A valine to leucine mutation in hypomorphic *Wolbachia* CidB reduces both deubiquitylation and cytoplasmic incompatibility

by

Daniel Effiong Akwa

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Approved by

John F. Beckmann, Assistant Professor Entomology and Plant Pathology
Nannan Liu, Professor Entomology and Plant Pathology
Paul A. Cobine, Professor Biological Sciences
Arthur G. Appel, Professor Entomology and Plant Pathology

Except where specified, data presented in this paper were obtained by me (Daniel Effiong Akwa) throughout the duration of my master's degree in entomology at the Department of Entomology and Plant Pathology - Auburn University between August 2019 and August 2021.

Abstract

Cytoplasmic incompatibility (CI) remains one of the most highly researched and applied phenotype conferred by the bacterium, *Wolbachia* on the host organisms it infects. For programs that rely on the CI phenomenon, the strength of CI expression is critical and must be maintained at high levels. Unfortunately, CI strength has been observed to vary and the determinants for the variability in cytoplasmic incompatibility (CI) strength remain unknown. While selection does not act to maintain CI strength, we show through a genomic and biochemical analysis that a valine to leucine mutation (V875L) in the deubiquitylating (DUB) domain of a *Wolbachia* CI-inducing gene reduces the cleavage capability and efficiency in cytoplasmic incompatibility deubiquitylating protein (CidB) responsible for causing CI. To describe this analysis, I focus on first re-introducing the CI phenomenon, its source, genetic factors, pathways, effects and real-world applications. Later, I talk about the discovery of hypomorphic (weak) CI and preliminary research done to narrow down probably determinants that might cause weak CI. Finally, I create a model to test the hypothesis that a certain valine to leucine mutation (V875L) in the DUB domain of the effector proteins of CI lead to varying strengths of CI commonly identified as hypormorphic CI in the wild.

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List of Abbreviations

BSA	Bovine Serum Albumin
CI	Cytoplasmic Incompatibility
CidA	CI Inducing Deubiquitylating Protein – Antidote
CidB	CI Inducing Deubiquitylating Protein
Cif	CI Inducing Factor
CifA	CI Inducing Factor – Antidote
CinA	CI Inducing Nuclease Protein – Antidote
CinB	CI Inducing Nuclease Protein
Cnd	CI Inducing Protein with both Nuclease and DUB domains
DCV	Drosophila C Virus
DENV	Dengue Nile Virus
DNA	Deoxyribonucleic Acid
DUB	Deubiquitylating
IIT	Incompatible Insect Technique
IPM	Integrated Pest Management
Nuc	Nuclease
PCR	Polymerase Chain Reaction
SIT	Sterile Insect Technique
TA	Toxin-Antidote
Ub	Ubiquitin
Ub_2	Di-Ubiquitin
UBL	ubiquitin-like protein

UBP	Ubiquitin-specific processing proteases
USP	Ubiquitin-specific processing proteases
WMP	World Mosquito Program
WT	Wild Type
ZIKV	Zika virus

Chapter 1: Introduction

1.1 *Wolbachia*

Wolbachia, originally known as "*Rickettsial*-like organisms" are rod-like, gram negative, obligate intracellular bacteria originally identified in the smears of testes and ovaries from *Culex pipiens* L. (Hertig & Wolbach 1924). *Wolbachia* were initially found densely packed in the smears of testes and ovaries from *Culex pipiens* mosquitoes. They can also be found to be infecting other somatic tissues in various host organisms, some of which include but are not limited to the abdomen of *Culicoides sanguisuga* L. commonly known as sand-fly (Hertig & Wolbach 1924), the muscle, retina and brain of *Drosophila melanogaster* (Min & Benzer 1997) and within hemocytes in *Armadillidium uulgare* Latr. (Martin et al. 1973). Specifically, *Wolbachia* are alpha proteobacterium generally thought to be endosymbiotic and capable of infecting insect species. *Wolbachia* can, but rarely infect insect species horizontally; either between members of the same species (intra-specifically) or across species boundaries (inter-specifically). The primary mode of infection passage is vertically, through maternal transmission through ovary-egg cytoplasm (Boyle et al. 1993; Hoffmann & Turelli 1997). Due to the versatility in its transmission, *Wolbachia* are widespread and can be found in most invertebrate populations in the wild (Werren et al. 2008), with more than 40% of arthropod species being infected with it (Zug & Hammerstein 2012). While *Wolbachia* can be found in both somatic and germline cells of insects, they are predominantly found in germline cells (gonads) in most insect species (Dobson et al. 1999). When present in these hosts, *Wolbachia* produce a variety of different phenotypes varying between an endosymbiotic or parasitic nature. Some of the phenotypes considered to be parasitic in nature include but are not limited to, CI (Yen & Barr 1973), degeneration of tissues including brain, retina, and muscle, culminating in early death (Min & Benzer 1997) and feminization (Martin et al. 1973; Juchault & Legrand 1985; Rigaud

1991). While some of the phenotypes considered to be endosymbiotic in nature include but are not limited to nutritional supplementation in bedbugs (Hosokawa et al. 2010). Another significant yet non-parasitic and non-endosymbiotic phenotype include its ability to reduced vector competence for the transmission of arboviruses in certain insects (Teixeira et al. 2008). It is important to note that while *Wolbachia* are capable of infecting insect species, there is no record of *Wolbachia* infections in humans or mammals. As such, this remains a greatly under-researched area with much promise. Based on the variety of phenotypes *Wolbachia* infections can confer on host organisms, *Wolbachia* can be applied in many aspects of agriculture and medical insect pest management; *Wolbachia* usage in IPM is increasing and will continue to play a major role in our near future. This is evident by the numerous amounts of research publications and experiments undergone to study different aspects of the organism (Table T1). This thesis is focused on the CI phenotype of *Wolbachia* infections.

	Hits
Wolbachia	40,300
Cytoplasmic Incompatibility	56,200
Cytoplasmic Incompatibility + *Wolbachia*	8,660
Reduced vector competence for the transmission of arboviruses + *Wolbachia*	2,420
Degeneration and early death + *Wolbachia*	1,610
Feminization + *Wolbachia*	3,560

Table T1: Google scholar hits returned for each search term (ret. July 1, 2021)

1.2 Cytoplasmic Incompatibility (CI)

CI, simply described as sterility in male sperm, is one of the most heavily researched phenotypes expressed by *Wolbachia* in host organisms, see table T1 (Yen & Barr 1973; Zug & Hammerstein 2012). CI is a reproductive parasitism phenotype first identified in populations of *Culex pipiens* which were unable to mate successfully (Laven et al. 1957). Since the discovery of CI, there exists many bacteria capable of manipulating insect host reproduction. Some of these bacteria include, *Rickettsiella* (Rosenwald et al. 2020), *Mesenet* (Takano et al. 2021), *Cardinium* (Hunter et al. 2003), and *Wolbachia* (Yen & Barr 1974). All of these cause CI and are capable of reducing the viability of uninfected host embryos fertilized by symbiont-modified sperm (Hoffmann & Turelli 1997; Beckmann et al. 2019; Chen & Hochstrasser 2020; Shropshire & Bordenstein 2020). It is important to note that while *Wolbachia* was first discovered in *Culex pipiens* mosquitoes (Hertig & Wolbach 1924), CI was discovered (Laven et al. 1957) independently of *Wolbachia*. Hence, the linkage between CI and *Wolbachia* was not discovered until an absence of CI was observed in populations of *Culex pipiens* after being treated with tetracycline antibiotic. Upon further investigation, the bacterium - *Wolbachia pipientis* was confirmed to be absent and thus the linkage was made (Yen & Barr 1973). CI contributes significantly to *Wolbachia's* status as the most commonly known endosymbionts in nature (Zug & Hammerstein 2012) because it increases their equilibrium infection frequency in populations; in turn, this can provide *Wolbachia* with more opportunities to spread and jump to alternate hosts.

