Suppression of *Wolbachia* mediated Cytoplasmic Incompatibility

by

Gagan Deep Sharma

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Approved by

Dr. John F. Beckmann, Assistant Professor of Entomology
Dr. Kathleen Martin, Assistant Professor of Entomology
Dr. Nannan Liu, Chair & Endowed Professor of Entomology

Abstract

Insect biocontrol applications often hinge upon insect reproduction. *Wolbachia* are maternally inherited intracellular bacteria that infect insects. *Wolbachia* sterilizes a mosquito sperm resulting in a lethal embryonic phenotype called cytoplasmic incompatibility (CI), wherein eggs from un-infected females fail to develop when fertilized from *Wolbachia*-infected males. Contrary to this, if males and females both are infected, embryos are viable. The molecular mechanism of CI involves *Wolbachia* deubiquitylating enzyme *CidB* and its cognate partner antidote, *CidA.*

In CHAPTER II, the process of identifying potential molecular targets and suppressors of *Wolbachia's CidB* is described. By finding alleles capable of suppressing CI, we will gain insights into the molecular pathways targeted by *CidB. In toto*, we propose that *CidB* targets Kap-α, nuclear-protein import and P32, protamine-histone exchange to induce CI.

Next question was whether *CidB* cleaves ubiquitin directly off Kap-α or P32 to induce CI. In CHAPTER III, functional mutations and genetic modifications in these two genes were explored to test the role of these two genes in the suppression of CI in *Drosophila*. The work done here is important for two reasons, firstly, they provide hints toward pathways targeted by CI. Secondly, these genes might co-evolve as resistance factors to CI. Importantly, suppression of CI in vectors will reduce the effectiveness of global mosquito control efforts harnessing *Wolbachia* and CI.

Dedicated to my mom, my sister and

to the loving memories of

my Dad

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Table of Contents

List of Tables

List of Figures

LIST OF ABBREVIATIONS

CDK1	Cyclin-dependent kinase 1
CHIKV	Chikungunya Virus
CI	Cytoplasmic Incompatibility
CMY	Cornmeal Molasses Yeast media
Cid	CI-inducing deubiquitylase
Cin	CI-inducing nuclease
DNA	Deoxyribonucleic acid
DENV	Dengue Virus
DUB	Deubiquitylating enzyme
EDP	Eliminate Dengue Project
EPA	Environmental Protection Agency
GFP	Green Fluorescent Protein
GTP	Guanosine Triphosphate
IIT	Incompatible Insect Technique
MTD	Maternal Triple Driver
MSA	Multiple Sequence Alignment
NEB	Nuclear Envelope Breakdown
NFW	Nuclease-Free Water
NGT	Nanos-Gal4-Tubulin driver
NLS	Nuclear Localization Signal

NPC	Nuclear Pore Complex
PCR	Polymerase Chain Reaction
ROS	Reactive Oxygen Species
SD	Segregation Disorder
SIT	Sterile Insect Technique
TA	Toxin-Antidote
UAS	Upstream Activation Sequence
UTR	Untranslated Region
WT	Wild Type
YFV	Yellow Fever Virus

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Bacteria Endosymbionts of Insects

Insects are the most diverse group of animals, implying that the numbers of insect species are more than any other group (Chapman, A.D., 2006). In a conservative estimate, at least 15-20% of insects develop symbiotic relationships with bacteria (Batra & Buchner, 1968). Sometimes endosymbionts form mutualistic relationships with their insect partner, whereas in some cases, they cause severe effects on various biological functions of their insect host (Feldhaar & Gross, 2009). The association between insects and bacteria can be obligate or facultative.

Obligate endosymbiosis (from the perspective of the host) means that the host cannot survive without the endosymbiont. Often, these symbionts provide their hosts with nutrients or perform other functions that are essential for their normal growth and development. An obligate intracellular relationship (from the perspective of the symbiont) implies that the symbiont cannot be cultured outside their hosts. Frequently, in an insect, obligate endosymbionts live in specialized cells known as bacteriocytes that may constitute a large organ structure called bacteriome. Obligate endosymbionts associated with bacteriocytes are frequently transmitted maternally by a mechanism known as “transovarial transmission” (or vertical transmission). In vertical or maternal inheritance, the symbiont directly infects the embryos within the maternal body, and this type of transfer of bacterial endosymbionts is widespread among arthropods (Mira & Moran, 2002). Other symbionts are facultative, which implies that they are not necessary for the host’s survival and that bacteria can usually replicate. Facultative endosymbionts do not reside in specialized host cells

but may occur extracellularly in hemocoel or other body tissues such as fat body, gut, nervous, or muscular tissue (Wernegreen, 2002).

Wolbachia (Rickettsiales: Anaplasmataceae), *Rickettsia* (Rickettsiales: Rickettsiaceae), and *Cardinium* (Bacteroidales: Bacteroidaceae) are the most common type of endosymbiotic bacteria found in arthropods including insects, mites, spiders, crustaceans as well as in some non-arthropods such as filarial nematodes (Stouthamer et al., 1999; Weinert et al., 2015; Werren et al., 2008). They are commonly referred to as reproductive manipulators because of the phenotypic effects they exhibit on their hosts. *Rickettsia* and *Cardinium* infect 24% and 13% of all arthropod species, respectively (Weinert et al., 2015), whereas *Wolbachia* alone infects nearly 53% of terrestrial arthropods (Zug & Hammerstein, 2012), 52% of aquatic insects (Sazama et al., 2019) as well as a significant number of filarial nematodes (Ferri et al., 2011). *Wolbachia* is the most prominent member of reproductive manipulators, thanks to its wide range of phenotypes it can induce.

1.2 History of *Wolbachia*

Wolbachia was firstly described in 1924 by Marshall Hertig and Simeon Burt Wolbach. These two observed it in crushed gonads of *Culex pipiens* (common house mosquito). These two studied micro-organisms (at the time broadly grouped into *Rickettsiae*). They examined many arthropods, including twenty-five males and females of *Culex pipiens* collected from Boston and Minneapolis. They observed “gram-negative, rod-like coccoid bodies” in the smears of testes, ovaries as well as in the eggs of *Culex pipeins* (Hertig, 1924). They called them “Harmless Rickettsia.” Hertig continued his study on this bacterium and later in 1936 formally named this parasite as *Wolbachia pipientis* in honor of Wolbach (Hertig, 1936).

Further, in 1936 Hertig published an article in which he mentioned two ways in which these bacteria can be transmitted in organisms. The most commonly observed method of *Wolbachia* transmission is via egg cytoplasm from mother to the offspring. Other method noted was individual to individual transmission, which is less frequently observed. He also noted that *Wolbachia* is found in testes and ovaries of hosts, as well as, in mature eggs, but not in mature sperm, which means they are eliminated during spermatogenesis from the germline. Thus, females remain the only source of *Wolbachia* transmission to offspring.

It is estimated that *Wolbachia* invaded arthropods approximately 200 million years ago (Gerth & Bleidorn, 2017). *Wolbachia* impacts the host arthropod in diverse ways, such as general nutrition (Hosokawa et al., 2010) and immunity. Importantly it was recently discovered to convey anti-viral protection against insect-vector borne disease (Dobson et al., 1999; Hedges et al., 2008; Kern et al., 2015; Werren et al., 2008; Zug & Hammerstein, 2012). *Wolbachia* impacts development pathways through stem cell proliferation and embryogenesis (Fast et al., 2011). In the stem cell niche, it is thought to speed up stem cell production, which would have the downstream advantage of making females more fecund, and thus more likely to maternally transmit *Wolbachia*. It has been posited, in some cases, to drive speciation events (Bordenstein et al., 2001; Jaenike, 2007a). The wide range of impacts on insect biology emphasizes the importance of studying *Wolbachia*.

1.3 *Wolbachia*-induced reproductive alterations

Wolbachia parasitic strains have evolved at least four ways to manipulate host reproduction which (in general) selectively favors infected females, thus ensuring their intergenerational

transmission and persistence in populations (Werren et al., 2008). These include parthenogenesis, feminization, male killing, and cytoplasmic incompatibility. They are outlined below.

