure 11. Mg$^{2+}$ and Mn$^{2+}$, but not Ca$^{2+}$, support the DNase activity of CinB$^{\text{wPip}}$. In all reactions, 1 µM CinB$^{\text{wPip}}$ was incubated with 15 nM DNA for 90 min in the presence of 5 mM divalent cations. To stop the reactions, EDTA was added to each reaction to a final concentration of 10 mM. Samples were run in a 0.8% agarose gel, and the gel was stained with ethidium bromide.
**Figure 12.** DNase activity of CinB<sup>wPip</sup> is weakly pH-dependent. Reaction pH was between 6.0 (left lane) and 10.5 (right lane) with 0.5 increments between adjacent lanes. In all reactions, 1 µM CinB<sup>wPip</sup> was incubated with 15 nM DNA for 90 min. To stop the reactions, EDTA was added to a 2x molar excess over Mg<sup>2+</sup>. Samples were run in a 0.8% agarose gel, and the gel was stained with ethidium bromide.
Figure 13. CinB<sup>W<Pi></sup> cleaved both single- and double-stranded 70-mer DNAs. CinB<sup>W<Pi></sup> at 1 µM was incubated with 0.5 µM Cy5-labeled DNA for 90 min. Reactions were set up as described in Figure 8. Samples were run in a 9% polyacrylamide/TBE gel and imaged on Typhoon FLA 7000.
**Figure 14.** Time-course experiment showing CinB\textsuperscript{wPip} cleavage of 45-nucleotide or 70-nucleotide Cy5-labeled single-stranded DNA. CinB\textsuperscript{wPip} at 1 µM was incubated with 0.5 µM Cy5-labeled DNA. Reactions were set up as described in Figure 8. Samples were run in a 9% polyacrylamide/TBE gel and imaged on Typhoon FLA 7000.
Figure 15. Mutation of either K636 or K279 eliminated CinB<sup>W<Pi></sup> cleavage of linearized pBluescript SK+ (~3 kb). The mutant proteins appeared to still bind DNA based on the signal remaining in the loading wells (top). In all reactions, 1 µM CinB<sup>W<Pi></sup> was incubated with 15 nM DNA for 90 min. To stop the reactions, EDTA was added to a 2x molar excess over Mg<sup>2+</sup>. Samples were run in a 0.8% agarose gel, and the gel was stained with ethidium bromide.
Figure 16. Catalytic K636A mutation eliminated DNase activity in CinB\textsuperscript{wPip} except at the highest concentrations, which might represent low levels of contaminating \textit{E. coli} nucleases. Lanes starting from the left: 0, 20, 50, 100, 200, 500 nM, 1, 2, 5, 10, 25, 50 \textmu M. In all reactions, 1 \textmu M CinB\textsuperscript{wPip} K636A was incubated with DNA for 90 min. To stop the reactions, EDTA was added to a 2x molar excess over Mg\textsuperscript{2+}. Samples were run in a 0.8% agarose gel, and the gel was stained with ethidium bromide.
Figure 17. CidB<sup>ωPip</sup> protein, even with a potentially reactivated CTND through a double point mutation (V686E, R688K), did not display nuclease activity but did display a weak DNA-binding activity based on the reduced mobility of the substrate DNA at high protein concentrations. Lanes starting from the left: 0, 20, 50, 100, 200, 500 nM, 1, 2, 5, 10, 25, 50 µM. In all reactions, 1 µM CidB<sup>ωPip</sup> was incubated with DNA for 90 min. To stop the reactions, EDTA was added to a 2x molar excess over Mg<sup>2+</sup>. Samples were run in a 0.8% agarose gel, and the gel was stained with ethidium bromide.
Figure 18. CinB<sup>WT</sup> showed no obviously increased activity against various structured DNA substrates. Ten substrates were used in this assay (from left to right: four-way junction, four-way junction with homologous cores, three-way junction, three-way junction with homologous cores, duplex, loop-out, mismatch, half-duplex 1, half-duplex 2, and single stranded DNA). Reactions were set up as described in Figure 8. Samples were run in a 9% polyacrylamide/TBE gel and imaged on Typhoon FLA 7000.
Figure 19. CinB<sup>Pip</sup> showed no RNase activity against either total RNA extract from *D. melanogaster* or yeast tRNA. 500 ng of RNA were used in each reaction. Linearized pBluescript SK+ was used as a positive control for nuclease activity. Reactions were set up as described in Figure 8. Samples were run in a 0.8% agarose gel, and the gel was stained with ethidium bromide.
iv. **Protein-Protein Interaction Between CinA and CinB**

An important aspect of the toxin-antidote model of CI is the binding specificity between the protein pairs within each *cif* operon as suggested by our previous affinity pull-down experiments (Beckmann, Ronau and Hochstrasser, 2017). Here we used isothermal titration calorimetry (ITC) to determine quantitatively the affinity between CinA^wPip^ and CinB^wPip^. ITC revealed a $K_d$ of $25 \pm 1.3$ nM, demonstrating strong binding between the cognate pair (**Figure 20**). We tested whether the tight association of CinA with CinB would inhibit CinB nuclease activity. Pre-incubation of CinB^wPip^ with excess CinA^wPip^ did not reduce DNase activity in our *in vitro* assays, suggesting that CinA rescues cells from CinB-induced toxicity through a distinct mechanism, e.g., cellular re-localization (**Figures 8 and 13**). This observation was not surprising inasmuch as co-incubation of CidA^wPip^ and CidB^wPip^ also did not limit the DUB activity of CidB^wPip^ (Beckmann, Ronau and Hochstrasser, 2017).
Figure 20. The ITC binding isotherm for binding of CinA<sup>wp</sup> to CinB<sup>wp</sup> yielded a $K_d$ of $25 \pm 1.3$ nM. This tight interaction provides support to the hypothesis that the cognate pair exists as a toxin-antidote system. Top panel shows raw injection data over time while the bottom panel shows integrated heats over the course of the reaction (n = 3). Experiment was performed by Judith Ronau.
v. Discussion