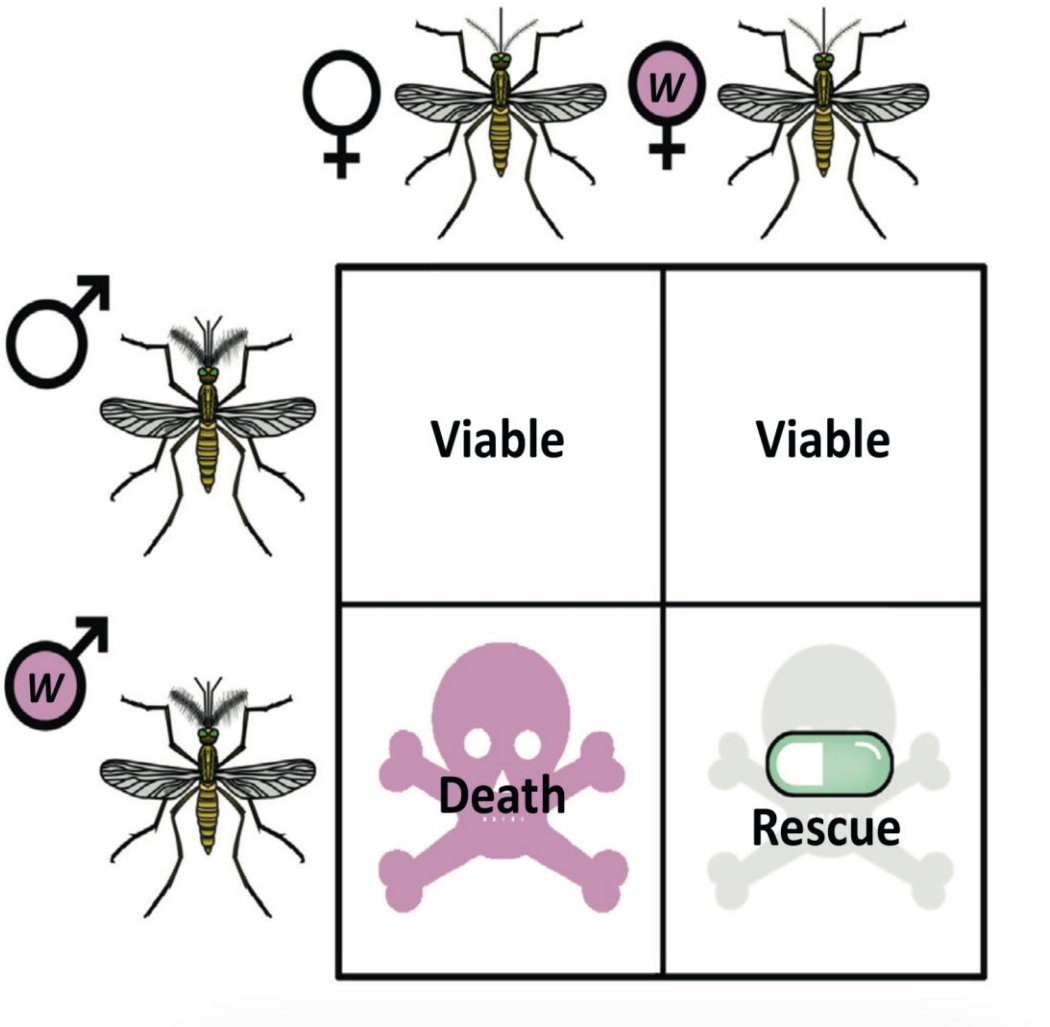

Figure 1: Toxin-Antidote model of CI. Crosses indicating compatibility between infected females and infected or uninfected males. Whereas compatibility only if cross is between uninfected males and uninfected females (Beckmann et al. 2019)

Note: *w* = *Wolbachia* infection

The mechanism of CI has been theorized to operate as a toxin-antidote system (Beckmann et al. 2019). When members of same insect species without a CI-causing *Wolbachia* infection mate, fertilization of the egg to form a zygote occurs successfully to produce viable offspring which can survive. When a CI-causing *Wolbachia* infection present in a male species is crossed with an uninfected female of the same species, the outcome is an unsuccessful fertilization of eggs by CI-sterilized sperm. This is because a toxin protein, denoted by "B" is hypothesized to be produced in the cytoplasm of the infected male species sperm cells which causes sterilization. As such, inviable offspring are produced which die due to their inability to survive/develop. This is evident as low or zero hatch-rate (assuming CI event is perfect) (Laven et al. 1957; Beckmann et al. 2019). Fortunately, when CI-causing *Wolbachia* infections are present in both male and female insect species, CI can be rescued. In this case, an antidote protein, denoted by "A" is theorized to be produced in the egg cells of infected females. Once produced, the antidote is capable of binding to the toxin and rescuing its toxicity effect, thereby allowing a successful fertilization of an infected egg to occur by the infected sperm (Beckmann et al. 2019). This evident as a normal hatch-rate. In the last possible mating scenario, when CI-causing *Wolbachia* infections present in the female species are crossed with uninfected males, the predicted outcome is a successful fertilization of infected eggs by uninfected sperm to form an infected egg. This observed event occurs, because of an absence of toxicity in the sperm, evidently allowing a normal fertilization to occur. Hence, the TA model of CI, see Figure 1 (Beckmann et al. 2019).

Also noted here is that *Wolbachia* infections can only be passed down from mother to offspring commonly known as maternal transmission (Hoffmann & Turelli 1997). While the TA

model of CI can be attained using a single strain (unidirectionally), CI can also be attained using two *Wolbachia* strains, bidirectionally (Bourtzis et al. 2003) and possibly more.

1.3 Applications of CI:

Based on the nature of CI mechanism and its popagation (Beckmann et al. 2019), CI tends to behave like a gene-drive mechanism. According to Alphey et al. 2020, "gene-drive is a process that promotes or favors the biased inheritance of certain genes from generation to generation". CI can be used to replace populations of insects with "better" or less damaging ones. A recent strategy aims to use CI to spread *Wolbachia*'s ability to reduce vector competence (Teixeira et al. 2008) into populations of *Aedes aegpyti*. Additionally, isolating and using just the toxin part of CI allows one to sterilize insects. Sterilization by *Wolbachia* can be used for the sterile insect technique (SIT). Thus, there are two main applications of CI, SIT and Gene Drive.

Sterile Insect Technique (SIT) is a vector biology technique championed as one of the pioneer successful methods implemented for area-wide insect population control and currently being incorporated into IPM programs around the world today (Klassen et al. 2021). Initially drawn-up as a plan to combat screwworm populations (Knipling 1955), SIT involves the release of sterile male populations of an insect species into the wild. Once released, these sterile males outcompete the population of non-sterile males capable of mating with females. This leads to a lower number of successful mating event occurring between pairs. Eventually, the assumption is that reduced offspring populations are observed due to a greater proportion of mating events happening between sterile males which cannot mate with females to produce offspring as compared to non-sterile males and females (Knipling 1955). One of the key components to the success of this technique revolves around the ability to produce sterile males without affecting their "fitness" (Knipling 1955). This was achievable during the pioneer procedure which

involved the use of cobalt radiation as a method of sterilization in male screwworms (Knipling 1955). Unfortunately, when this radiation procedure is repeated in other insects such as mosquitoes at radiation level that would result in above 70% sterility in males, some adverse effects on the "fitness" level of the insects such as, reduced longevity as a result of radiation-induced somatic damage, reduced mating ability and reduced competitiveness accompanied those irradiation levels. (Proverbs 1969; Helinski et al. 2009). Other shortcomings of SIT by irradiation include harmful effects on humans, making this a cost un-effective procedure. To circumvent this, CI-inducing *Wolbachia* infections have been introduced as an alternative method of sterilization during SIT application in mosquitoes (Dobson et al. 2002; Zheng et al. 2019).

This specific approach is commonly identified as Incompatible Insect Technique (IIT) and is preferable for numerous reasons. First of which include the fact that healthy sterile males can be released as there are no known side effects of CI-inducing *Wolbachia* infections on mosquito species (Dobson et al. 2002; Zheng et al. 2019). This means, all male species with CI-inducing *Wolbachia* infections will have the same "fitness" levels as normal WT male species, thereby not hindering their ability to effectively outcompete non-sterile males and eventually lead to an overall reduction in insect offspring populations (Knipling 1955). Secondly, IIT has a life-spanning advantage over traditional SIT by irradiation. This is because unlike sterility through irradiation which may not affect subsequent generations, sterility through CI-inducing *Wolbachia* infections can be passed on due to the ability for *Wolbachia* infections to be transmitted horizontally, intraspecifically (between members of the same species) or interspecifically (across species boundary) and vertically through maternal transmission in host eggs (Boyle et al. 1993; Hoffmann & Turelli 1997). The key here is that one can rear sterile

males in the factory, and they are only sterile outside in the wild where *Wolbachia* isn't present. Another key point is that for this technique to work infected females cannot be released into the wild.

A practical example of the application of CI-inducing *Wolbachia* infections being used as a method of SIT (IIT) include the Debug Fresno Project by Verily (Debug Project 2017). This project, run by Verily which is in turn is owned by Google was aimed at reducing the *Aedes aegypti* populations in neighborhoods in Fresno County, California using CI-inducing *Wolbachia* infections (IIT) in bio-engineered mosquitos. This project resulted in an observed reduction in the quantity of mosquitoes, which was 95.5% lower in discharge regions when contrasted with non-discharge regions, with the most geologically detached area arriving at a 99% decrease at peak mosquito season (Crawford et al. 2020). After these results were observed and analyzed, the Debug Fresno program was deemed successful and expanded. Recently, the EPA approved IIT in half the American states with a recent mass release in Miami (Dobson 2021) and other countries such as China, Singapore, etc (Debug Project 2020).

While CI-inducing *Wolbachia* infections are mostly used as an effective SIT to cause a reduction in insect populations, population control is not always the desired effect. In some cases, a certain population of organisms with a particular phenotype are considered more valuable. As such, this population with the phenotype in question are considered more desirable than the population without the phenotype. An example of a phenotype we (humans) find valuable, is the ability for certain *Wolbachia*-infected vectors to have reduced vector competence for the transmission of certain viruses in their host. This phenotype was first observed while studying *Wolbachia* bacterial strains associated with *Drosophila melanogaster* which could resist infection from *DCV* (Teixeira et al. 2008). Later on, it was observed that, while there were no

naturally occurring *Wolbachia* strains in *Aedes aegypti* species, an avirulent *w*Mel strain could be introduced. Once introduced, avirulent *w*Mel can confer both "CI" and "reduced vector competence for the transmission of certain viruses" phenotype (Walker et al. 2011; Van den Hurk et al. 2012; Bian et al. 2013; Ye et al. 2015; Aliota et al. 2016; Dutra et al. 2016; Pereira et al. 2018; Moreira et al. 2019).