1.3.1 Parthenogenesis

Wolbachia-induce parthenogenesis in some species like parasitic wasps such as *Trichogramma* (R. Stouthamer et al., 1999), mites (Weeks & Breeuwer, 2001), and thrips (Arakaki et al., 2001) however, it is much less common than CI. Multiple mechanisms are conflated into a broad category of parthenogenesis which causes confusion amongst the dissimilar mechanisms. To elaborate, the simple definition of parthenogenesis is to replicate another organism without input from a sexual mate, but this can happen via multiple mechanisms and in different sexual/genetic contexts. In the diploid form of parthenogenesis diploid egg, with inhibition of meiosis will grow into clone of the mother. This is a diploid form of parthenogenesis. In haplo-diploid parthenogenesis, sex is determined by diploid vs haploid status. More specifically, in some cases, two pairs of chromosomes cause female development while haploids, having one pair of chromosomes, develop into males. The opposite can also be true. In these organisms, *Wolbachia* infection in the haploid egg cells results in the doubling of a haploid set of maternal chromosomes, causing the eggs to develop into females (Richard Stouthamer & Kazmer, 1994). Thus, the females infected with *Wolbachia* will produce twice as many offspring as the non-infected ones. Thus, parthenogenesis can result from two distinct mechanisms in the egg, one where meiosis is inhibited and another where haploid egg DNA is replicated to become diploid.

In a general sense, parthenogenesis is a means of doubling the output of females to the exclusion of male sex determination. Downstream, *Wolbachia* are then inherited at double the frequency and produce twice as many offspring (all females) as the non-infected ones.

Mechanisms causing *Wolbachia*-induced parthenogenesis might utilize failure of chromosome separation or a cycle of incomplete cytokinesis, but the exact mechanisms remain ambiguous (Serbus et al., 2008).

1.3.2 Feminization

In some populations, *Wolbachia* converts the males into fully functional females, able to reproduce hence increases its transmission. Feminization was first characterized studied in Isopods (Crustaceans) by Thierry Rigaud and his colleagues at the University of Poitiers in Poitiers, France, in the 1990s. In Isopods, females are heterogamous (ZW sexual chromosomes), whereas the males are homogamous (ZZ sexual chromosomes). After *Wolbachia* infection, bacteria proliferate within the androgenic glands, inhibits their function, and eventually, genetic males (ZZ) develop into females (Vandekerckhove et al., 2003). *Wolbachia*-induced feminization is known in several insect species such as *Eurema hecabe* {Order: Lepidoptera (Hiroki et al., 2002)} and *Zyginidia pullula* {Order: Hemiptera (Negri et al., 2006)}. Currently, the exact mechanism of how *Wolbachia* causes feminization in insects is unclear. Recent studies suggest that it may be due to the interference of *Wolbachia* in the sex-determination pathways (Narita et al., 2007), the involvement of prophage WO gene within *Wolbachia's* genome (Pichon et al., 2012) or improper segregation of sex chromosomes (Kern et al., 2015).

1.3.3 Male-killing

Gregory Hurst and his colleagues in 1999 studied male-killing due to *Wolbachia* infection, which led to the death of male embryos in two species i.e., *Adalia bipunctata* (ladybug) and *Acraea encedon* (the butterfly) at the University College London in London, United Kingdom. *Wolbachia*-induced male-killing occurs primarily during embryogenesis. The evolutionary hypothesis is that

selective killing of male offspring at early embryonic stages leads to the provisioning of female embryos with more energy or in common terms, more food for the surviving female progeny. Until now *Wolbachia*-induced male-killing has been observed in numerous arthropod species including flour beetle {*Tribolium madens* (Fialho & Stevens, 2000)}, fruit flies {*Drosophila innubila, D. bifasciata, and D. subquinaria* (Dyer & Jaenike, 2004; Hurst & Johnson, n.d.; Jaenike, 2007b)}, butterflies {*Hypolimnas bolina* (Duplouy et al., 2010)}, ladybird beetle {*Coccinella undecimpunctata* (Elnagdy et al., 2013)} and moths {*Ostrinia furnacalis* (Fujii et al., 2001)}. Interestingly, in *Spiroplasma* of *Drosophila melanogaster*, male killing was attributed to a deubiquitylating otu domain protein called *Sp*AID (Harumoto et al., 2018). The evolution of this protein seems ancestrally linked to CI inducing deubiquitylases (Gillespie et al., 2018). Therefore male-killing and CI may mechanistically be linked somehow.

1.3.4 Cytoplasmic incompatibility

CI is the most common reproductive alteration induced by *Wolbachia* (Serbus et al., 2008). Marshall, in 1938 observed a strange phenomenon where the British *Culex pipiens* mosquitoes, when crossed to French *Culex pipiens* were unable to produce a viable offspring even though they laid eggs. This indicates there was something inside the eggs that prevented them from hatching (Marshall, 1938). Hannes Laven from the Institute of Genetics of Johannes Gutenberg-University in Mainz, Germany, discovered a similar phenomenon in *Culex pipiens* where after performing several crossing experiments from isolates collected across Europe, he observed that some combinations produced few or sometimes no offspring. He further demonstrated that there was no physical barrier for the sperm as it entered the egg successfully. The paternal and maternal nucleus were not fusing (no karyogamy), and the paternal genome was not playing any role in the

development of inviable embryos. It was determined that this was due to the mother's influence (Laven, 1957). Laven stated that the incompatibility factor which led to the development of inviable embryos must be in the cytoplasm, and henceforth named this phenomenon as "Cytoplasmic Incompatibility." CI is defined as a conditional sperm sterility which can be rescued by a maternal antidote. In this way, CI is defined as a toxin-antidote system (Beckmann et al., 2017, 2019a; Beckmann & Fallon, 2013; Beckmann et al., 2019; Hurst, 1991; Poinsot et al., 2003a). In 1967, Laven used "Cytoplasmic Incompatibility" as a biological control tool to eradicate *Culex pipiens fatigans* mosquitoes, a vector of filariasis in Burma (now Myanmar) by releasing incompatible males which is similar to sterile insect technique (SIT) developed by Edward F. Knipling and Raymond Bushland which they used for the eradication of screwworm fly from North and Central America (Novy J.E., 1979). In 1971-1973, Janice Yen and Ralph Barr of UCLA found that cytoplasmic incompatibility was induced by the intracellular bacteria, *Wolbachia* (Yen and Barr, 1971). They proved this by treating the mosquitos with tetracycline, after which, the crosses became compatible. The potential application of *Wolbachia* includes its usage as a biocontrol agent for the control of insect populations and especially disease vectors (Yen and Barr, 1973).

In summary, CI is defined as the embryonic lethality that occurs when the sperms modified by *Wolbachia* fertilize eggs that are not infected with *Wolbachia*. However, in the reciprocal cross, i.e., the cross between an infected female and uninfected male or the cross between male and female infected with the same *Wolbachia* strain will produce viable *Wolbachia*-infected offspring (Figure 1.1). CI can be unidirectional or bidirectional. Unidirectional CI occurs when a single *Wolbachia* CI-inducing strain infects the host population, whereas bidirectional CI occurs when

the sperm from a male infected with a different and incompatible *Wolbachia* strain fertilizes the egg infected with the different *Wolbachia* strain (Figure 1.2).