Recent studies have revealed the central role of the *cidA-cidB* operon in both CI induction and rescue (Beckmann, Ronau and Hochstrasser, 2017; LePage *et al.*, 2017; Shropshire *et al.*, 2018; Shropshire and Bordenstein, 2019; Beckmann *et al.*, 2019c). CidB is a DUB, and this enzymatic activity is crucial for its ability to promote CI.

Previous findings led to the hypothesis that the *cinA-cinB* locus, with a pair of genes paralogous to *cidA-cidB*, might also be involved in *Wolbachia*-induced CI despite lacking a known DUB domain. Here we have shown that CinB is a nuclease, specifically a DNase. It remains possible that the apparent lack of RNase activity reflects the absence of a crucial cofactor or appropriate reaction conditions. The DNA-cleaving activity we have detected is of broad specificity but weak, at least under the conditions tested. An unregulated, highly active nuclease would likely be harmful to *Wolbachia*; we note that expression of CinB in *E. coli* is not obviously deleterious to growth. It is possible that the enzyme is more potent against particular DNA sequences or structures. For example, during the exchange of protamine for histones that occurs in the male pronucleus in the nascent zygote, transiently uncoated paternal DNA may be prone to forming cruciforms or other structures that are preferred substrates for CinB. Among the earliest signs of CI are chromosome condensation defects in the male pronucleus (Callaini, Dallai and Riparbelli, 1997; Landmann *et al.*, 2009).

Both the NTND and CTND of CinB are highly conserved across many *Wolbachia* strains. Interestingly, our *in vitro* enzyme analysis suggests the nuclease activity of CinB requires both of its nuclease domains to be active. It is possible that the two domains feature a mechanism where one domain is involved in substrate recognition while the
other is responsible for the actual phosphodiester bond cleavage. Another possibility is that both nuclease domains recognize and cleave DNA substrates in a cooperative manner such that mutation in one nuclease domain is sufficient to inhibit the overall function of the protein. Many PD-(D/E)xK nucleases function as homodimers, which also brings together a pair of PD-(D/E)xK domains (Knizewski et al., 2007). Structural analysis will be needed to gain a deeper understanding of the exact CinB reaction mechanism.
Chapter IV: Analysis of the wPip cin Operon in Saccharomyces cerevisiae


i. Introduction

The first evidence suggesting that cifA and cifB genes form a toxin-antidote pair came from expression of these genes in yeast S. cerevisiae. Expressing either CidB\textsuperscript{wPip} or CinB\textsuperscript{wPip} in yeast induces a temperature-sensitive growth defect that can only be rescued when the cognate A protein from the same wPip Wolbachia strain is co-expressed (Beckmann, Ronau and Hochstrasser, 2017). The yeast lethality and rescue phenotypes were also later recapitulated using the cidA-cidB genes from the wHa strain of Wolbachia (Beckmann \textit{et al.}, 2019c). Catalytic protease domain cysteine-to-alanine mutation in CidB\textsuperscript{wPip} or a D-E-K catalytic triad triple mutation to alanines in the CinB\textsuperscript{wPip} C-terminal nuclease domain eliminated the proteins’ toxicity in yeast (Beckmann, Ronau and Hochstrasser, 2017). Here we report that single amino acid to alanine mutation in either the N-terminal or C-terminal nuclease domains in CinB\textsuperscript{wPip} is sufficient in inhibiting its toxicity in yeast. This result, together with our \textit{in vitro} enzymatic analysis, suggests that CinB is a nuclease toxin that requires two active nuclease domains.

A recent high-copy suppressor screen utilizing a yeast genomic tiling library identified karyopherin-α, a nuclear import receptor, as a strong suppressor of the toxicity
of CidB<sup>W</sup><sub>Pip</sub> in yeast, providing useful insights on the molecular mechanism of CI caused by <i>cid</i> operon (Beckmann <i>et al.</i>, 2019c). Here we report our preliminary data on a similar suppressor screen looking for potential suppressors of CinB<sup>W</sup><sub>Pip</sub>-induced toxicity in yeast. Lastly, our ITC experiment indicated strong protein-protein interaction between CinA<sup>W</sup><sub>Pip</sub> and CinB<sup>W</sup><sub>Pip</sub>. However, CinA binding did not inhibit the nuclease activity of CinB <i>in vitro</i>, suggesting that the rescue effect of CinA is likely caused by some other mechanism. One possibility is that CinA binding re-localizes CinB nuclease leaving it inaccessible to its target substrate. Here we also report some of our preliminary data on the localization of CinA and CinB in yeast along with our future directions.