The next question is, how can '*w*Mel' CI-causing genes drive the "reduced vector competence for the transmission of certain viruses" phenotype in *Aedes aegypti* species? Because avirulent *w*Mel genes in *Aedes aegypti* species are able to execute both phenotypes. As such, CI becomes the driving factor for the "reduced vector competence for the transmission of certain viruses" phenotype across generations. This is due to the ability of CI-causing *Wolbachia* strains manipulate its host's reproduction in order to select for its (CI-causing *Wolbachia*) own propagation, i.e select for its own kind (Beckmann et al. 2019). Evidently driving natural occurring *Wolbachia* to high equilibrium frequencies (Turelli 1994; Jaenike 2009; Kriesner et al. 2016; Cooper et al. 2017). Hence, both the CI and reduced vector competency phenotype coupled together contribute to allows *w*Mel to be used as a method of biological control for containing the spread of deadly diseases caused by arboviruses.

A currently implemented example of this application gene drive being used as an application of CI is the World Mosquito Program (WMP). This project is funded by The Bill and Melinda Gates foundation through the Grand Challenges in Global Health Program (Grand Challenges). The goal of the WMP program is to combat the spread of mosquito-borne arboviruses such a DENV, ZIKV, chikungunya virus and yellow fever virus using safe *Wolbachia* bacterial infections. The program, which began earlier in the decade aims at protecting up to 75 million people in the next 5 years. Currently, the WMP is being implemented

in over 11 countries spread across the world and affecting close to 6million people worldwide. In areas of its implementation such a Yogyakarta, there has been a 77% reduction in dengue incidence cases and an 86% reduction in Dengue hospitalizations when compared to untreated areas (WMP). This speaks to magnitude of the impact of the *Wolbachia* bacterium in our society of today and beyond.

While the use of "gene drive" focused programs as an application of CI has proven to be successful in controlling the spread of diseases by mosquito-borne arboviruses such a DENV, ZIKV, chikungunya virus and yellow fever virus (WMP), there exists other methods of disease prevention and control for mosquito-borne arboviruses. Some of these disease prevention and control strategies include but are not limited to vaccination, use of bug sprays and repellant and the elimination of vector (such as *Aedes aegypti*) breeding sites.

1.4 *Aedes aegypti* mosquito

Mosquitoes are known to vector diseases such as malaria, filariasis, dengue hemorrhagic fever, zika, west nile virus, etc making them to be easily concluded as the most dangerous creatures on the earth (WHO 2017; CDC 2019). With the most notable mention being malaria. Nearly half of the world population is at risk of getting a malaria infection (CDC 2019). In 2016, there were around 216 million reported cases of malaria and an estimated 445,000 deaths reported even though there has been a downward trend over the years (WHO 2017). Other notable facts include that about 3 billion people live in areas with increased risk of dengue (CDC 2019). Lymphatic filariasis transmitted through mosquitoes affects more than 120 million people in 72 different countries and comes with symptoms that can be irreversible (CDC 2019). The impact of mosquitoes and the diseases they spread have been hypothesized to kill more people

than most wars in history (IDPH 2017). This makes mosquito population control undoubtedly one of the key areas of research concerns for both our century and the next to come.

Of all the mosquito species available, the *Aedes aegypti* mosquito, commonly known as the "yellow fever mosquito" is one of the deadliest mosquitoes present. This title is attributed to it based on it being the most common vector for yellow fever virus and *DENV*, as well as other arboviruses such as *ZIKV*, Chikungunya virus, and so on (CDC 2019). *Aedes aegypti* are holometabolous insects stemming from the fact that they have 4 main developmental stages: egg, larvae, pupae, adult. This species of mosquito follows the R-selection strategy of reproduction, and their females are able to undergo 3 gonotrophic cycles before they die, laying around 100 eggs each time depending on various conditions. This also classifies them as having an iteroparous timing of reproduction. It takes 7-10 days for these mosquitoes to develop fully from egg to adult stage, all of which is spent adapted to an aquatic habitat except at the adult stage. The female mosquitoes have a total life span of about 40-80 days with no significant difference when feed either only 5% sucrose diet when compared to a mixed blood and sugar diets (Harrington & Helinski 2011). Based on a study using a model representing the evolution of the expectation of life according to parity rates shows that the longest life expectation is obtained at 27°C (Goindin et al. 2015). Male mating history and size has proven to affect the female fecundity of *Aedes* aegypti mosquitoes with subsequent mating and lower size leading to lower fecundity (Harrington & Helinski 2011). Once eggs are laid, based on environmental conditions, these eggs are able to resist desiccation and stay at the egg stage for up to nine months, after which they can hatch if re-exposed to favorable conditions (Fischer et al. 2019). This explains why attempts to eradicate *Aedes* aegypti populations by temporary eliminating breeding sites through routine community sanitation can prove somewhat ineffective. According to Brown, due

to harsh conditions coupled with the onset of the slave trade, *Ae. aegypti* were introduced into the New World from Africa, from where it subsequently spread globally to tropical and sub-tropical regions of the world (Brown et al. 2014). Subsequently after Brown's hypothesis, other people went on to look at certain factors that could create a general description of the population spread based on certain factors such as temperature suitability for oviposition (Brady et al. 2014), pathogen geographical distribution amongst other factors but these do not sufficiently discriminate where the species may or may not persist (Kraemer et al. 2015). The overall trend appears to be that the mosquito accumulates at more warm temperatures in opposition to cold temperatures. This stands to correlate with the fact that *A. aegypti* easily succumbs fatally at 0^{o}C and 37^{o}C, and as such does not thrive in dry hot climates and cold weather (WHO 2017). Looking at how far *Aedes* aegypti can travel, flight range studies have proven that most female *Aedes aegypti* spend most of their lives where they emerge as adults only moving an average distance of 181m from feeding sites (Brown et al. 2017). Female *Aedes Aegypti* mosquitoes mainly exhibit a hematophagous feeding behavior with preference on human blood. In contrast, the males exhibit a herbivorous feeding behavior feeding mainly on nectar and plant juices making them part of a nectar feeding guild. The females also require a blood meal to be able to produce offspring through a process called vitellogenesis in a gonotrophic cycle (Dimond, Lea, et al. 1955). Nevertheless, Female mosquitoes have been proven to be able to complete the anautogenous process in a lab experimental setup with restricted or no blood access (Ponlawat & Harrington, 2005). In the process of feeding, female Aedes Aegypti mosquitoes are known to transmit pathogens (Powell & Tabachnick, 2013).

Based on vector capability, life cycle, breeding and feeding pattern, the applications of CI-causing *Wolbachia* infections is preferred in the control of the *Aedes aegypti* mosquito

populations. At the cellular level, for CI applications to be effective, the genes which code for the proteins that execute the CI phenotype in reproductive cells have to be efficiently conserved when transferred from parent to offspring in order to maintain its functionality. So, the strength of CI is crucial to the efficacy of various programs (Dobson et al. 2002; Hoffmann et al. 2011; O'Neill et al. 2018; O'Neill 2018; Zheng et al. 2019).

This thesis focuses on taking a closer look at the biochemistry behind CI in order to possibly explain how it can be maintained or give clues on how CI DNA factors might fall apart in the wild.

1.5 CI Genes

Wolbachia genes responsible for controlling CI operate as a 2-gene operon system, *cifA/B* (Hoffmann et al. 1990; Beckmann & Fallon 2013; LePage et al. 2017; Beckmann et al. 2017; Shropshire et al. 2019; Shropshire et al. 2021). The first gene, denoted as A represents a gene sequence capable of producing an antidote protein which is able to bind to the next protein, B to rescue CI induction (Beckmann et al. 2017; Shropshire et al. 2018; Chen & Hochstrasser 2020). The second gene, denoted as the B gene is responsible for producing the toxin protein, which is the causative agent of CI in its host (LePage et al. 2017; Beckmann et al. 2017). CI genes (*cifA/B*) in turn code for protein. Based on the functions of the catalytic domain in these proteins, two possible pathways of CI mechanism have been identified. These are a nuclease pathway or de-ubiquitylating pathway. When the CI genes (*cifA/B*) code for a DUB protein, we call the gene products CidB and CidA. And when the CI genes (*cifA/B*) code for a nuclease protein, we call the gene products CinA and CinB. Another possible combination is *Cif*s which can code for proteins with both a nuclease and DUB catalytic active site, indicated by Cnd (Beckmann et al. 2019).

Although these genes are transferred as a 2 gene operon system vertically and horizontally, they have different sites of production. The B causative agent protein is expressed in the sperm, causing cytoplasmic changes which result in inviable sperm. While the A causative agent protein is expressed in the eggs. The bacterium which can live in either male or female gonads, can only survive and be passed to offspring through the egg cells. Hence, the idea of its maternal transmission. Theory indicates that selection does not act to increase or maintain CI (Turelli 1994), which varies considerably among even very closely related *Wolbachia* (Martinez et al. 2015; Cooper et al. 2017; Turelli et al. 2018). For example, CI strength differs significantly among model *w*Mel from *D. melanogaster* and closely related *w*Mel-like *Wolbachia* in the *D. yakuba* clade (*w*Yak, *w*San, and *w*Tei) that *w*Mel diverged from in the last 30,000 years (Martinez et al. 2015; Cooper et al. 2017; Cooper et al. 2019).

In my thesis, I focus on the CidB protein which cleaves Ubiquitin (Ub) molecule(s) covalently attached to another Ub molecule or substrate (Beckmann et al. 2019).