1.4 Eliminate Dengue program (EDP)

One initial strategy was to attempt to utilize a pathogenic *Wolbachia* infection to reduce the lifespan of *Aedes aegpyti* mosquitos, but *A. aegypti*, the main vector of dengue virus (DENV), does not have a native *Wolbachia* symbiont. In an initial attempt the *wMelPop* strain of *Wolbachia* was heterologously introduced via microinjection into *Ae. aegypti* and it reduced their lifespan (McMeniman et al., 2009), but the mosquitos also displayed reduced fitness when released in small-scale fields (Ritchie et al., 2015). After that *Ae*. *Aegypti* was infected with a less pathogenic strain, *wMel* strain (*Wolbachia* strain from *D. melanogaster*) which did not greatly impact the fitness of the mosquitoes (Frentiu et al., 2014; Hoffmann et al., 2011; Walker et al., 2011). Along with DENV, the *wMel* strain also confers protection against yellow fever virus (YFV) and chikungunya virus (CHIKV) in *Ae. Aegypti* (Aliota et al., 2016; Hurk et al., 2012). When these *wMel* infected mosquitoes were released into a small number of neighborhoods in Australia, cytoplasmic incompatibility effectively replaced the native population with the infected mosquitos. The infected mosquitos were no longer capable of transmitting DENV (Hoffmann et al., 2011). Thus, one implementation of *Wolbachia* infections for biocontrol is a strategy of population replacement, which relies upon *Wolbachia*'s innate gene drive system, cytoplasmic incompatibility.

Another application of *Wolbachia* for biocontrol is in the sterile insect technique (SIT). These applications involve release of lab reared *Wolbachia* infected male mosquitoes into the wild. This approach has been approved by the US Environmental Protection Agency (EPA) as a tool

against the Asian tiger mosquito (*Aedes albopictus*) in 20 states of United States and Washington DC.

The most fundamental difference between the two approaches is, in population replacement, *Wolbachia*-infected females, as well as male mosquitoes, are introduced, whereas, in the latter one, only *Wolbachia*-infected males are introduced. The introduction of *Wolbachia*-infected female mosquitoes is required for the success of the population replacement method as *Wolbachia* is maternally inherited, and the goal of this approach is to infect and replace the targeted population. The success of the program has inspired a comprehensive effort to find applications for *Wolbachia* in other disease vectors. For example, the anopheline mosquitoes, which are the major carriers of malaria, are particularly important. In recent times, several species of *Anopheles* that might harbor a variety of *Wolbachia* strains have been discussed, which may confer CI and disrupt disease transmission (Gomes et al., 2018). However, there still remain several challenges before *Wolbachia* can be proposed as a tool for malaria control. At the moment, applications are focused on insect-borne virus control in *Aedes*.

1.5 Models to describe Cytoplasmic Incompatibility

Considerable efforts have been focused on studying the underlying mechanism of CI due to *Wolbachia's* role in disease control and insect ecology. Comprehensive cytology studies of embryonic defects in model insects offer suggestions of how CI causes embryonic fatality after fertilization. In 1968, Ryan and Saul published the earliest important cytological research on CI in *Nasonia vitripennis* and found that paternal chromosomes appeared abnormal with respect to chromatin condensation (Breeuwer & Werren, 1990). Another experiment performed in *Culex pipiens* by Erich Jost in 1970 demonstrated that in CI embryos, karyogamy is inhibited, thus

preventing the paternal chromosomes from contributing to embryonic development (Jost E., 1970). This reproduced similar experiments by Laven (Laven, 1957), but instead, he utilized novel genetic markers to reach his conclusion. These two studies led to the earlier conclusion that embryonic death from CI results due to defects in the chromatin of paternal insect sperm.

Since the 1990s, researchers have tried to specify what exactly *Wolbachia* does to insect sperm, an inability of paternal chromatin to condense certainly contributes to CI induction. This was cytologically characterized with clear microscopy in *Nasonia* (Breeuwer & Werren, 1990). In CI, majority of the embryonic deaths are associated with flaws in first mitotic division (Tram et al., 2006) which includes the inhibition of the cell-kinase CDK1 activity in the male pronucleus that catalyzes the metaphase to anaphase transition and delayed paternal nuclear envelope breakdown (Tram, 2002). There is impaired maternal histone deposition onto the paternal genome following protamine removal (Landmann et al., 2009). These cell cycle delays are accompanied by chromosomal abnormalities, especially in the paternal DNA, which includes impaired condensation and inability to segregate correctly that further leads to bridging and shearing of chromosomes during anaphase (Callaini et al., 1997; Reed & Werren, 1995; Ryan & Saul, 1968). A similar kind of CI cytology has been observed in various insects (Tram et al., 2003).

Despite a lack of evidence on precisely what sperm alteration used to causes the defects listed above, researchers turned to conceptual models that might describe CI-related defects, based on the position that (1) *Wolbachia* somehow "modifies" the sperm to cause severe defects in the timing and progression of mitosis, i.e., at early stages of spermatogenesis, and (2) females with the same strain can "rescue" this modification (Werren, 1997). Werren formalized these ideas through the "mod-rescue" model, which includes two functions: modification, where a sperm is modified

and rescue (in the egg) where the modification is restored to its original state. The conceptualization of CI as two distinct functions mod-resc; modification-rescue was important in that it provided a framework for experimental testing. These systems are compatible with a synonymous means of describing CI, the Toxin-Antidote (TA) model. In a TA model, sperm from *Wolbachia*-carrying males kill embryos with a toxin upon fertilization, unless the egg carries the antidote (Hurst, 1991). Although this *mod resc* (or toxin-antidote model) is a useful general idea, the specific nature of *mod* or toxin mechanisms have not been completely characterized and may in fact be redundant (Poinsot et al., 2003b).

Furthermore, there are additional conceptualizations which describe how CI might work to induce and rescue insect sterility. These additional models are (i) "slow-motion" model and (ii) "titration–restitution" model (iii) "lock-and-key model". The slow-motion model suggests that during the first mitosis, paternal chromosome condense and produces an anaphase-like appearance slower than maternal chromosomes due to the *mod* factor produced by *Wolbachia*, which results with cell cycle mis-timing between the maternal and paternal pronuclei. Here *mod* merely delays and does not entirely block entry into mitosis (Callaini et al., 1997). This model is further expanded to suggest that there is also delay in the nuclear envelope breakdown for the paternal material which creates asynchrony between the paternal pronucleus and maternal cytoplasm (Ferree & Sullivan, 2006; Serbus et al., 2008; Tram, 2002). *Wolbachia* causes a similar modification in maternal chromosomes when present in the egg; causing rescue and restores the synchronous cycle between maternal and paternal complements (Poinsot et al., 2003b).

The "titration–restitution" model posits that *Wolbachia* titrates certain essential components from the sperm during spermatogenesis. If the same strain of *Wolbachia* is present in

eggs, it will offset these criteria factors, allowing for the normal progression of embryogenesis. According to this model, “modification” and “rescue” can be determined by the same gene(s) if the change from titration to restitution is triggered by infected female hosts after fertilization or by separate genes if one gene codes for titration factors and the other codes for titration inhibition, resulting in restitution (Poinsot et al., 2003b).

According to the lock and key model, *Wolbachia* put a lock on the paternal genome, and only the female who is infected with the same *Wolbachia* strain has a necessary key to unlock these after fertilization, thereby rescuing the mitotic defects (Poinsot et al., 2003b). The uniqueness of the lock-and-key model is that it takes the framework of a toxin-antidote system but explicitly implies that a physical interaction occurs between the lock and its key. This is in contrast to mod-resc models which need not specifically implement rescue via any physical binding of the two components. Although each of the above models has its own merits, most current datasets support a system that follows the rules of Toxin-Antidote models with its lock-and-key specification (Beckmann et al., 2017, 2019a, 2019c; Beckmann & Fallon, 2013; Beckmann et al., 2019).

1.6 Causal factors for CI

Before the CI inducing genes were discovered various hypotheses, with little concrete evidence filled the literature. An initial study compared the host gene expression of infected vs. non-infected hosts, which showed that host histone chaperone Hira (Zheng et al., 2011) and the *D. melanogaster* JhI-26 gene (Liu et al., 2014) each might participate in CI, but these studies could only account for ~30% of the CI phenotype. In addition, work also centered on the large number of *Wolbachia* proteins containing ankyrin repeat domains, which are typically involved in protein-protein interactions and, therefore, a tempting host modification candidate. Multiple studies have

investigated the link between these proteins and cytoplasmic incompatibility, but no direct CI link has been found (Duron et al., 2007; Papafotiou et al., 2011; Yamada et al., 2007). *Wolbachia* infections purportedly leads to increased ROS levels in testes and ovaries, which further damages spermatid DNA, so reactive oxygen species (ROS) was also associated with CI-induction (Brennan et al., 2012).