**ii. Cin Operon Expression in Yeast**

To determine if the toxicity of CinB<sup>W</sup><sub>Pip</sub> expression in yeast is due to its nuclease activity, we generated a panel of mutants with mutations in catalytic residues of either the NTND or CTND. Simultaneously changing all three CTND catalytic residues to alanines (3A) or individually (D614A, E634A or K636A) was sufficient for eliminating the CinB-induced growth defect (<b>Figure 21A</b>). Mutation of the corresponding NTND catalytic residues also eliminated CinB<sup>W</sup><sub>Pip</sub>-induced toxicity, suggesting that the toxin function of CinB<sup>W</sup><sub>Pip</sub> requires both nuclease domains to be active (<b>Figure 21B</b>). Changes in protein levels due to the mutations in either domain cannot account for the loss of temperature-dependent lethality (<b>Figures 22-24</b>). Interestingly, CinB seemed to undergo proteolytic cleavage in yeast cells, creating two fragments. While the N-terminal fragment is stable, the C-terminal fragment appeared to undergo further degradation in cells and thus could not be detected by Western blot analysis (<b>Figure 23 and Figure 24</b>). Nonetheless, these
results together with our enzymatic analysis are consistent with the hypothesis that both N- and C-terminal nuclease domains are important for the function of CinB toxin.
Figure 21. CinB<sup>xPip</sup> toxicity in the <i>S. cerevisiae</i> BY4741 strain. All genes were N-terminally FLAG-tagged and cloned into pYES2, a galactose-inducible expression vector. Six-fold serial dilutions were conducted and yeast were allowed to grow for two days before imaging. Expression of wild-type CinB caused a temperature-dependent growth defect. Such growth defects were not observed in yeast expressing CinB with predicted inactivating point mutations in either the CTND (A) or NTND (B). All serial dilutions were done in triplicate with independent transformants.
Figure 22. Relative expression levels of WT and mutant CinB proteins in yeast.

Equivalent numbers of yeast based on OD$_{600}$ were lysed, and the lysates were resolved by SDS-PAGE and immunoblotted for CinB by α-FLAG antibody; PGK served as a loading control. A fraction of CinB is proteolytically cleaved in yeast and the N-terminal fragment is labeled with an asterisk. (see Figures 23 and 24).
Figure 23. Effect of doubly tagged Flag-CinB<sup>wPip</sup>-HA on *S. cerevisiae* (BY4741) growth. Growth assay was performed as Figure 21. Expression of Flag-CinB<sup>wPip</sup>-HA induced the same temperature-sensitive grow defect as the singly tagged Flag-CinB<sup>wPip</sup>. The growth defect was abolished when yeast expressed the mutated derivative, Flag-CinB<sup>wPip-K636A</sup>-HA, suggesting the C-terminal HA tag did not interfere with the toxin-like function of CinB.
**Figure 24.** Western blot analysis of yeast expressing either Flag-CinB\(^{\text{Pip-HA}}\) or the catalytic K636A mutant derivative, confirming that the inactivating mutation did not reduce the expression level of CinB. The protein undergoes an apparent proteolytic cleavage in yeast cells, as suggested by a N-terminal species at ~37 kDa in the anti-Flag blot. However, a C-terminal fragment of CinB was not seen in anti-HA blot, indicating that this fragment, if indeed synthesized, might have been further degraded.
iii. **CinB Suppressor Screen Utilizing a Yeast Genomic Tiling Library**

*(Preliminary Findings)*

The fact that nuclease activity of CinB is responsible for its toxicity in yeast led us to hypothesize that the growth defect caused by CinB might be due to CinB-induced DNA damage, resulting in genomic instability. Using a yeast high-copy genomic tiling library, we sought to identify yeast genes that can suppress CinB\(^{\text{wPip}}\)-induced growth lethality, hoping to gain insight into the molecular mechanism behind its function. The screen was performed in two conditions. In one condition, after transformation of the genomic library, colonies were allowed to form before the expression of CinB\(^{\text{wPip}}\) was induced by galactose. Unfortunately, this condition resulted in possible homologous recombination of the *URA3* selection marker between the two plasmids used in the screen, leading to a high number of false-positive results (*Table 1*). In the second screening condition, CinB\(^{\text{wPip}}\) expression was induced right after the transformation of the library plasmids by plating directly onto galactose. Suppressor plasmids recovered under this more stringent condition showed no homologous recombination of *URA3* marker (*Table 2*). The screen was performed only once under each condition. Future work involves doing the screen in triplicate under the more stringent conditions followed by subcloning the individual genes from each candidate plasmid to identify the responsible suppressor.
**Table 1.** Yeast suppressor plasmids identified under the condition where after the transformation of genomic tiling plasmids colonies were allowed to grow for a few days before the expression of CinB\(^{wPip}\) was induced by galactose. Candidate suppressor plasmids were sequenced to verify if homologous recombination of *URA3* occurred between the library plasmids and pRS416gal1.
Table 2. Yeast suppressor plasmids identified under the condition where CinB\textsuperscript{w\textsubscript{Pip}} expression was induced right after the transformation of the library plasmids. None of the candidate suppressors obtained under this condition had homologous recombination of \textit{URA3} occurred between the library plasmids and pRS416gal1.
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iv. Localization of CinA and CinB in Yeast (Preliminary Findings)