1.6 Ubiquitin

Ubiquitin, a vital component of the ubiquitin modification cycle is a key component of most cellular processes in humans and beyond. Either found as a single unit or attached to another molecule, the addition or removal of a Ub or ubiquitin-like proteins (UBLs) molecule can signal a wide variety of cellular process including but not limited to apoptosis, DNA repair, endocytosis, cellular signaling and protein quality control (Hershko & Ciechanover 1998; Ronau et al. 2016). Examples of UBLs include SUMO, NEDD8, and ISG15 which are involved in protein regulation (Welchman et al. 2005). The process of adding a ubiquitin molecule is mediated by a group of molecules; E1 (Ubiquitin Activating Enzyme), E2 (Ubiquitin Conjugation Enzyme) and E3 (Ubiquitin Ligase). These cause the covalent attachment of Ub

molecules to a substrate protein singly (mono-ubiquitylation) or to other Ub molecules (poly-ubiquitylation) generally by an amide (isopeptide) bond between the C-terminal carboxyl group of ubiquitin and the lysine side chain of the protein substrate or Ub. Based on the type of the E1, E2 and E3 molecules, the arrangement pattern of Ub linkages at the lysine residue will differ (Ronau et al. 2016). An example of the different linkage patterns of Ub at the lysine residue include K63, K48, K11. Specifically, covalently linking K48 to a protein molecule signals its degradation and K63 poly-ubiquitylation can function in signal-transduction cascades (Welchman et al. 2005). Another portion of the ubiquitin modification cycle involves the process of removing the Ub molecule from a substrate or from another Ub molecule (in the case of poly-ubiquitylation). This involves the action of a DUB protein which must have an active catalytic site specific to the linkage pattern of the added Ub or UBLs molecule (Ronau et al. 2016). Both the specificity in the covalent attachment and cleavage of Ub molecule combines to describe the idea behind the 'specificity' of the ubiquitin modification cycle.

Based on previous research, CidB has a higher affinity and efficiency for K63- & K48-linked ubiquitin chains (Beckmann et al. 2017). Hence, my research focuses on finding the effect of a single valine to leucine point mutation on the DUB domain of the WT CidB which is a DUB protein.

1.7 DUB Proteins

A DUB protein enzyme is a major component of the Ub modification cycle involved in cleaving Ub molecules or ubiquitin-like proteins (UBLs) molecule covalently attached to other Ub molecules (in the case of poly-ubiquitylation) or a substrate molecule by hydrolysis of the isopeptide bond linkage, see figure 3 (Hu et al. 2005; Ronau et al. 2016). This cleavage process is responsible for signaling a cascade of major cellular processes including but not limited to

apoptosis, DNA repair, endocytosis, cellular signaling and protein quality control (Hershko and Ciechanover 1998; Ronau et al. 2016). Ubiquitin-specific processing proteases (UBPs or USPs) is the largest of at least five main classes of DUB enzymes, with UBPs/USPs having over 60 members identified in the human genome.

CidB protein is classified as a DUB protein enzyme due to its ability to its ability to remove a Ub molecule covalently attached to another Ub molecule or substrate (Beckmann et al. 2017) and can be specifically classified as USP.

1.8 Summary of collaborators contribution (Beckmann et al. 2021)

To determine how much and why naturally segregating mutations in *w*Mel-like *cifs* influence CI strength, the two divergent *cif* operons observed in *w*Yak that infects *D. yakuba* in triplicate were cloned and sanger sequenced to confirm prior reports (Cooper et al. 2019). Specifically, *w*Yak was observed to have two pairs of *cif* loci: a Type 1 pair with a deubiquitylase (DUB) domain (cid^{wYak}) that is homologous to cid^{wMel} and a Type 4 pair with nuclease domains (cin^{wYak}) that is homologous to cin^{wPip} in *w*Pip that infects *Culex pipiens* (Cooper et al. 2019).

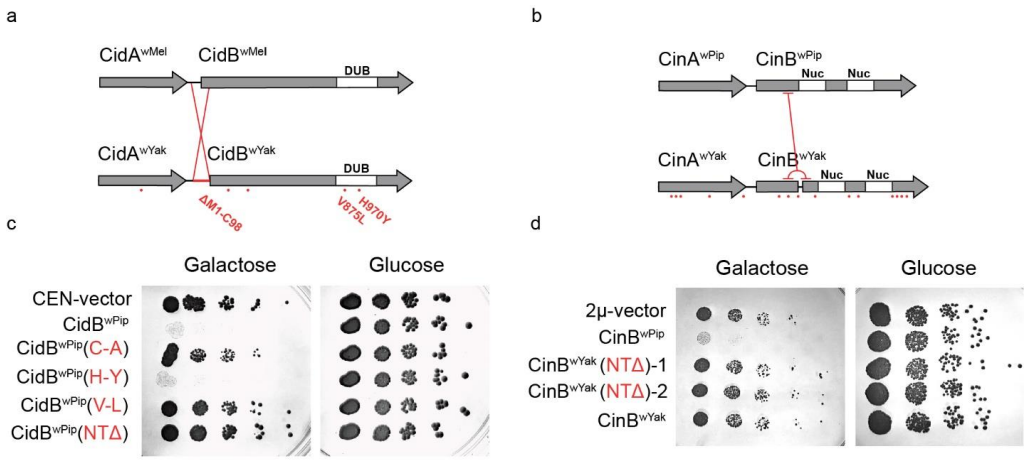

Figure 2: Analysis of *cif* permutations in *w*Yak, *w*Mel, and *w*Pip. a. Six divergences distinguish Cid^{wMel} from Cid^{wYak} including five nonsynonymous mutations and an inversion that truncates the protein's N-terminus. Two mutations are within the DUB domain. b. 16 divergences distinguish Cin^{wPip} from Cin^{wYak} including 15 nonsynonymous mutations and a tandem duplication yielding a frameshift and premature stop codon. c. Serial dilutions of analog mutations within yeast expression constructs show (V-L) and (NTΔ) eliminate toxicity compared to wild type toxic control, CidB^{wPip}, and a negative catalytic inactive CidB^{wPip} (C-A). CEN-vector is an empty pRS416gal1 negative control. d. CinB^{wYak} alleles in yeast didn't produce phenotypes for three variant constructs including CinB^{wYak}(NTΔ) - beginning after the tandem duplication and the wild type sequence containing the tandem duplication. (NTΔ)-1 and 2 are the endogenous sequence or codon optimized respectively. CinB^{wPip} is a toxic control and an empty 2μ-vector is pYes2, a negative control. Serial dilutions were performed at 34°C and 35°C respectively for c. and d. Media is synthetic defined lacking uracil with galactose or glucose as inducer and repressor. Figures are representative of triplicates (Beckmann et al. 2021).

In search of sequence variation that differentiated *w*Mel-like *cifs*, *cid*^{wYak} was aligned to *cid*^{wMel} and *cin*^{wYak} was aligned to to *cin*^{wPip} , see figure 2 (Cooper et al. 2019; Beckmann et al. 2021). In total, six coding permutations differentiate *cid*^{wMel} from *cid*^{wYak}, including five nonsynonymous mutations (one in *cidA*^{wYak} and four in *cidB*^{wYak}), and an inversion at a CCG palindrome on the 5' end of *cidB*^{wYak} that truncates CidB^{wYak}. Two of the nonsynonymous mutations in *cidB*^{wYak} fell within the DUB domain known to be catalytically active for CidB^{wMel} (V875L, valine to leucine; H970Y, histidine to tyrosine) (Beckmann et al. 2017). *cin*^{wYak} was differentiated from *cin*^{wPip} by 16 coding permutations, including 15 nonsynonymous mutations (five in *cinA*^{wYak} and ten in *cinB*^{wYak}), and a tandem duplication resulting in a frameshift and premature stop codon splitting CinB^{wYak} into two parts. No mutations were observed to fall within the tandem nuclease domains (Nuc) known to be catalytically active for CinB^{wPip} (Chen et al. 2019). For both CifB^{wYak} copies, upstream methionines possibly initiate translation of N-terminally truncated CifB proteins.

It was proposed that, naturally observed *cif* sequence variation, particularly in functional domains, contributes to CI-strength variation. And to test this hypothesis, the effects of natural *w*Yak *cif* mutations were screened on a CI model in yeast. In this model, CifB proteins induced yeast toxicity and CifA co-expression rescued it (Beckmann et al. 2017; Chen et al. 2019; Beckmann, Sharma et al. 2019). Since catalytic inactive DUB mutants are unable to induce CI, researchers focused on *w*Yak *cif* mutations found in the DUB domain (Beckmann et al. 2017). They also hypothesized that N-terminal truncations weaken CI, because this pattern is observed recurrently in putatively pseudogenized *cifB* genes (Meany et al. 2019; Martinez et al. 2021). In yeast serial dilutions, the *w*Yak V875L (V-L) DUB mutation and a ΔM1-C98 N-terminal deletion (NTΔ) independently eliminated CidB-induced toxicity, while the H970Y (H-Y) DUB

mutation did not differ from the CidB control. (Phenotypic ablation can be attributed to reduced CidB deubiquitylation, which was test below.) For Cin, wildtype CinB^{wYak} and an ΔM1-K126 N-terminal deletion (NTΔ) lacked CinB- induced toxicity. It was concluded that *w*Mel-like *cif* functions were disrupted by natural mutations in their functional domains and through truncation of N-termini (NTΔ) of their CifB proteins. These variants observed in *w*Yak *cifs* represent candidate mutations for involvement in CI-strength variation.