The genes by which *Wolbachia* causes CI were identified in 2013 (Beckmann & Fallon, 2013). Based on the hypothesis that the CI toxin (not the bacteria itself) should be present in the mature sperm of *Wolbachia*-infected males of *Culex pipiens* mosquitoes, a sperm proteomic study was conducted to identify possible CI-inducing protein candidates (Beckmann & Fallon, 2013). This approach finally identified peptides encoded by wPip_0282 (or wPa_0282) in *Wolbachia* modified sperm from dissected *C. pipiens* spermathecae using liquid chromatography tandem mass spectrometry. The wPip_0282 was initially predicted to be a toxin gene within a TA operon pair but was later in-fact revealed to be the antidote. It is a part of the two-gene operon system due to its synteny and co-transcription with another downstream *Wolbachia* gene, i.e., wPip_0283, later found out to be the toxin (Beckmann & Fallon, 2013).

Biochemical analyses, heterologous yeast expression, and *Drosophila* transgenic expression of the *w*Pip (*Wolbachia* from *Culex pipiens*) CI genes showed that wPa_0283 is a CI-inducing DUB (deubiquitylating enzyme) and wPa_0282 was an inhibitor of toxicity induced by that protein domain in yeast. These specific genes are now annotated as *CidB* (CI inducing DUB) and *CidA* .A combination of methods and models systems was used to demonstrate that these genes (*cidA* and *cidB*) and their homologs in *Wolbachia* from *Drosophila* are responsible for induction and rescue of CI (Beckmann et al., 2017; LePage et al., 2017). *CidB* is always encoded downstream

of *cidA* (Beckmann & Fallon, 2013) and thus the syntenic structure of the operon mirrors that found in toxin-antidote systems (Beckmann et al., 2019a). Mutagenic analysis of $CidB_{wPip}$ at the active site cysteine (C1025A) eliminated CI in transgenic insects (Beckmann et al., 2017). *CidB* always encoded downstream of *cidA* (LePage et al., 2017). Thus, the CI phenotype was directly linked to deubiquitylating activity. Ubiquitin is a small protein that post-translationally modifies protein substrates and has many different functions. Ubiquitylation of proteins is reversed by cellular DUBs which specifically cleave ubiquitin from ubiquitin-modified proteins (Ronau et al., 2016). Importantly, these two proteins were found to interact with each other in a cognate-specific manner (Beckmann et al., 2017) in accordance with predictions set forth in the lock and key model described above (Poinsot et al., 2003b).

These factors, when expressed in uninfected transgenic *Drosophila* males and crossed with wild type females, recapitulate CI during the first embryonic mitosis (Beckmann et al., 2017; LePage et al., 2017). An interesting conflating of these data is that sometimes CI can be induced by transgenic expression of just the toxin and sometimes it requires expression of both A and B proteins (Chen et al., 2019; Shropshire & Bordenstein, 2019). Ongoing research is investigating these divergences and seeks to understand the role of A proteins in the induction of CI.

1.7 Tackling the unknown: discovering a mechanism for CI

Wolbachia-induced CI has the potential to control vector population (especially the mosquitoes) and protect millions of people from arboviral diseases such as dengue, Zika, West Nile and Yellow fever (Dorigatti et al., 2018; Dutra et al., 2016a; Slatko et al., 2014). Understanding the mechanism, upon which these control efforts rely, is an important step toward

more effective *Wolbachia* based biocontrol strategies. As described above the *Wolbachia CidA* and *CidB* proteins were recently found to be central to CI, but no *CidB* targets were known yet. We do not understand what cellular mechanisms *CidB* manipulates to induce CI. A simple experimental means of identifying molecular enzyme pathways is to isolate and identify suppressors of phenotypes. Because *CidB* induces strong phenotypes in both yeast and *Drosophila* we were able to utilize these systems to identify suppressors. In CHAPTER II, we describe the process of identifying potential molecular targets and suppressors of *Wolbachia's CidB.* In CHAPTER III, we focused on determining whether *CidB* cleaves ubiquitin directly off Kap-α or P32 to induce CI. The work done here is important for two reasons, firstly, to provide hints toward pathways targeted by CI and secondly, they might co-evolve as resistance factors to CI. Importantly, suppression of CI in vectors will reduce the effectiveness of global mosquito control efforts harnessing *Wolbachia* and CI.

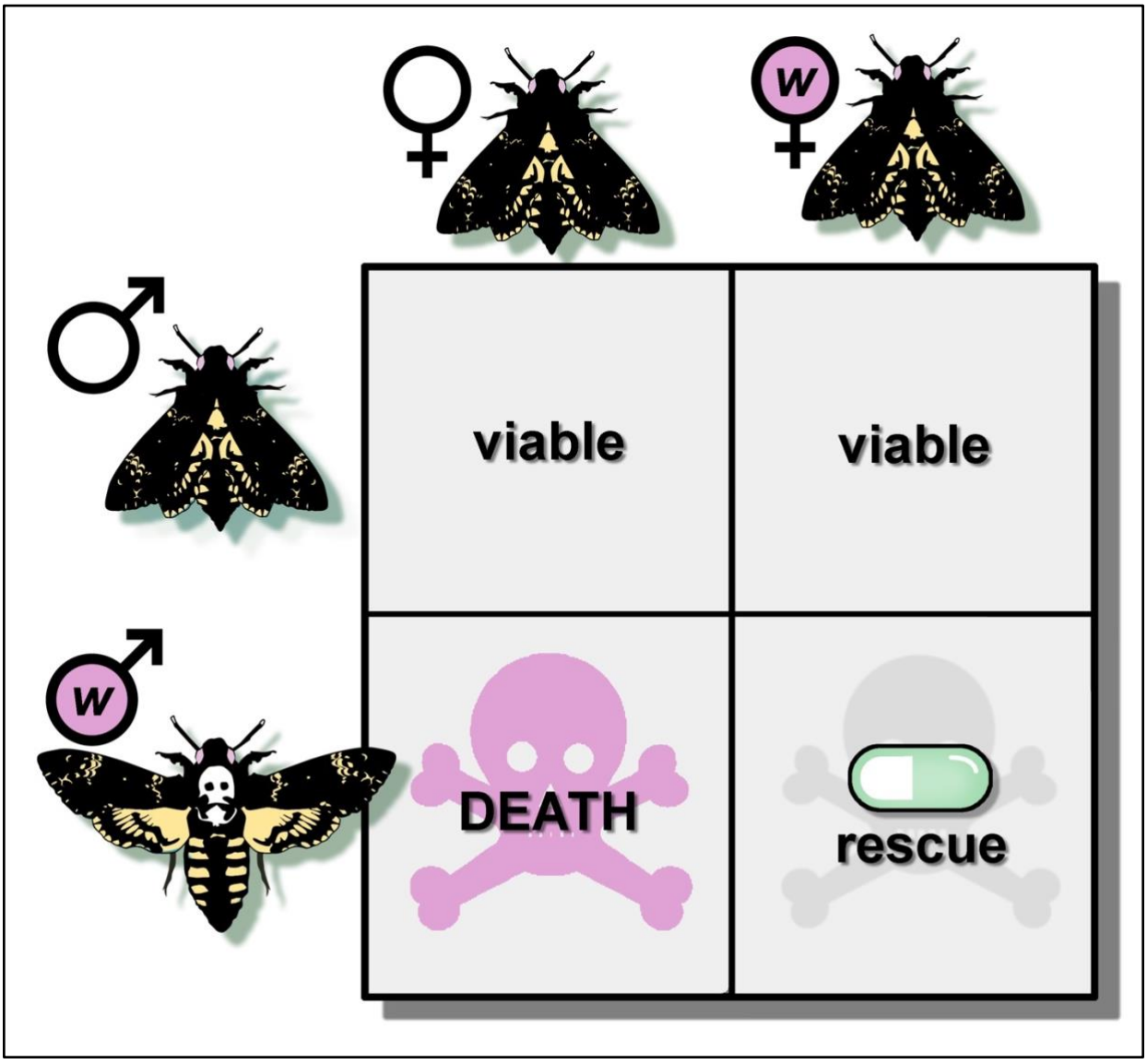

Figure 1.1. *Wolbachia*-induced cytoplasmic incompatibility If the females are infected with *Wolbachia* (w, pink), then they will be compatible with both *Wolbachia* infected as well as uninfected males, whereas if the females are not infected with *Wolbachia*, then they will be compatible with uninfected males only. Abbreviation: w, *Wolbachia*-infected (Figure altered from Beckmann et al., 2019a)