To determine the localization of CinA/B^wPip and CidA/B^wPip in yeast, we tagged CinA^wPip and CidA^wPip with mCherry and tagged CinB^wPip and CidB^wPip with eGFP. N-terminal mCherry or eGFP fusions disrupted the proper toxin-antidote behavior of both cif operons in yeast (Figures 25 and 26). In contrast, C-terminal mCherry and eGFP fusions behave similarly to the untagged proteins in toxicity and rescue assays (Figure 27 and Figure 28). However, preliminary data indicated that the expression levels of these proteins in yeast were too low to be detected by live-cell microscopic imaging. Future directions involve either genomic integration of these genes for more consistent protein expression or doing immunofluorescence imaging with fixed yeast cells. We created 3xFlag-tagged CinB^wPip and HA-tagged CinA^wPip in yeast plasmids and showed that these tags did not disturb the toxin-antidote behavior between CinB and CinA, and thus are suitable to use for immunofluorescent imaging (Figure 29).
Figure 25. Effect of N-terminal fluorescent protein fusion of CifPip on S. cerevisiae (BY4741) growth. Growth assay was performed as Figure 21. All CifAPip proteins are N-terminal mCherry fusions while all CifBPip proteins are N-terminal eGFP fusions. The eGFP fusion CifBs lost their ability to induce temperature dependent growth lethality in yeast. Co-expression of mCherry-CinAPip with either eGFP-CinBPip or eGFP-CidBPip induced growth defect, which was not observed if the proteins are untagged, suggesting the N-terminal fluorescent protein fusions created artificial phenotypes that are related to the protein tags.
Figure 26. Western blot analysis of yeast expressing either eGFP-CifB<sup>W</sup>Pip (left) or mCherry-CifA<sup>W</sup>Pip (right). Uninduced samples were grown in glucose media and served as negative controls. Equivalent numbers of yeast based on OD<sub>600</sub> were lysed, and the lysates were resolved by SDS-PAGE and immunoblotted for either CifB by anti-GFP (left) or CifA by anti-mCherry (right) antibodies. The expected sizes for eGFP-CidB and eGFP-CinB are about 161 kDa and 107 kDa, respectively. And the expected sizes for mCherry-CidA and mCherry-CinA are 84 kDa and 78 kDa, respectively.
**Figure 27.** Effect of C-terminal fluorescent protein fusion of Cin^wtPip^ on *S. cerevisiae* (BY4741) growth. Growth assay was performed as Figure 21. CinA and CinB without fluorescent protein fusion are N-terminally Flag-tagged. Though weaker than Flag-CinB, CinB-eGFP fusion protein still possesses toxicity at 36°C. Furthermore, CinA-mCherry fusion is able to rescue yeast from growth defect induced by either Flag-CinB or CinB-eGFP fusion, indicating that the rescue behavior of CinA was not altered by the C-terminal mCherry tag.
**Figure 28.** Effect of C-terminal fluorescent protein fusion of Cid\textsuperscript{wPip} on *S. cerevisiae* (BY4741) growth. Growth assay was performed as **Figure 21.** CidA and CidB without fluorescent protein fusion are N-terminally Flag-tagged. CidB-eGFP fusion showed slightly stronger toxicity at 37°C compared to Flag-CidB. CidA-mCherry fusion is able to rescue yeast from growth defect induced by either Flag-CidB or CidB-eGFP fusion, indicating that the rescue behavior of CidA was not altered by the C-terminal mCherry tag.
**Figure 29.** Effect of 3xFlag-tagged CinB<sup>^Pip</sup> and HA-CinA<sup>^Pip</sup> on *S. cerevisiae* (BY4741) growth. Growth assay was performed as **Figure 21.** Unlabeled CinA and CinB are N-terminally Flag-tagged. Both 3xFlag-CinB and CinB-3xFlag exhibited similar temperature-dependent growth lethality as Flag-CinB. Furthermore, HA-CinA is able to rescue yeast from growth defect induced by 3xFlag-CinB, CinB-3xFlag, or Flag-CinB.
v. Discussion

Consistent with data from our in vitro nuclease assays, the temperature-sensitive growth defect in S. cerevisiae caused by CinB also requires both of its N- and C-terminal nuclease domains to be active. Furthermore, we showed that single amino acid to alanine mutations in either of its nuclease domains is sufficient for inhibiting its toxicity in yeast. These data together with our enzymatic analyses highlight CinB as a dual nuclease domain toxin whose toxicity is linked to its nuclease activity.

Despite the strong protein-protein interaction between CinA\textsuperscript{wPip} and CinB\textsuperscript{wPip}, pre-incubation of CinB\textsuperscript{wPip} with 10-fold molar excess CinA\textsuperscript{wPip} did not reduce DNase activity in our in vitro assays. Similarly, co-incubation of recombinant CidA\textsuperscript{wPip} and CidB\textsuperscript{wPip} also did not limit the DUB activity of CidB\textsuperscript{wPip}, suggesting that CifA rescues cells from CifB-induced toxicity through a mechanism not involving direct inhibition of enzyme activities (Beckmann, Ronau and Hochstrasser, 2017). One hypothesis we favor is that CifA binding changes the cellular localization of CifB thus hindering it from accessing its substrates and preventing toxicity to the cells. To test this hypothesis, we created CifA-mCherry fusion and CifB-eGFP fusion and examined the protein localization in yeast. We showed that N-terminal fluorescent protein fusion disrupts the proper toxin-antidote behavior of the Cif proteins while C-terminal fusions maintains their toxin-antidote-like property. Unfortunately, the expression level of these proteins was too low for live-cell imaging in yeast. Future work should exploit strategies to increase expression level of the protein or different imaging methods such as immunofluorescent imaging with fixed yeast cells.
Chapter V: Cin Operon in Transgenic Drosophila Melanogaster