To test the hypothesis that (V-L) and (NTΔ) mutations observed in Cid^{wYak} reduce Cid^{wMel} CI strength, researchers next expressed transgenes in *Wolbachia*-free *D. melanogaster* males using the Gal4/UAS system, see figure 3 (Beckmann et al. 2021). When driving transgenes with the nanos-Gal4-tubulin (NGT) driver in males (LePage et al. 2017; Beckmann, Sharma et al. 2019; Shropshire, Kalra et al. 2020), the Cid^{wMel} construct caused strong transgenic CI (hatch rate = 44% ± 16% SD, *N* = 26), measured as the percent of embryos that hatch from a mating pair's clutch, relative to the hatch produced by uninfected controls (hatch rate = 97% ± 3% SD, *N* = 29). As predicted by researchers, introducing the (V-L) mutation into Cid^{wMel} disrupts CI (hatch rate = 77% ± 10% SD, *N*= 36), reducing CI strength by an average of 2.6-fold (95% CI = 1.8 - 4.9-fold). This further suggests the DUB contributes to CI induction, as predicted (Beckmann et al. 2017). A maternal cytoplasmic *w*Mel infection fully rescues both Cid^{wMel} (99% ± 2% SD, *N* = 30) and weakened Cid^{wMel}(V-L) CI (98% ± 3% SD, *N* = 32). The Cid^{wMel}(NTΔ) construct does not cause CI (hatch rate = 95% ± 7% SD, *N* = 31), indicating that the N-terminus of CidB is also essential for CI. While *w*Yak causes sporadic and weak CI in *D. yakuba* (Cooper et al. 2017), neither the Cid^{wYak} (97% ± 5% SD, *N* = 36) nor the Cin^{wYak} (97% ± 5% SD, *N* = 36) complete constructs induce CI with the weak NGT driver. Therefore, researchers increased transgenic expression using the stronger Maternal Triple Driver (MTD)

driver (Petrella et al. 2007) and repeated all crosses. Boosted Cid^{wMel} and Cid^{wMel}(V-L) expression yielded hatch rates near zero, demonstrating that hypomorphic CI produced by Cid^{wMel}(V-L) under the NGT driver increases to Cid^{wMel} intensity if sufficiently overexpressed. This is in contrast to the (NTΔ) mutant, in which CI is ablated even under boosted expression. These results suggested that the (V-L) variant is a true enzymatic hypomorph. Under similar conditions, the positive Cin control (Cin^{wPip}) induced weak CI, whereas neither Cid^{wYak} nor Cin^{wYak} complete constructs produced a phenotype. In addition, under the boosted driver, both transgenic phenotypes from Cid^{wMel} and Cid^{wMel}(V-L) were fully rescued by crossing to a maternal (cytoplasmic) *w*Mel infection. Notably, while Cid^{wYak} could not induce CI, when expressed in females, it fully rescued strong CI induced by a cytoplasmic *w*Mel infection in young males. This agreed with their theory and past results that indicate selection does not act to maintain CI induction but does act to preserve rescue functions (Turelli 1994; Meany et al. 2019; Martinez et al. 2021). It was hypothesized that variation in CI strength results from mutational effects on CidB's DUB domain.

To test this, we made His6-tagged-recombinant-protein expression constructs to purify truncated CidB^{wMel} DUB domains. These were then compared with constructs bearing amino acid substitutions found in *w*Yak (H-Y; and V-L; in addition to a catalytic mutant C-A). DUBs were purified by affinity chromatography, quantified by densitometry, and subjected to downstream deubiquitylation kinetics assays (Fig. 3). Previous reports suggest that CidB preferentially cleaves lysine-63 (K63) linked di-Ubiquitin as opposed to lysine-48 (K48) (Beckmann et al. 2017).

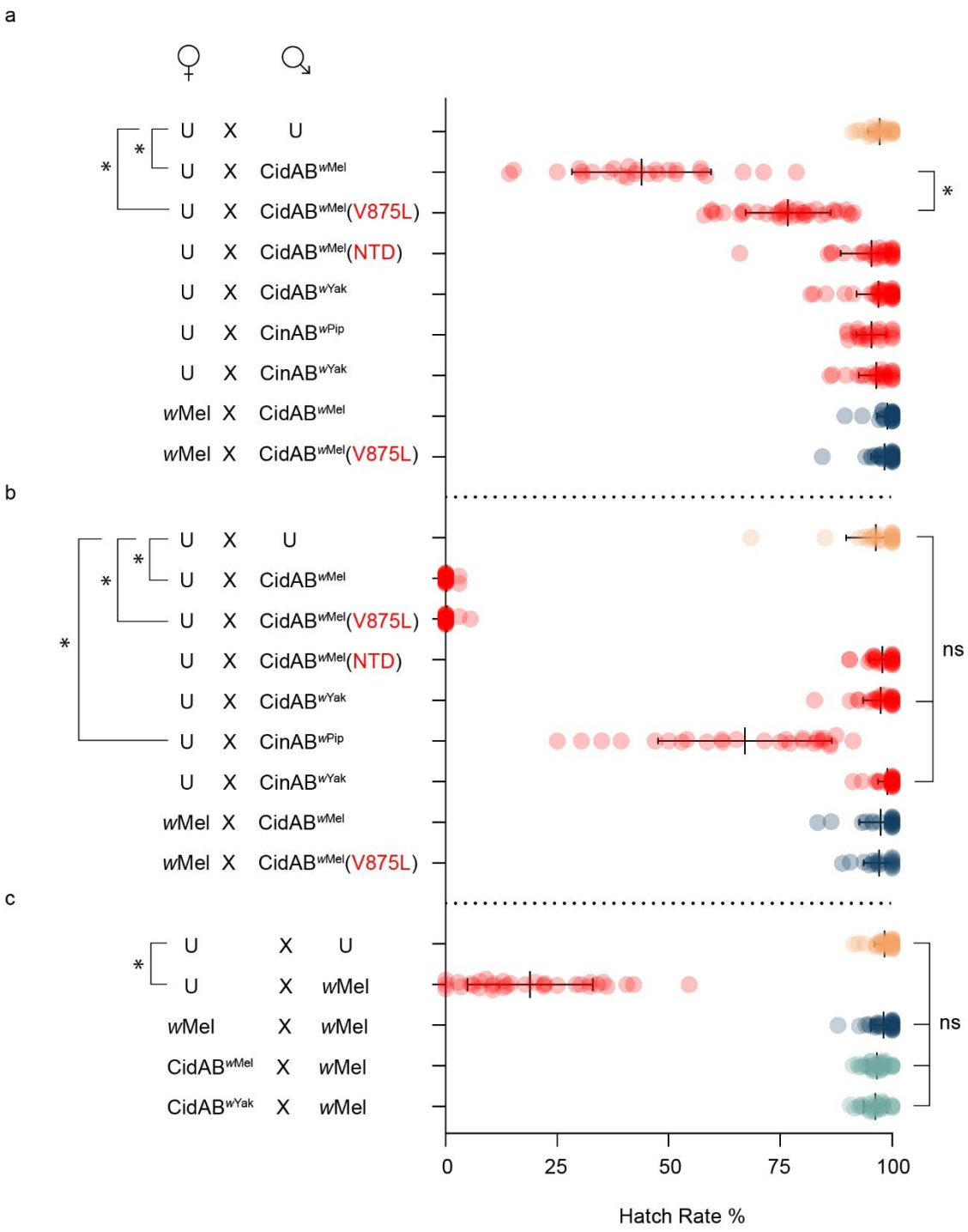

Figure 3: Analysis of mutations in *D. melanogaster* model. A. Design of transgene constructs. T2A is a viral sequence causing translation of two proteins. Red dots indicate amino acid changes. (M99) and (M127) label start codons after NTΔ. 1) positive CI control - the *cid*^{wMel}

backbone. 2) *cid*$^{w\text{Mel}}$ (V-L) point mutant. 3) *cid*$^{w\text{Mel}}$ (NTΔ) starting at M99. 4) *cid*$^{w\text{Yak}}$ analog - in effect, *w*Yak wildtype. 5) positive CI control - the *cin*$^{w\text{Pip}}$ backbone. 6) *cin*$^{w\text{Yak}}$ analog - in effect, wYak wildtype. 7) pUASp-attb insertion vector. B. Transgenic CI with the weak NGT driver. Males expressing *cid*$^{w\text{Mel}}$ cause strong CI relative to uninfected (U). Males expressing *cid*$^{w\text{Mel}}$(V-L) cause hypomorphic CI. *w*Mel cytoplasmic infection rescues CI from males expressing *cid*$^{w\text{Mel}}$ or *cid*$^{w\text{Mel}}$(V-L). C. CI analysis with boosted expression using an MTD driver. Males expressing *cid*$^{w\text{Mel}}$ or *cid*$^{w\text{Mel}}$(V-L) cause strong CI that cytoplasmic *w*Mel infections in females fully rescue. Males expressing *cin*$^{w\text{Pip}}$ cause weak CI. D. Transgenic rescue. *w*Mel cytoplasmic infection in males causes strong CI. *w*Mel cytoplasmic infection in females rescues *w*Mel-induced CI. Expression of operons *cid*$^{w\text{Mel}}$ or *cid*$^{w\text{Yak}}$ in females fully rescues *w*Mel-induced CI. Raw hatch rates are plotted over means and standard deviations. (*) is $P < 10^{-6}$, and (ns) is not significant. *P*-values for (B) and (C) are calculated from one-tailed Wilcoxon tests with Bonferroni correction for multiple comparisons, while *P*-values for (D) are calculated by a Kruskal-Wallis analysis with Dunn's multiple comparison test (Beckmann et al. 2021).