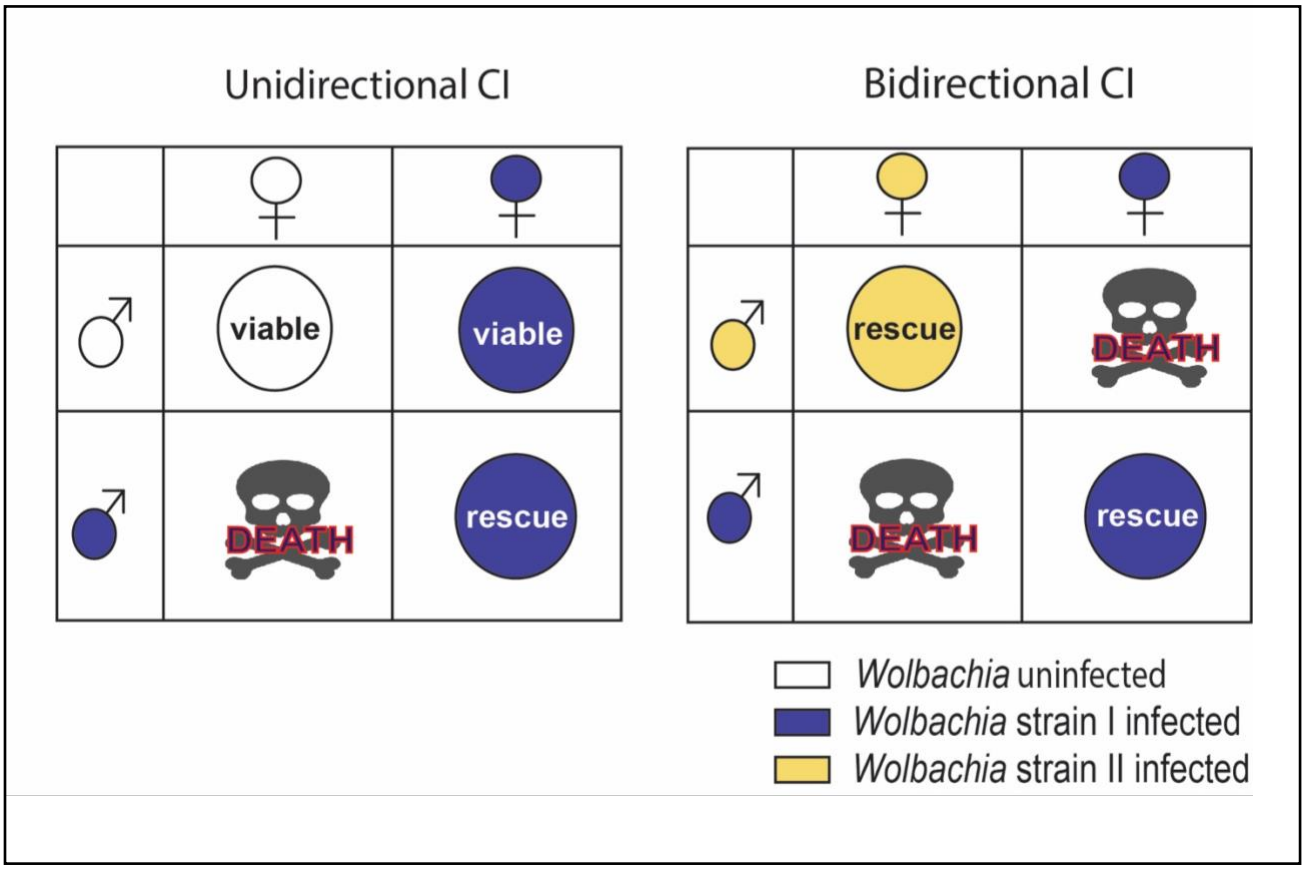

Figure 1.2. Unidirectional and Bidirectional CI. Unidirectional CI results from mating of infected males and uninfected females. All other crosses are compatible whereas bidirectional CI results from mating of males infected with *Wolbachia* strain I and females infected by *Wolbachia* strain II (and vice versa).

CHAPTER 2

THE *WOLBACHIA* CYTOPLASMIC INCOMPATIBILITY ENZYME CIDB TARGETS NUCLEAR IMPORT AND PROTAMINE-HISTONE EXCHANGE FACTORS†

2.1 Abstract

Insect biocontrol applications often hinge upon insect reproduction. *Wolbachia* are maternally inherited intracellular bacteria that infect insects. *Wolbachia* sterilizes mosquito's sperm resulting in a lethal embryonic phenotype called cytoplasmic incompatibility (CI), wherein eggs from un-infected females fail to develop when fertilized from *Wolbachia*-infected males. Contrary to this, if males and females both are infected, embryos are viable. CI can be rescued and suppressed under certain conditions. The molecular mechanism of CI involves *Wolbachia* deubiquitylating enzyme *CidB* and its cognate partner antidote, *CidA*. Ubiquitin is a post-translational modification that commonly regulates substrate stability and localization. The substrate targets *CidB* acts upon to induce CI are unknown. As a strategy to uncover this target, our research is focused on identifying host genes with the ability to modulate CI penetrance. By finding alleles capable of suppressing CI, we will gain insights into the substrate targets *CidB* acts upon to induce CI which are unknown. To complement this work, a high-copy suppressor screen

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of *CidB* toxicity was firstly performed in *Saccharomyces cerevisiae* (Baker's yeast) to test *CidB* toxicity in yeast. The most robust suppressor was karyopherin-α, a nuclear-import receptor, which requires nuclear localization- signal binding. A protein-interaction screen of *Drosophila* extracts also identified karyopherin-α and a protamine-histone exchange factor, P32 as primary interacting partners of *CidB.* The work of my thesis was to test the hypothesis that these proteins might be involved in CI. Through transgenic expression of *Drosophila* karyopherin-α and P32, we tested if either of these proteins could suppress CI. CI penetrance was measured by counting egg hatch-rates in CI crosses. Both the formerly mentioned genes do suppress wild and transgenic CI phenotypes. The hypothesis is that *CidB* targets nuclear-protein import and protamine-histone exchange to induce CI.

2.2 Introduction

As described above, the work of chapter 2 unfolds within the context of a broader study with multiple researchers. Our broad goal was to identify pathways targeted by CI. Below I will summarize what was known about the molecular mechanisms of CI in excerpts from our publication:

"When insects mate and form a viable zygote there are important structural changes that must take place to convert DNA derived from the paternal genome (sperm) into DNA that is capable of combining with the maternal genome (karyogamy). The insect zygotes follow well described developmental pathways (Loppin et al., 2015; Schmidt et al., 2017). An early step is nuclear envelope breakdown (NEB) of the sperm-derived male pronucleus. The small, highly basic

protamine proteins, used to package paternal DNA at high density are stripped from the DNA (Balhorn, 2007; Loppin et al., 2015; Rathke et al., 2014; Tirmarche et al., 2014, 2016) and nucleosomes are then assembled with maternal histones (J. Liu et al., 1997; Loppin et al., 2015). The protamine-histone transition utilizes specific histone chaperones such as P32 and Nap1 (Emelyanov et al., 2014; Emelyanov & Fyodorov, 2016). Subsequently, male and female pronuclei come together (but do not fuse) and undergo DNA replication. In the first zygotic mitosis, the two sets of chromosomes condense, align on the metaphase plate, separate in anaphase in parallel and then finally intermingle (Tram et al., 2003).