i. Introduction

Transgenic fruit fly studies have shown that the cid operons from both wPip and wMel Wolbachia strains can induce embryonic lethality with early-stage developmental defects similar to CI embryos (Beckmann, Ronau and Hochstrasser, 2017; LePage et al., 2017). Transgenic female flies expressing either cidAwMel and cidA-cidBwMel can rescue both transgenic CI caused by cidwMel and natural CI caused by wMel-infected male flies (LePage et al., 2017; Shropshire et al., 2018). Prior to this work, there was no direct evidence proving that the cin operon is also capable of inducing CI and rescue.

Sequence comparison indicated the cin operon was a distant paralog of the cid operon (Beckmann and Fallon, 2013). Similar to cid, the cin operon also induced toxicity and rescue when expressed in yeast S. cerevisiae (Beckmann, Ronau and Hochstrasser, 2017). Most importantly, the fact that some strong CI-inducing Wolbachia strains, such as the wNo strain that infects Drosophila simulans, contain only cin but not cid type operons and that neither operon is present in wAu, a close relative of wMel that does not induce CI, strongly suggested that the cin-type operon should also be able to induce CI independent of the cid operon. Here we report that expression of the cin operon in
transgenic male flies can indeed induce post-zygotic male sterility and embryonic defects typical of CI. Importantly, transgenic cinA can rescue defects in egg hatch rates when expressed in females.

**ii. Hatch Rate Analysis of Transgenic Fruit Flies**

To test the ability of the cin operon to induce CI in the absence of Wolbachia infection, we created transgenic D. melanogaster lines containing cin^wPip genes by site-directed PhiC31-mediated integration (Groth et al., 2004). Recent studies utilized two distinct strategies to generate transgenic Cid-expressing flies (Beckmann, Ronau and Hochstrasser, 2017; LePage et al., 2017; Shropshire et al., 2018). Here we attempted both. In the first, cinA^wPip and cinB^wPip genes were integrated into separate chromosomes (UAS:cinA/UAS:cinA; UAS:cinB/UAS:cinB) while the second strategy utilized a fusion of cinA^wPip and cinB^wPip genes linked by a T2A viral peptide-coding sequence (UAS:cinA-T2A-cinB/UAS:cinA-T2A-cinB) that causes the ribosome to terminate and immediately restart translation on the T2A sequence, resulting in the synthesis of two separate proteins from one transcript (Figure 30). All fly lines were verified to be uninfected with native Wolbachia isolates by PCR amplification of cidA^wMel gene (Figure 31). Both the Nanos-Gal4-Tubulin (NGT) driver and maternal triple driver (MTD-Gal4) were used for specific expression of the Gal4 transcription factor in fly germline cells, stimulating transcription of the cin transgenes through their Gal4-responsive upstream activation sequences (UAS) (Rorth, 1998; Petrella, Smith-Leiker and Cooley, 2007; White-Cooper, 2012). The MTD driver can increase the transcript levels of cidA^wMel by over 1000-fold relative to expression with the NGT driver, allowing transgenic cidA^wMel females to rescue CI
Figure 30. Two strategies were used to generate transgenic flies: (A) $cinA^{w_{Pip}}$ was inserted into the second chromosome and $cinB^{w_{Pip}}$ was inserted into the third chromosome ($UAS:cinA/UAS:cinA; UAS:cinB/UAS:cinB$), or (B) $cinA^{w_{Pip}}$ and $cinB^{w_{Pip}}$ were linked by a T2A viral sequence (yellow) and inserted into the third chromosome ($UAS:cinA-T2A-cinB/UAS:cinA-T2A-cinB$).
Figure 31. PCR verification of Wolbachia infection status in both *D. melanogaster* and *D. simulans*. Top: Positive controls for PCR amplification using the actin gene. Bottom: PCR amplifications of either *cinA<sup>WMel</sup>* for *D. melanogaster* or *cinB<sup>WNo</sup>* for *D. simulans* (last two lanes) showing that none of the *D. melanogaster* stocks used was infected by *wMel* Wolbachia and only *wNo (+)* stock was infected by *wNo Wolbachia*. 
induced by male flies infected with wMel (Shropshire et al., 2018). The level of CI was determined from the percentage of embryos that hatched into larvae.

When crossed to wild-type (WT) females, males transgenic for cinA<sup>WpIp</sup> alone under the control of NGT driver did not affect hatch rates, whereas males transgenic for cinB<sup>WpIp</sup> alone produced a ~30% hatch rate reduction (Figure 32 Top Panel). This reduction was partially but significantly suppressed if the catalytic lysine-636 residue was mutated to alanine. Consistent with hatch rate data for transgenic cidA<sup>WMel</sup> and cidB<sup>WMel</sup> (LePage et al., 2017), male flies transgenic for both cinA<sup>WpIp</sup> and cinB<sup>WpIp</sup> (expressed with the NGT driver) induced a stronger reduction in hatch rates (~50-60%) when compared to males transgenic for cinB<sup>WpIp</sup> alone (Figure 32 Middle Panel). Both transgenic strategies generated a similar level of hatch rate reductions when using the NGT driver. Importantly, the catalytic lysine-636 to alanine mutation significantly weakened (by ~30%) the effect on hatch rates caused by transgenic UAS:cinA/+ NGT:Gal4/+; UAS:cinB/+ males, suggesting the reduction in hatch rates depended on the nuclease activity of CinB<sup>WpIp</sup> (Figure 32 Middle Panel).