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Chapter 2 (Thesis statement and specific aims)

2.1 Summary of preliminary events:

Based on preliminary studies conducted, it is theorized that CI drives natural occurring *Wolbachia* to high equilibrium frequencies (Turelli 1994; Jaenike 2009; Kriesner et al. 2016; Cooper et al. 2017) and has two applications in transinfected vector systems. Firstly, mass releases of wMel-infected, CI-causing males can suppress the naturally uninfected or incompatible mosquito embryos; and this sterile insect technique (SIT) crashes vector populations via CI (Dobson et al. 2002; Zheng et al. 2019). This is now EPA approved in half the American states with a recent mass release in Miami (Dobson 2021). Secondly, as a means for researchers use CI to drive *w*Mel *Wolbachia* that naturally infect *Drosophila melanogaster* through transinfected vector populations, where stable germline infections can reduce vector competence for Zika and DENV (Walker et al. 2011; Van den Hurk et al. 2012; Bian et al. 2013; Ye et al. 2015; Aliota et al. 2016; Dutra et al. 2016; Pereira et al. 2018; Moreira et al. 2019).

Both applications rely upon CI, which is predicted to be lost (Cooper et al. 2017). The molecular executioners of CI that are encoded within two gene operons called *cifs* were identified. These CI factors, though being diverse, share common ancestry (Martinez et al. 2015; Cooper et al. 2017). These *cifs* can be further differentiated based on how they execute CI through different pathways which is dependent on the function of the protein which they encode. *cifs* could be a cid, cin or cnd; which denote deubiquitylating, nuclease or a combination of both of their biochemical activities that underlie. When looking at a particular pathway, Cid (deubiquitylase pathway), the CidA protein encoded by the A region of the 2 gene operon is the antidote. While CidB protein, encoded by the B region of the 2 gene operon is the deubiquitylating toxin (Beckmann et al. 2019). The CI inducing deubiquitylase, CidB, is loaded in sperm and executes the embryo unless the egg contains a Wolbachia-encoded antidote, CidA.

Theory predicts that selection does not act to maintain cif operons, suggesting CI field use might be on borrowed time and their operons will eventually fall apart in the wild (Cooper et al. 2017). Indeed, recent analyses characterized pseudogenized *cif* remnants in genomes of non-CI causing *Wolbachia* (Martinez et al. 2015; Cooper et al. 2017). These have been found in *w*Yak-clade *Wolbachia* (*w*Yak, *w*San, and *w*Tei) that infect Drosophila yakuba-clade hosts (*D. yakuba, D. santomea, and D. teissieri*) and are very closely related to *w*Mel (0.11% divergence across the genome). These "*w*Mel-like" *Wolbachia* show weakened hypomorphic CI. Their corresponding cid locus known to cause CI in *w*Mel has been pockmarked with mutations in *w*Yak (Beckmann et al. 2021). Also, *w*Yak-clade *Wolbachia* have a second set of CI loci absent in *w*Mel, but nearly identical to cin loci found in *w*Pip and other B-group Wolbachia that diverged up to 50 million years ago from *w*Yak, *w*Mel and other A-group *Wolbachia*. And recent data indicates that *Wolbachia*-specific ISWpi1 transposons mediate the horizontal transfer of these *cif* loci between WO prophage regions within divergent *Wolbachia* genomes together, these data highlight the utility of *w*Mel-like *Wolbachia* infecting the *D. yakuba* clade as a model for understanding the life, death, and resurrection of CI loci.

My overall goal here is to dissect the genetic basis of "*w*Mel-like" CI death and resurrection and to identify mutations that modify and break CI. This measures the durability of *w*Mel-CI vector control tools. I propose the following thesis and specific aims to address this urgent problem:

2.2 Thesis statement and aims

Based on collaborative works presented in chapter 2, I will proceed to look at the effect of the mutations theorized in CidB, that when present at the protein cellular level, will support the notion that a valine to leucine mutation in hypomorphic *Wolbachia* CidB protein reduces both deubiquitylation and cytoplasmic incompatibility

Specific Aims:

- Characterize effect of multiple natural mutations found within the DUB catalytic domain of CidB across different substrates (K63 and K48 Ub).
- Quantify the degree by which cleavage differs using densitometry.

2.3 Research:

DNA manipulation: Native *cif* operons identified in cloned by PCR using Phusion polymerase (New England Biolabs) into plasmids/vectors. In general, various PCR amplicons produced throughout the study are visualized using 0.8-2% agarose gels with ethidium bromide. Mutants were made using site directed mutagenesis or SLIM as described prior (Diao & White 2012; Conner et al. 2017; Gillespie et al. 2018). All construct inserts and variants were fully sanger sequenced to confirm DNA fidelity. Final plasmid constructs are transformed into Top10F' *E.coli* by standard chemical transformation and stored in glycerol stocks at -80°C.

Cobalt Affinity Protein Purification: A high expressing truncated DUB domain from $CidB^{wMel}$ corresponding to residues H717-R1128 of native sequence was cloned from genomic DNA into the pBadA (arabinose inducible, ampicillin selection) *E.coli* expression plasmid. A start codon and N-terminal His6 tag was inserted upstream (construct Cu31). Various mutants

were built by site directed mutagenesis off this plasmid backbone including (C-A), (V-L), and (H-Y). Plasmids were transformed by electroporation (Eporator, Eppendorf) into the arabinose compatible expression strain BL21-AI™ (ThermoFisher). For expression, a starter culture was inoculated into 8 liters LB ampicillin and shook at 37°C. Cultures were induced at OD_{600} 0.5 by addition of arabinose to final concentration of 0.02%. Temperature was then shifted to 18°C and cultures were shook all night long. Cultures were pelleted by centrifugation in a Sorval RC5B plus floor centrifuge using a SLA-3000 rotor at 16,9000 g for 10 mins. Cell pellets were placed on ice and resuspended in 5 ml binding wash buffer (50 mM sodium phosphate pH 8.0, 300 mM sodium chloride, 0.01% tween-20, 10 mM Imidazole). To assist lysis, samples were incubated on ice with a pinch of egg white lysozyme (VWR) for 30 minutes. Samples were then passed twice through French press then centrifuged at ~5,000 G for 6 hours in a Heraeus Multifuge X1R (Thermo Scientific) at 4°C. Supernatants with soluble protein extracts were then passed twice through 0.45 μm syringe filters (Acrodisc, Pall) and transferred into 15 ml conical tube with 2 ml HisPur cobalt beads (ThermoFisher). Tube was inverted at 4°C with for 30min. Contents were transferred to a disposable column and pushed through using a peristaltic pump. Column was then washed with 40 column volumes of binding wash buffer. Proteins were eluted off the column by addition of 1 ml elution buffer (50 mM sodium phosphate pH 8.0, 300 mM sodium chloride, 0.01% tween-20, 300 mM Imidazole). Column was then capped and inverted for 15 min at 4°C. Eluent was pushed through the column and collected into a fresh tube, followed by a final wash with 1 ml elution buffer yielding 2 ml total eluent. Samples were simultaneously concentrated, and buffer exchanged by centrifugation in a centrifugal filter (Amicon Ultra-4; 3K NMWL). Elution Buffer was exchanged to a storage buffer (50 mM Tris-HCl pH 7.6, 150 mM NaCl and 1 mM dithiothreitol (DTT), 30% glycerol). Purified proteins were aliquoted into 1.5 ml

tubes and stored at -80°C. Protein yields were calculated by comparison to a standard bovine serum albumin (BSA) curve; using densitometry of Coomassie stained SDS-PAGE gels with the Gel Doc Ez Imager (Biorad).

Densitometry (figure 4): The principle behind protein calibration using densitometry involves running a range of samples of a protein with known protein concentrations and a range of samples of a desired protein(s) with unknown protein concentration levels side-by-side on an SDS-PAGE gel. Once ran and imaged, a software can then be used to compare the absorbance readings of both ranges to appreciate the concentration of the desired protein(s) that is unknown. In this experiment, BSA was used as the protein with known protein concentration values when appreciating the concentration of CidB enzyme. While mono-Ub was used as the protein with known protein concentration when appreciating enzyme kinetics of the cleavage activity of the DUB active site. To find the range of BSA concentrations that would be suitable, a dilution gradient of purified CidB protein samples was ran on an SDS-PAGE gel. This accommodates for two things. Firstly, the amount of CidB in the purified protein sample at the purification stage was unknown. So, it was imperative to accommodate for a suitable protein concentration quantity that could be accurately quantified. If an abundance of protein was expressed and purified, the concentration had to be lowered to a suitable range that could be visible on a gel without smearing. Or, if the amount of protein produced was too little, aliquoted samples had to be concentrated to be in a visible range that can be accurately quantified. The second reason for accommodating the unknown protein quantity, is to enable the researcher (me) pick a suitable range of known protein concentration (BSA or mono-Ub) that would be optimal for calibrating the unknown protein concentration. Once ran and imaged, ImageJ software was used to obtain absorbance readings for all the proteins samples ran on the SDS-PAGE gel. The absorbance

values were then transferred to Microsoft excel and a linear regression model (y=mx+c) was obtained using BSA concentration values that were cross referenced with their absorbance readings. Using the linear relationship between BSA concentration values that were cross referenced with their absorbance readings, the values for the unknown concentrations of CidB were appreciated.