In CI zygotes, the earliest detected abnormality is impaired maternal H3.3 histone deposition onto the paternal DNA following protamine removal (Landmann et al., 2009). Paternal pronuclear NEB is delayed and activity of the cell-cycle kinase CDK1, which normally drives the metaphase-to-anaphase transition, is inhibited in the male pronucleus (Tram, 2002). Condensation of the paternal chromosomes is delayed or impaired, often leading to chromosome shearing and bridging during anaphase (Callaini et al., 1997; Reed & Werren, 1995; Ryan & Saul, 1968). This is fatal in diploid insects. Similar CI cytology has been documented in diverse insects (Tram et al., 2003). The phenotypic consistency across species suggests that *Wolbachia*-induced CI targets conserved cellular machinery required for cell and nuclear division (Callaini et al., 1997; Landmann et al., 2009; Reed & Werren, 1995). CI might directly disrupt the protamine-histone exchange (Landmann et al., 2009); other extra-nuclear sperm factors have been ruled out as targets (Presgraves, 2000). From the results in the current study, we propose that key CI targets include nuclear transport factors (karyopherins) and protamine-histone exchange factors.

In transgenic Cid models, the phenotypes described above and the downstream incompatibility of CI depends on the *CidB* deubiquitylase (DUB) activity (Beckmann et al., 2017). Therefore, here we focus on identification of *CidB* targets after identifying genes using both physical and genetic interaction screens (Beckmann et al., 2019). We use transgenic *Drosophila* to identify dosage suppressors of *CidB*-derived toxicity. We identified karyopherin-α (Kap-α/importin-α), as one of the the suppressors of the CidB toxicity. Kap-α is a conserved nuclear-import receptor for proteins with classical nuclear localization signals (NLSs) (Chen & Madura, 2014). After substrate recruitment, Kap-α associates with karyopherin-β and escorts cargo through nuclear pores (Chook, 2001). Nuclear Ran-GTP binding releases the cargo, and the karyopherins recycle to the cytoplasm (Goldfarb et al., 2004). CidB-Kap-α interaction connects CI induction and nuclear transport. Our study also highlights *CidB* association with protamine-histone exchange chaperones P32 and Nap1. Importantly, cognate *CidA* antidote binding to the *CidB* toxin eliminates these interactions. These discoveries identify the first potential CI molecular targets that comport with prior cytological observations (Ferree & Sullivan, 2006; Landmann et al., 2009) .

2.3 Material and methods

2.3.1 Fly rearing

Flies were reared on 4.6% cornmeal (w/v), 7.8% molasses (w/v) media, 2.3% yeast (w/v) (CMY) media. Firstly, experiments were performed at temperatures ranging from 21^0C to 25^0C (Figure 2.2) for temperature optimization. For the analysis of CI, F_0 crosses were initiated by crossing homozygous Gal4 driver females (NGT- Nanos-Gal4-Tubulin) to UAS-transgene males

(CidA-T2A-CidB$_{wMel}$). All the F_0 crosses were kept at 22^0C to control for any temperature-dependent maternal effects. Temperature was only temporarily lowered to 18^0C for overnight virgin collection and were stored at room temperature after collections.

2.3.2 Transgenic *Drosophila* and transgenic CI crosses

DNA for the cidA-T2A-cidB$_{wMel}$ operon (Beckmann et al., 2017) in addition to D.m.Kap-α1 were codon optimized for Drosophila and ordered from Genscript (Piscataway, NJ). Some constructs were purchased from Genscript Drosophila cDNA libraries. To begin CI suppression analysis we cloned or obtained clones for seven transgenes (Srp1, Hrp1, RTT103, Kap-α1, Kap-α2, P32 and Nap1) in pUC57 vector and sub-cloned into pUASp-attB (Rørth, 1998; Takeo et al., 2012). This vector appends the K10 3' UTR, which is known to localize transcripts to the *Drosophila* oocyte (Serano and Cohen, 1995). Final constructs were either fully sequenced or sequenced on ends by sanger sequencing and verified by restriction enzyme digests. BestGene Inc (Chino Hills, CA) was contracted for embryo microinjection of *D. melanogaster* #9744 (attP site on chromosome three) and ΦC31 integrase-mediated transgene insertion. At least 200 *D. melanogaster* embryos per gene were injected by BestGene (Chino Hills, CA), and on the basis of *w+* eye color transformants were selected. Crossing of cidA-T2A-cidB$_{wMel}$ operon-transformed male flies with females from strain #4442 carrying the nanos-Gal4-tubulin 3' untranslated region (NGT) driver induced CI (Figure 2.2). This served as a phenotypic confirmation of transgene expression and accords with previous results (Beckmann et al., 2017; LePage et al., 2017).

2.3.3 *Drosophila* hatch rate assays

For the temperature optimization, firstly, f_0 crosses were crossed with homozygous Gal4 driver females (NGT- Nanos-Gal4-Tubulin) to UAS-transgene males (CidA-T2A-CidB$_{wMel}$) because the Gal4 gene and UAS-target (gene X) are separated into two distinct transgenic lines. Hence, the target gene X is turned on only in the progeny of the f_0 cross. Then heterozygous f_1 males i.e., $_{NGT}$CidA-B$_{wMel}$ were crossed to the f_1 WT virgin female flies at temperatures shown in fig. 2.1 F_1 flies, which were heterozygous for both the NGT driver and the Gal4-UAS-transgene, were aged 3–4 days at restrictive temperatures (Figure 2.2) and crossed one to one, male and female, in a 3.5-ounce, round bottom, polypropylene *Drosophila* stock bottle. An apple juice-agar plate (made by 104.4g of Dextrose, 52.1g of Sucrose, 39.6g of Agar, 48 mL of 1.25N NaOH, 810 mL of apple juice to 968 mL of de-ionized water followed by autoclaving) with a small amount of yeast paste smeared in the center of plate; was placed in the bottle opening and affixed with tape. Bottles were placed in incubator at respective temperatures. After 36 hr., we discarded the original apple juice plates and replaced with freshly yeasted apple juice plates. Flies were allowed to oviposit for 24 hr. before removing the plate. Eggs were given 36 hr. to hatch while being incubated at the respective temperatures. Hatch rates were evaluated by microscopy and by counting hatched and unhatched egg totals. Any crosses with less than ten total embryos were excluded out from the hatch rate assay. Flies used in this study were white Canton-S (wCS; WT); nanos-Gal4-tubulin, #4442; MTD-Gal4, #31777, which has multiple GAL4 inserts on all three large chromosomes, including nanos-Gal4, nanos-Gal4:VP16, and otu-Gal4 and is infected with *Wolbachia*; and UASp-Kap-α2, #25400 (Mason et al., 2002). Fly lines were created by us or obtained from the Bloomington Stock Center (Bloomington, IN).