Transgenic cinA-T2A-cinB males induced a greater hatch rate reduction (nearly 90%) when under the control of the strong MTD driver. Importantly, we observed rescue of these low hatch rates by crossing transgenic cinA-T2A-cinB<sup>WpIp</sup> males to females that were transgenic for cinA<sup>WpIp</sup> (Figure 32 Bottom Panel). This strengthens and generalizes the hypothesis of cifA as the antidote gene in the different cif operon systems. Unexpectedly, transgenic UAS:cinA/+; UAS:cinB/+ males from five independent homozygous lines all failed to lower hatch rates under the MTD-Gal4 driver when crossed to WT females; this is discussed below.
**Figure 32.** Expression of the *cinA-cinB*<sup>wpip</sup> genes in flies induces CI-like embryo killing and rescue phenotypes. (Top panel) Crosses with flies transgenic for either *cinA*<sup>wpip</sup> or *cinB*<sup>wpip</sup> alone expressed using the NGT driver (highlighted in orange). Data in burgundy represents CI-inducing crosses while green represents either rescue or weakening of CI induction by the CinB-K636A mutation. All control crosses are shown in gray. n = 40-64. (Middle panel) Crosses with flies transgenic for the entire *cinA-cinB*<sup>wpip</sup> operon under control of the NGT driver. n = 38-58. Error bars in (C) and (D) represent standard deviation of the mean; **P < 0.01, ***P < 0.001, ****P < 0.0001 by ANOVA with multiple comparison between all groups. (Bottom panel) Transgenic CI in crosses with flies transgenic for the entire *cinA-cinB*<sup>wpip</sup> operon expressed using the strong maternal triple driver (MTD, highlighted in red) could be rescued by transgenic *cinA*<sup>wpip</sup> females. Vertical lines represent medians. n = 23-57. ****P < 0.0001 by two-tailed Mann-Whitney U test.
iii. Cytology of Transgenic and Natural CI Induced by cin Operon

We next determined whether the hatch-rate reduction caused by transgenic expression of the $cinA$-$cinB^{wPip}$ genes could be traced to embryonic defects similar to those seen in natural CI. The cytology of fly embryos infected with Wolbachia strains known to contain only a $cin$ operon has never been reported. Therefore, we first analyzed embryos from $D. simulans$ infected by $wNo$, a Wolbachia strain containing a $cin$ but no $cid$ operon, for comparison to the cytological analysis of our transgenic flies (Figure 31) (Gillespie et al., 2018; Lindsey et al., 2018). When mated with uninfected females, the $wNo$-infected male flies induced strong embryonic hatch rate reduction similar to its previously reported CI penetrance (Figure 33) (Mercot et al., 1995; Poinsot et al., 1998). The $wNo$-infected flies also exhibited a range of cytological defects similar to those previously reported for $wMel$-induced CI with the exception that no regional mitotic failure was observed (Figure 34) (LePage et al., 2017).

Most importantly, all transgenic crosses that resulted in reduced hatch rates induced CI-like embryonic defects in embryos collected after 1-2 h development (Figures 35 and 36). Similar to the $wNo$ infection control, about half of the embryos from the cross between transgenic $cinA$-T2A-$cinB$ males under the MTD-Gal4 driver and WT females exhibited an abnormal cytological phenotype. Together, these data establish a role for the Wolbachia $cinA$-$cinB$ genes as an independent CI-inducing gene system and support the hypothesis that $cifA$ genes are specifically required for the rescue of CI, similar to their ability to suppress $cifB$ toxicity in yeast.
Figure 3. wNo infected male *D. simulans* produced a ~80% hatch rate reduction when crossed to uninfected females. Error bar represents standard deviation of the mean. n = 30-33. ****$P < 0.0001$ by two-tailed Mann-Whitney U test.
**Figure 34.** Representative images of propidium iodide-stained embryos from incompatible crosses between *w*No-infected males and uninfected females showing (A) an unfertilized embryo, (B) a normal embryo after 1 h development, (C) a normal embryo after 2 h development, (D) an embryo with early mitotic failure, and (E) an embryo showing anaphase chromatin bridging.
**Figure 35.** Embryos from crosses between transgenic *D. melanogaster* males expressing the *cinA-cinB\textsuperscript{WPlp}* operon and wild type females show CI-like cytology. Representative images of propidium iodide-stained embryos from females showing (A) an unfertilized embryo, (B) a normal embryo after 1 h development, (C) a normal embryo after 2 h development, (D) an embryo with early mitotic failure, and (E) an embryo showing anaphase chromatin bridging.
Figure 36. Quantification of embryo cytology. wNo (-) and wNo (+) represent *D. simulans* uninfected or infected by wNo, respectively. wNo infection was confirmed by PCR amplification of the *cinB*<sup>wNo</sup> gene (see Figure 31). For the transgenic *D. melanogaster* crosses (bottom), only the *cinA*-T2A-*cinB* crosses under the MTD-Gal4 driver (highlighted in red. NGT-Gal4 driver highlighted in orange) strongly phenocopied the natural CI cytology. The number of embryos examined in each cross is shown. Embryos exhibited normal cytology after 1-2 h were grouped together and are shown in teal. ****$P < 0.0001$ by Chi-Square test comparing normal (Figures 34B and 34C) and abnormal (Figures 34A, 34D and 34E) cytological phenotypes.
iv. Discussion