Ubiquitin Cleavage Assays: Mono-Ubiquitin (Ub), di-Ubiquitin (Ub_2), poly-Ubiquitin (all Boston Biochem), and purified CidB were thawed on ice. Ubiquitin digestion assays (total volume 20 µl) were mixed in Ubiquitin cleavage buffer (50 mM Tris HCL pH 7.6, 20 mM KCl, 5 mM $MgCl_2$, 1 mM DTT) to a final concentration of 0.2 µM enzyme and 2 µM substrate. Samples were incubated at 37°C for 5 min. on a rotator. Reactions were quenched by addition of 5µl of 4x Laemmli SDS-PAGE buffer. Samples were warmed for 20 min on hot plate at 40°C (according to manufacturer specifications). Samples were then run on 15% SDS-PAGE gels and stained using a standard silver stain kit (Fast Silver, G Biosciences). Michaelis-Menten kinetics were measured by holding enzyme concentration constant while varying substrate concentrations from 0.5 µM to 4 µM. These gels were silver stained and the change in concentration of mono-Ubiquitin [product] was compared to a standard mono-Ubiquitin curve using ImageJ software. Values were transferred to Graphpad Prism software and plots were created using default Michaelis-Mentin algorithm analysis.

Results and Discussion:

Initial tests showed decreased deubiquitylation from the $CidB^{wMel}$(V-L) when compared to $CidB^{wMel}$ and $CidB^{wMel}$(H-Y) (Figure 5A). I then repeated the tests with K48 linked di-Ubiquitin and poly-Ubiquitin substrates and observed the same patterns, though to a lesser degree (Fig. 5B and 5.3). The K48 substrate data were more variable, and the divergences were

not as strong compared to K63. Therefore, I quantified the hypomorphic activity of CidB^{wMel}(V-L) using timed 5-minute digests of both K63 and K48 linked di-Ubiquitin in conjunction with Michaelis- Mentin kinetics to measure the precise reduction in enzyme efficiency (Fig. 5C, D, and E). The (V-L) point mutation strongly reduces CidB^{wMel} ability to bind to K63 (Fig. 51E), while the ability to bind to K48 is almost unchanged (minimal ratio change in K*M* see value 1.1 in Fig. 51E). Similarly, the ability to cut K63 is more strongly impaired than the ability to cut K48 (comparing ratio changes in kcat values, see values 8.3 vs 1.6 in Fig. 5E). Overall CidB^{wMel}(V-L)'s enzyme efficiency is reduced 2.4-fold for the K63 substrate and ~1.5 fold for K48. This indicates that CI is likely induced by an interaction involving K63 Ubiquitin linkages because the CidB^{wMel}(V-L) mutant also reduced transgenic CI penetrance. These new data support a previous hypothesis that CI is induced by cleavage of K63 linked chains from an unknown substrate, perhaps P32 or Karyopherin-α (Beckmann et al. 2017; Beckmann, Sharma et al. 2019).

In summary, the *w*Yak cid mutations that were predicted to influence CI strength do, with one (V-L) reducing CidB^{wMel} CI strength by an average of 2.6-fold, and an N-terminal truncation ablating CidB^{wMel} CI by a yet unknown mechanism. Parallel reduction in DUB activity on K63 by an average of 2.4-fold directly correlates CI induction to DUB function. Since wYak causes sporadic and weak CI (Cooper et al. 2017), and because complete CidwYak and CinwYak did not individually induce CI here, my data suggest three possible hypotheses for the existence of relatively weak wYak CI: these operons interact to produce weak CI (Shropshire et al. 2020); expression from native *Wolbachia* contributes to and/or modulates *cif*-induced CI; and/or host genomes modulate CI strength (Reynolds & Hoffmann 2002; Beckmann, Sharma et al. 2019; Shropshire et al. 2020), a pattern documented in natural D. yakuba-clade crosses (Cooper et al.

2017). Regardless, I predict that DUB contributions are significant, and my results demonstrate how single mutations may directly weaken CI through their effects on DUB enzymatic activity.

Importantly, my discovery of a hypomorphic mutation that preferentially cleaves different ubiquitin linkages will help identify the penultimate target of CI. On what timescale is CI disrupted? Disruptions of *cifB* are prevalent within published genomes and appear to underlie a common process of pseudogenization (Martinez et al. 2021), leading terminally toward rescue only phenotypes as predicted by theory (Turelli 1994). This process appears to be concurrently playing out in wRi, wYak, and wMau. In these systems wRi is an early-stage example, still bearing strong CI (with 2/3 cifs putatively pseudogenized) (Hoffmann et al. 1986; Beckmann & Fallon 2013; LePage et al. 2017; Shropshire et al. 2021; Martinez et al. 2021); wYak is at an intermediate stage, with both *cifs* putatively pseudogenized (Cooper et al. 2019), yet still capable of weak and sporadic CI (Cooper et al. 2017); and wMau is at a terminal stage, retaining only rescue functions with *cifB* fully psuedogenized in the last few hundred thousand years (Meany et al. 2019). My results demonstrate that individual mutations in *cifs* can influence the strength of CI caused by wMel-like Wolbachia that diverged in the last 30,000 years. Additional sampling of closely related *Wolbachia* that differ in CI phenotype is needed to better understand the timing of *cif* degradation, which is clearly relevant for *Wolbachia* biocontrol strategies in mosquito systems (Dobson et al. 2002, Hoffmann et al. 2011, Turelli, & Barton 2017; Ross et al. 2019). Despite weak purifying selection, CI is incredibly common in nature (Shropshire, Leigh et al. 2020). This motivates additional analyses of *cif* function, unrelated to CI, that might lead to selection pressures that indirectly preserve CI. The prevalence of CI-inducing strains in nature also provides the opportunity for them to move horizontally among host species (Hurst & McVean 1996), as demonstrated for wMel-like and wRi-like *Wolbachia* (Conner et al. 2017;

Turelli et al. 2018; Cooper et al. 2019). As previously noted (Martinez et al. 2021), such clade selection may also act on *cif* genes themselves, which are associated with mobile elements that mediate horizontal *cif* transfer among divergent genomes (LePage et al. 2017; Gillespie et al. 2018; Cooper et al. 2019). Indeed, theoretical analysis supports the plausibility of this process of clade selection on *Wolbachia* and *cifs* to explain the contradiction of pervasive CI in nature and *cif* degradation (M. Turelli, personal communication with J.F. Beckmann).