2.3.4 DNA extraction

DNA was extracted by a Bead Ruptor Method {modified from the original "Yeast Genomic DNA Prep" protocol (Robert J. Tomko Jr., 2009)} from all the transgenic flies used in the experiments. To describe: Ten male *Drosophila melanogaster* flies (stored at -80^{0}C) were taken in a 200 mL screw-cap tubes. 200 µL of Buffer A (2mL of 2% Triton X-100, 5mL of 20% (w/v) stock in dH_2O of 1% SDS, 2mL of 5M stock in dH_2O of 100mM NaCl, 1mL of 1M stock in dH_2O of 10mM Tris pH 8.0 and 200µL of 500mM stock in dH_2O of 1mM EDTA) was added into it. Then 200µL Phenol: Chloroform: Isoamyl alcohol (25:24:1) and ~200µL of 425-600µm acid-washed glass beads were added (Phenol solubilized the proteins whereas chloroform solubilized the lipids, and isoamyl alcohol acted as an anti-foaming agent. These three made up the organic phase, and DNA was in the aqueous phase). Screw caps were tightened, and tubes were placed in the Bead Ruptor Elite machine, and the cycle was run (set at 20sec on, 20sec off x 4). Samples were placed on ice. 200µL of dH_2O was added and samples were centrifuged at 13,000 rpm for 10 minutes to separate phases (organic/aqueous). The top 400µL of the aqueous phase was transferred to a new tube. Phenol: Chloroform: Isoamyl alcohol phase was disposed of according to chemical safety guidelines. 800 µL (2 volumes) of 100 % ethanol and 120 µL (=10% total volume) Sodium Acetate pH 5.4 were added. This is standard ethanol precipitation. Samples were held overnight at -20ºC for the DNA precipitation. Nucleic acids were pelleted by centrifugation at 13,000 rpm for 10 minutes next day. The supernatant was discarded, and the pellet was washed in 1 ml of 70% ethanol and centrifuged at full speed for 10 minutes. The supernatant was discarded again (carefully, to avoid dislodging the pellet of DNA). Tubes were allowed to sit on the benchtop for

a minute to allow any residual ethanol to collect at the bottom of the tubes and aspirated. Dried the pellet at 42ºC heat block for 15 min (checked for dry pellet; it turned clear), then resuspended the pellet in 100 µL TE (Tris-EDTA) or water. For the sonication, 100 µL (suspend) + 900 ul of nuclease-free water (NFW) was added to make the final volume 1000 µL. Finally, samples were divided as 200 µL (working stock stored at -20ºC) + 800 µL (sample stock stored at -80ºC).

2.3.5 Polymerase Chain Reaction (PCR)

All the fly lines used in the experiment were free of *Wolbachia*, were verified using PCR and primers, recognizing a conserved region in the *Wolbachia* VirD4 gene (Figure 2.8). As a positive control, we amplified the D. *melanogaster* histone H3 gene. The PCR reaction (50 µl) contained 31.5 µl of Nuclease-Free Water (NFW); 1 X of 5x Phusion HF Buffer; 200 µM of 10 mM of DNTps; 0.5 µM each of the forward and the reverse primers; 2 µL (<250 ng) of template DNA and 1.0 units/50 µl reaction of Phusion DNA polymerase. The forward primer and reverse primers for the VirD4 from the Type IV SS Conserved region from wPpe with wRi were AC009 and AC010 respectively (Table 1.1). We followed manufacturer's protocol (New England BioLabs Inc., MA) for the PCR reaction. The thermocycling protocol for reaction was an initial denaturing step at 98°C for 5 min (hot start), 85°C for 30 seconds (paused and Phusion DNA polymerase added at this step), 35 cycles of final denaturing step 95°C for 10 seconds, annealing at 64.3°C for 30 seconds, followed by initial extension at 72°C for 15 seconds with a final extension at 72°C for 5 minutes. For the positive control i.e., histone H3 gene forward and reverse primers were JFB 646 and JFB 647 respectively (Table 1.1). The thermocycling protocol for reaction remains the same as described above for the VirD4 gene except for the annealing temperature, which is 59°C

and the initial extension time, which is 10 seconds. PCR products were run on 2% agarose gels and photographed with UV light illumination.

2.3.6 Statistical analysis

All statistical analyses were conducted in GraphPad Prism 8.4.2. One-way ANOVA with multiple comparison followed by Dunn's test of multiple corrections was performed. Outliers were removed by the ROUT method. All *P* values are reported.

2.4 Results

2.4.1 Transgenic CI Induction strongly depends on temperature

To induce CI, two methods were utilized. The first is by transgenic expression of the CI factors *cidA* and *cidB* in males crossed to wild-type females. The second is by a wild-type *w*Mel infection (in males) crossed to uninfected wild-type females. A precondition for studying suppression of CI was to optimize CI induction conditions for maximum reproducibility and data precision, thereby lowering variance in the data. To study the gene expression and drive tissue-specific conditional expression of the gene of interest Gal4/UAS system is widely used in *Drosophila* (Brand & Perrimon, 1993). This system has two parts. These two components i.e., Gal4 and UAS, are held in separate parental fruit fly lines as chromosomal insertions. The first one is Gal4, a transcription factor gene from yeast (*Saccharomyces cerevisiae)*, which is part of a larger Galactose metabolism genetic unit, Gal4 up-regulates transcription of any genes downstream of an Upstream Activating Sequence (UAS). Therefore, the second part of the

bimodal system is the UAS which can be inserted 5' (upstream) of any transgene of interest. In fly genotypes that have both components, Gal4 binds to the UAS and turns on transcription of the gene of interest (or the transgene) in *Drosophila*. Functionally, to activate a transgene requires crossing a homozygous Gal4 line with a homozygous UAS-transgene line. All resulting F1 progeny will then bear the heterozygous genotype of Gal4/UAS-transgene; effectively turning it on. In genotypes where a UAS-transgene is present, but also lacking Gal4, the transgene remains in a relatively silent state {though this is not complete silence and leaky transcription does occur; (Beckmann et al., 2017)}. Schematic representation of this system is shown in figure 2.1.

One hypothesis is that temperature is a factor that influences expression of Gal4/UAS systems (which we used to drive transgene expression). Gal4 has temperature dependency showing maximum and minimum activity at 29^0C and 16^0C, respectively. So by varying the temperature, a wide range of expression levels of the responder genes can be achieved in *Drosophila* (Duffy, 2002). We envisioned that suppression of CI might be strong and also it could be weak. A weak suppressor isn't necessarily less relevant or less informative. As such, we hoped to optimize our system such that we could detect both weak and strong CI suppression. To optimize expression temperatures the following criteria was used: strong CI will produce hatch rates of 0% whereas weak CI will produce hatch rates around 83%. Hatch rate (HR) of 83% was observed at 21^0C suggesting that the CI transgenes were suppressed at colder temperatures. Thereafter, increasing temperatures reduced hatchrates. We observed 35% at 22^0C, 21% at 23^0C (Figure 2.2), and around zero percent at 25^0C (Figure 2.2).

CI induction is strongly dependent on temperature Although higher temperatures caused larger reductions in the hatch rates, it was determined that 22_0C was the optimal temperature for observing partial suppressive effects (and possibly enhancement effects) on CI.

2.4.2 Overexpressed *Drosophila* karyopherin's and protamine-histone chaperones suppress CI

From the yeast *CidB* suppressor screen experiments, there were three strong suppressors that suppressed the $cidB_{wPip}$ toxicity in *Saccharomyces cerevisiae* (Beckmann et al., 2019). These were Srp1, Hrp1, and Rtt103. Srp1 encodes yeast karyopherin-α or importin-α a protein that is linked to nuclear import (Chen & Madura, 2014). Hrp1 encodes an RNA-binding protein and is involved in the 3'-end processing of mRNA precursors and mRNA export from the nucleus to the cytoplasm (Kessler et al., 1997). Rtt103 is a transcriptional regulator and acts as a termination factor for RNA polymerase II (Nemec et al., 2017; Srividya et al., 2012). Srp1 was the most robust suppressor. An independent proteomic pull-down screen identified the *Drosophila melanogaster* ortholog of Srp1, Kap-α, as a physical binder of *CidB*. *D. melanogaster* has four paralogous of karyopherin-α gene which are α1, α2, α3, and α4 (Hler et al., 1999; Phadnis et al., 2012; Pieper et al., 2018). Kap-α1 was chosen for analysis because it is closest in sequence to yeast Srp1, and Kap-α2 was the top *CidB** (*CidB's* DUB domain is inactivated) interactome hit (Beckmann et al., 2019). Additionally, P-32, and Nap1, were identified from the *Drosophila* interactome screen analysis as potential *CidB* substrates or cofactors (Beckmann et al., 2019). Those two proteins interact within a complex that remodels sperm chromatin (Emelyanov et al., 2014). Therefore,

these two hits were also of interest to me because of a theoretical link to CI phenotypes occurring on sperm chromatin; paternal chromosomes fail to condense properly for the first cell cycle (Landmann et al., 2009).