Recent studies have shown that the *cidA-cidB* operon can induce both CI and rescue when transgenically expressed in *D. melanogaster* (Beckmann, Ronau and Hochstrasser, 2017; LePage *et al.*, 2017; Beckmann *et al.*, 2019c; Shropshire *et al.*, 2018; Shropshire and Bordenstein, 2019). Our work here demonstrates the sufficiency of the *cinA-cinB* operon for both inducing CI by expression in males and rescuing CI through expression (of *cinA*) in females. Cytology of CI embryos induced by the *cin*-type operon alone had not been previously characterized. Here we show that transgenic CI embryos induced by *cinA-cinB* operon exhibited similar cytological defect as naturally occurring CI embryos from *D. simulans* infected by w*No Wobachia* strain, which contains only the *cin* nuclease-type operon. Together our transgenic fruit fly data characterized the *cin*-type operon as CI-inducing genes independent to the *cid*-type operon. The rescue of transgenic CI through expression of *cinA* in females further highlights the role of *cifA* genes as the antidotes in the CI-inducing operon systems.

Surprisingly, *UAS:cinA/+; UAS:cinB/+* males did not induce embryonic lethality when under the control of the MTD-Gal4 driver, unlike *UAS:cinA-T2A-cinB/UAS:cinA-T2A-cinB* males, in our transgenic CI crosses. It is possible that the operon-like structure of *cinA-T2A-cinB* better mimics the natural expression ratio of the *cinA* and *cinB* genes, which was shown to be important in the *cid* operon (Bonneau *et al.*, 2018a). If relative expression of CinA were too high in *UAS:cinA/+; UAS:cinB/+* flies under the MTD-Gal4 driver, it could dampen CinB toxicity in CI crosses; alternatively, insufficient CinA during male spermiogenesis might selectively kill sperm precursors with high CinB levels. Another unexpected finding from our transgenic fly analyses was that MTD-
driven expression of *cinA*-T2A-*cinB* in females caused a high level of embryonic lethality. It is likely that such embryonic lethality is due to the toxicity from expressing the operon at a very high level.
Chapter VI: Summary and Discussion

CI is the most common form of *Wolbachia*-induced reproductive manipulations and has long been utilized in mosquito-borne disease control to reduce the spread of Dengue and Zika viruses, and other human pathogens. For many decades, the molecular basis of *Wolbachia*-induced CI had been a mystery. In 2017, the *cidA-cidB* operon was reported by two groups to be sufficient in *Wolbachia*-induced CI (Beckmann, Ronau and Hochstrasser, 2017; LePage *et al.*, 2017). The *cin* operon, a distant paralog of the *cid*-type operon, was also proposed to be another CI-inducing operon that contains putative nuclease domains. The fact that some strong CI-inducing *Wolbachia* strains contain only *cin* but not *cid* operons and that neither operon is present in *Wolbachia* strains that do not induce CI led us to hypothesize that the *cin* operon should also be able to induce CI independently of the *cid* operon.

Here we have shown that transgenic expression of the *cinA-cinB* operon in male *D. melanogaster* indeed recapitulates CI-like embryonic lethality when these males are mated with wild-type uninfected females. Importantly, such transgenic CI can be rescued by expression of *cinA* in uninfected female flies. The rescue of transgenic CI by *cinA* alone is fully consonant with our earlier finding that *cinA* suppresses *cinB*-induced toxicity in yeast (Beckmann, Ronau and Hochstrasser, 2017). These results validated the role of the *cin*-type operon in inducing CI and of *cinA* in rescue in CI-inducing *Wolbachia* strains lacking *cid* operons.

Interestingly, when mated with wild-type uninfected females, transgenic males expressing *cinB* alone only induced a weak embryonic hatch rate reduction compared
to transgenic cinA-cinB<sup>wPip</sup> males. This result is, however, similar to previously reported study with cid operon from wMel Wolbachia strain where expression of neither cidA<sup>wMel</sup> or cidB<sup>wMel</sup> alone in transgenic males was sufficient in inducing CI (LePage et al., 2017; Shropshire et al., 2018; Shropshire and Bordenstein, 2019). It is intriguing how in both cases the cifA gene can contribute to CI while expressed in males and rescue while expressed in females. One possibility is that CifA and CifB protein complex forms a toxin that targets male-specific host factor in sperms and can be reversed later by CifB binding to maternally-supplied CifA. One way to test this hypothesis is by creating mutations in the CifA-CifB interface to disrupt protein-protein interaction. If the hypothesis holds true, one can expect such mutations to abolish the ability of CifA-CifB transgenic males to induce CI and the rescue effect of CifA in transgenic females.