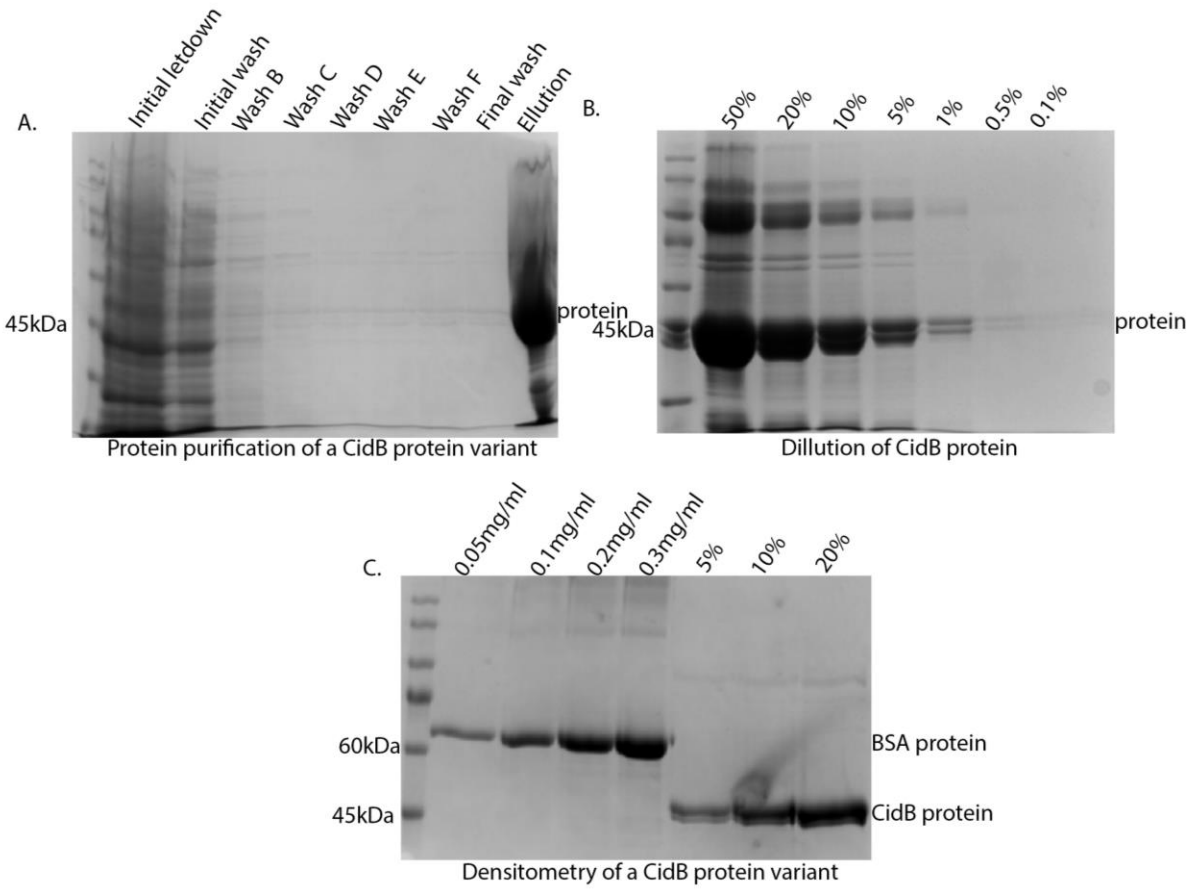
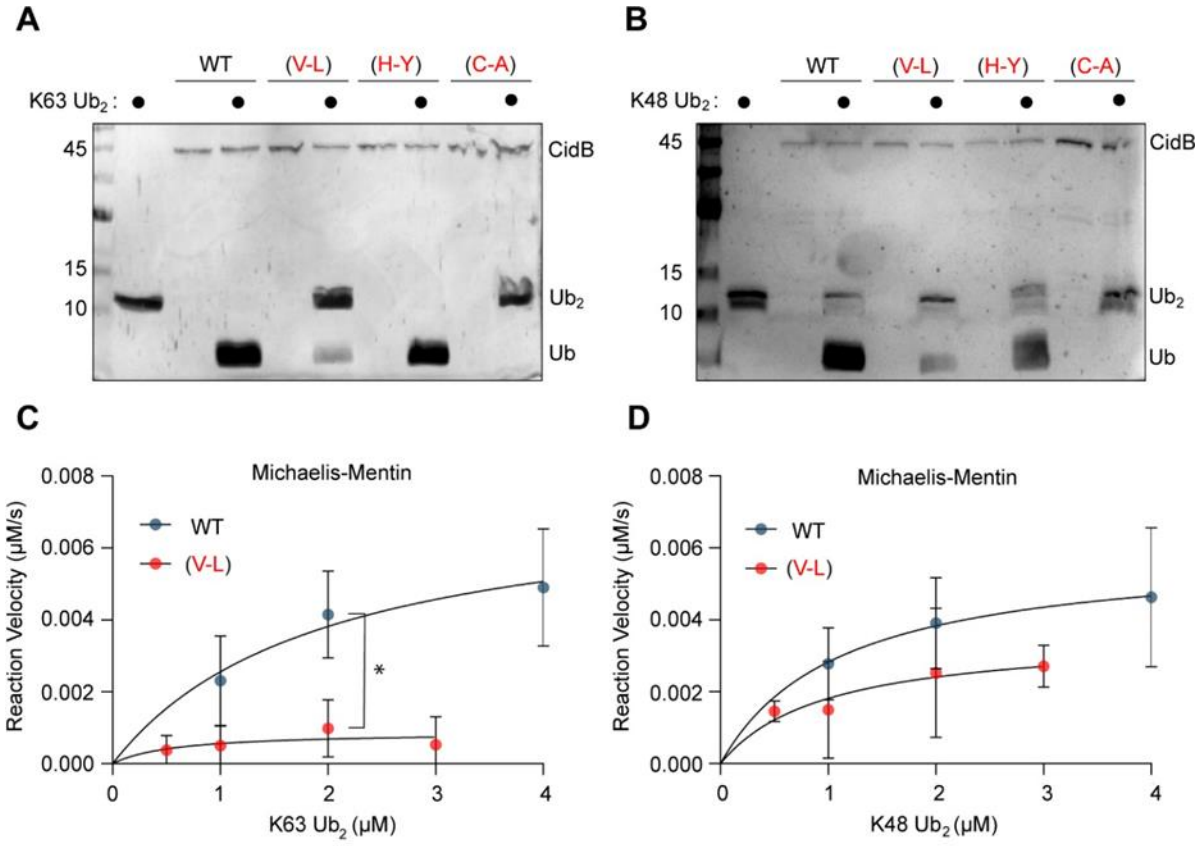


Figure 4: Protein purification, dilution and densitometry of a CidB protein variant



E

Substrate	Enzyme	KM (μM)	Vmax (μM/s)	kcat(1/s)	kcat/KM
K63 Ub_2	WT	1.948	0.0075	0.0375	0.0193
K63 Ub_2	(V-L)	0.571	0.0009	0.0045	0.0079
	ratio WT/(V-L):	3.4	8.3	8.3	2.4
K48 Ub_2	WT	1.109	0.0060	0.0300	0.0271
K48 Ub_2	(V-L)	0.975	0.0036	0.0180	0.0185
	ratio WT/(V-L):	1.1	1.7	1.6	1.4

Fig. 5. Ubiquitin cleavage assays with CidB^{wMel} variants. CidB^{wMel} proteins are truncated to the DUB domain (residues H717-R1128). (V-L) and (H-Y) are substitutions found in CidB^{wYak}. (C-A) is catalytic mutant negative control. Silver stained SDS-PAGE analysis of one-hour digests with K63 linked di-Ubiquitin (Ub2) (A) and K48 linked Ub2 (B). The (V-L) mutant has reduced DUB activity compared to wildtype (WT). Markers are kDa. Michaelis-Mentin graphs showing reaction velocity vs substrate concentration from timed 5-minute digests for K63 (C) and K48

(D). (V-L) reaction velocities are reduced compared to wildtype. At substrate concentrations of 2 μM the difference is statistically significant (P = 0.008, unpaired t-test with Welch's correction). E. Quantification of kinetic parameters for WT and (V-L) enzymes. Enzyme efficiency (kcat/KM) of (V-L) mutants are reduced 2.4-fold and ~1.5 fold from the wildtype for K63 and K48, respectively.

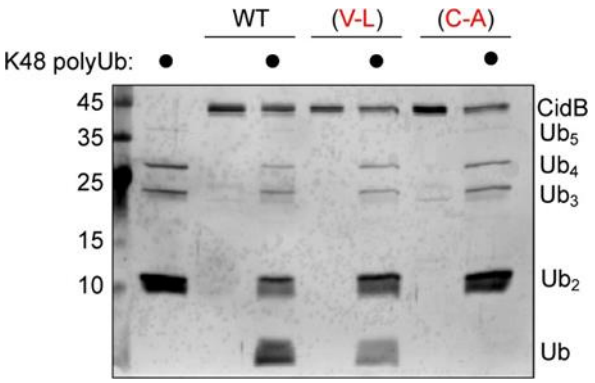


Fig. 6. Poly-Ubiquitin cleavage assays with CidB^{wMel} variants. All CidB^{wMel} proteins are identical to those described in Fig. 3. A-B. Silver stained SDS-PAGE analysis of one-hour digests with poly-K48 linked Ubiquitin (Ub2-5). The (V-L) mutant shows reduced DUB activity in comparison to wildtype (WT).

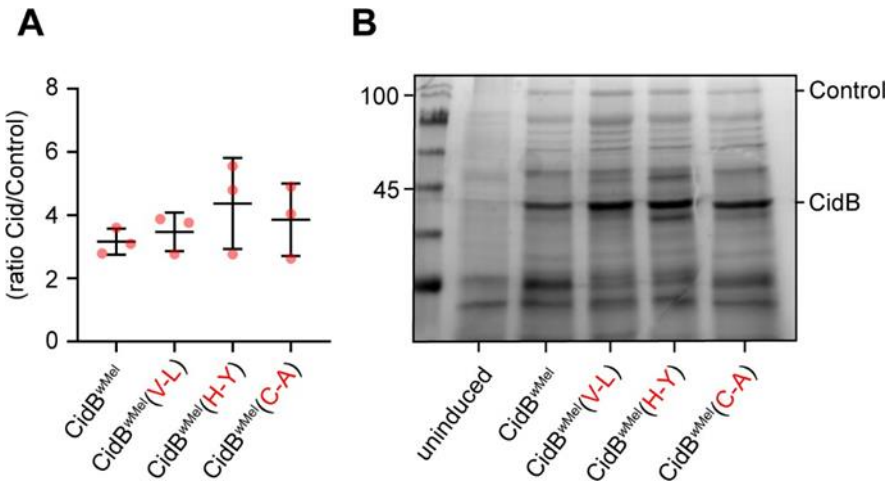


Fig. 7. Variant CidB DUB domain protein levels after recombinant protein expression in *E.coli.* A. Quantified ratios (CidB / 120 kDa Control band) of silver-stained SDS-PAGE gel bands (Beckmann et al. 2021). B. Example silver-stained SDS-PAGE gel. Integrated densities are measured for corresponding gel bands in ImageJ and plotted in Graphpad Prism. The gel is representative of triplicates. All mutants tested are not expressed less than native CidB$^{w\text{Mel}}$ DUB sequence.

2.4 Future Perspectives:

The first major improvement would be to replicate these results in a full CidB protein model rather than just the truncated DUB domain. Looking at how protein structure and function behaves in the complete model will give a better insight into the differences in functioning of WT CidB in comparison to mutated CidB variants. Another major improvement in this research experiment would be to analyze the biological area where these CidB proteins carry out their function to cause CI. Understanding the chemical components of this biological area (inside the sperm cells of host species), will help to better simulate experimental scenarios closer to the theoretical environment in the wild. This will give a better perspective on the nature of the protein and offer other possible factors that might lead to hypomorphic CI in the wild.

To take this experiment further, replication of the valine to leucine mutation in CI-causing *Wolbachia* strains could be done to find similarities and differences in its efficacy, strengths of hypomorphicity, and even the effects of abiotic factors based on different host organisms impacted.

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