Our transgenic cin<sup>wPip</sup>-induced CI embryos showed the same cytological defects as embryos from CI crosses between wNo-infected male D. simulans and uninfected females, further highlighting the central role of the cin-type operon in inducing CI in Wolbachia strains, such as wNo, that lack cid operons. The embryonic defects caused by cin operon are similar to previously reported cytological defects induced by cid-type operons with the exception that regional mitotic failure was only observed in cid- but not cin-induced CI embryos (LePage et al., 2017). Such differences can potentially be related to different underlying molecular mechanisms in how the two types of toxin induce CI. To unravel the exact molecular mechanism of CI, it will be important to identify the substrates of both the DUB and the nuclease toxins in both Wolbachia-infected flies and transgenic flies expressing the cif operons.
Our *in vitro* nuclease activity assay confirmed CinB to be a DNase that contains two nuclease domains and has activity towards both single- and double-stranded DNAs. The nuclease activity of CinB is also essential for its toxicity in yeast and its ability to induce CI in when transgenically expressed in fruit flies. Though not detected in our study, it remains possible that CinB has RNase activity against certain RNAs. Importantly, the DNase activity of CinB requires a fully functional catalytic D-E-K triad in both of its nuclease domains. It is possible that the two domains combine in a mechanism where one domain is involved in substrate recognition while the other is responsible for the actual phosphodiester bond cleavage. Another possibility is that both nuclease domains recognize and cleave DNA substrates in a cooperative manner such that mutation in one nuclease domain is sufficient to inhibit the overall function of the protein. Many PD-(D/E)xK nucleases function as homodimers, which also brings together a pair of PD-(D/E)xK domains (Knizewski *et al.*, 2007). Structural analysis will be needed to gain a deeper understanding of the exact CinB reaction mechanism.

We have also measured a tight physical association between CinA and CinB (*Figure 20*). Nevertheless, this interaction did not inhibit the catalytic activity of the nuclease, a result similar to our finding with CidA and CidB where association of the cognate pair failed to suppress the DUB activity of CidB against model substrates such as ubiquitin polymers (Beckmann, Ronau and Hochstrasser, 2017). The rescue mechanism might instead be caused by CinA or CidA association changing the cellular localization of the cognate B toxins or their ability to bind their critical targets *in vivo*. A recent study found that the catalytic inactive CidB protein interacts with both karyopherin-α (Kap-α), a nuclear import receptor, and the P32 histone chaperone from *Drosophila melanogaster*.
protein extracts (Beckmann et al., 2019c). These data suggest that CidB could potentially localize in the nuclei and CidA binding might hinder CidB from entering nuclei and accessing its target substrates. Nonetheless, future work should focus on examining the localization of CifAs and CifBs proteins in fruit flies infected by *Wolbachia* or that are transgenic for *cif* operons. It would also be worthwhile to look at the yeast localization of CifA-CifB protein pairs that are capable of inducing both toxicity and rescue.

While details of the CinB nuclease’s mode of action remain to be worked out, our results highlight a novel mechanism for CI that is likely to be broadly relevant to *Wolbachia*-induced CI in many different arthropods. An interesting question is why some *Wolbachia* carry both *cin* and *cid* loci, as is true for wPip, or have B genes predicted to encode active nuclease and DUB activities in the same polypeptide (CndB class) ([Figure 2](#)) (Gillespie et al., 2018; Beckmann et al., 2019b). The CI loci are usually part of WO prophage regions, and repeats or partial repeats are common. Thus, these paralogs may be subject to rapid evolutionary changes that allow shifts between DUB-dominated and nuclease-dominated CI mechanisms in response to host adaptations to the endosymbiont. Finally, PD-(D/E)xK nucleases may have roles in other host-parasite interactions. The selfish genetic element *Medea*, for instance, which kills embryos expressing *Medea* maternally but lacking the gene in the zygote, also encodes a putative nuclease of this class (Lorenzen et al., 2008).
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## Appendix II: Drosophila Lines Used in This Study

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### Appendix III: Primers Used in This Study

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<tr>
<th>Primer name</th>
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<th>Sequence 5' to 3'</th>
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<tr>
<td>JFB058</td>
<td>F</td>
<td>GAGAAGTTGGCGGTT-ATGTTGGTTATAAATGCTACTGATC</td>
<td>Site-directed mutagenesis to change Aspartate (D) to Alanine (A) at the DEK site of CinB&lt;sub&gt;W&lt;/sub&gt;Pip</td>
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<td>CATAACCGCCAACCTTCTCCACCACCTATTTGAAATTTC</td>
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<td>GAATAGCGCTAAAAATTGCTAAGAAAGGAGAATTGG</td>
<td>Site-directed mutagenesis to change Glutamate (E) to Alanine (A) at the DEK site of CinB&lt;sub&gt;W&lt;/sub&gt;Pip</td>
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<td>Site-directed mutagenesis to change both E and K to Alanine (A) at the DEK site of CinB&lt;sub&gt;W&lt;/sub&gt;Pip</td>
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<td>JFB146</td>
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<td>HC200</td>
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<td>GAGCTTCAGCAGGT-ACTGGTGAGATAAGT-ACAGTG</td>
<td>Primers to make quickchange CinB K279 =&gt; A</td>
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<td>Four-way junction sequences (4J, 1+2+3+4; 4Jh, 2+5+6+7; 4Jhs, 5+7+8+9); sequence 2 and 5 have Cy5 label versions</td>
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<td>Sequences for duplex (D, 2+10), looped-out (L10, 2+11), mismatched (G/A, 2+12) and half duplex DNAs (Hd1, 2+5; Hd2, 2+6)</td>
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