As described above transgenic cidA-B_{wMel}-induced CI was optimal at 22°C. Since both the *CidB* suppressor screen for yeast and the interactome screen for *Drosophila* identified karyopherin-α, we determined whether increased dosage of karyopherin-α genes in fruit fly mothers might suppress CI. Overexpression of either *Drosophila* karyopherin-α paralogs in the female germline partially suppressed CI caused by transgenic expression of CidA-B_{wMel} in males. *D.m* Kap-α2 was a stronger and more significant ($p<0.0001$) suppressor than *D.m.* Kap-α1 (Figure 2.4). We also tested yeast Srp1 and Hrp1 in a similar manner and found they were not able to suppress the transgenic CI. As a negative control, we also tested effects of transgenic GFP expression under similar conditions. GFP would not be expected to suppress CI. To our surprise, GFP also caused a partial suppression that was not statistically distinguishable from the weak karyopherin-α1 suppression, but it was distinguishable from karyopherin-α2's suppression. To summarize, weak suppression induced by a negative control caused us to rule out weak transgenic CI suppression of karyopherins as valid. We next tested whether maternal overproduction of P32 or Nap1 could suppress transgenic CI and found that overexpression of P32 showed highly significant suppression even relative to the GFP control, increasing egg hatch rates by ~30%. Suppression was equivalent to the rescue observed with transgenic expression of the actual *CidA* antidote. (Figure 2.4).

Importantly, when we measured the suppressive effects of karyopherin-α and P32 overexpression in the female germline in mating's with male flies carrying *wMel* bacterial infections, partial but highly significant suppression was observed for both P32 and karyopherin-α (Figure 2.4). Importantly, under this context, we did not observe false negative suppression by GFP. We conclude that the suppression by karyopherin-α and P32 was relevant to natural CI.

2.4.3 Non-specific suppression of synthetic CI by GFP

When we used transgenic GFP as a negative control, it also caused a weak suppression in synthetic CI (~15%). Moreover, this suppression was not statistically distinguishable from the karyopherin-α2 suppression (Figure 2.4). But when we used transgenic GFP as a negative control and crossed with males infected with *wMel*, it did not suppress the wild CI (Figure 2.5) and the hatch rate difference was not statistically significant from the WT cross. So, in that case non-specific suppression did not result when crossed to females expressing GFP.

2.4.4 *CidA* is an antidote and *rescues* embryonic viability

Females expressing $CidA_{wMel}$ by themselves rescue CI from males under both transgenic $cidAB_{wMel}$ operons or *wMel* infections contexts (Figure 2.4 and 2.5). These supports and extends previous studies which showed similar rescue effects with $CidA_{wMel}$ or $CinA_{wPip}$ transgenic expression in female (Chen et al., 2019; Shropshire et al., 2018; Shropshire & Bordenstein, 2019). These results support the general toxin-antidote model which states that *CidA* is an antidote that can rescue the activity of the *CidB* toxin (Beckmann et al., 2019a).

2.4.5 CI suppression by karyopherin's is species specific

The yeast (*Saccharomyces cerevisiae*) karyopherin-α protein i.e., Srp1 is a most robust suppressor $CidB_{wPip}$ toxicity (Beckmann et al., 2019). We also tested whether this particular ortholog could suppress CI in fruit flies. Thus, we overexpressed *S.c.* Kap-α/Srp1 in addition to the other karyopherin's mentioned above in female insect germlines. Unlike Drosophila karyopherin-α2, yeast Srp1 failed to suppress the CI in the *Drosophila melanogaster* context. Hatch rates were not statistically different from the WT cross or the GFP negative control (Figure 2.6). In sum, we concluded that suppression by karyopherin's is species specific.

2.4.6 Karyopherin's have maternal toxicity

One hypothesis was that we might be able to boost suppression by increasing the maternal overexpression of our transgenes to extremely high levels. To test this, we turned to the stronger maternal triple driver (MTD) line which provides robust germline expression throughout oogenesis (Petrella et al., 2007) due to the insertion of three independent Gal4's. However, when karyopherin-α was overexpressed in females, it caused embryos to die independently of CI. We reasoned then, that this driver would confound our measurements by conflating death by CI, with death by maternal overexpression toxicity of karyopherin's. When we used a weaker driver (NGT; the Gal4 line used for the all the above work), the suppression effects were significant but relatively small (Figure 2.7). Thus, we concluded that our measurements had utilized the most optimal Gal4 driver system.

2.5 Discussion

The *Wolbachia CidA* and *CidB* proteins were recently found to be central to CI, but no *CidB* targets were known. Two orthogonal screens of *CidB* genetic and physical interactions in *S. cerevisiae* and *D. melanogaster*, respectively, identified the nuclear-import receptor karyopherin-α (Kap-α). Kap-α bound to *CidB* and genetically suppressed *CidB*-derived defects when overexpressed. The Kap-α NLS-binding site was required for suppression of *CidB* toxicity. *CidB* also binds Drosophila P32 and Nap1, which promote protamine-histone exchange. Overexpression of either Kap-α or P32 in female insect germlines suppressed natural as well as the transgenic CI. We also show that *CidA* in mother flies is sufficient to rescue both transgenic and wild CI.

2.5.1 Mechanistic models of CI induction and rescue

It is not yet clear if the Kap-α and P32, are deubiquitylated by the *CidB* enzyme or how this could help account for their functions in CI. Srp1/Kap-α (and Hrp1, another top hit) are known to be ubiquitylated in yeast based on proteomic surveys (Swaney et al., 2013). One highly speculative model invokes *CidB* cleavage of ubiquitin from both Kap-α and histone chaperones such as P32 (or the histones themselves), reducing their functionality. Histone H2A and H2B are well characterized as ubiquitylated proteins, and histone H2B was identified in the *CidB**-binding screen. Its ubiquitylation may promote histone H3.3 loading and nucleosome formation. There is evidence for ubiquitin-H2B and histone chaperones cooperating in replication-independent nucleosome assembly (Wu et al., 2017).

Ubiquitylation of Kap-α may also be important for its ability to promote nuclear import of a key maternal protein(s) involved in protamine-histone exchange (or for a nuclear non-transport function of Kap-α) (Oka & Yoneda, 2018). Our crosses suggest both Kap-α and P32 are limiting in CI embryos because transgenic expression of either suppresses *Wolbachia*-induced incompatibility. In regard to the above model, *CidB* deubiquitylation of ubiquitin-modified histones, histone chaperones and/or Kap-α would be envisioned to impair histone deposition {but not protamine removal (Landmann et al., 2009)}. Overexpressed Kap-α might enhance import of histone chaperones or ubiquitylation factors to overcome the activity of *CidB*. Similarly, overexpression of histone chaperones such as P32 could enhance nucleosome assembly. Determination of exactly how the proteins we have identified contribute mechanistically to CI is an important goal for future studies.

The fact that the antidote, *CidA*, contributes to both CI induction and rescue is seemingly at odds with its designation as an antidote. However, this dual functionality is characteristic of toxin-antidote (TA) operons (Yamaguchi et al., 2011). Previously described model envisioned co-translation of *CidA* and *CidB* followed by *CidA-B* protein complex formation, possibly after passage through a type IV secretion system into the host cytoplasm (Beckmann et al., 2017; Beckmann & Fallon, 2013). We postulated that *CidA* antidote functionality has a dual purpose. One function is to prevent premature toxicity of *CidB* during spermiogenesis. *CidA* may even promote localization of the toxin into sperm. Rapid degradation of antidote, also characteristic of TA operons, in the egg would activate the relatively stable *CidB* toxin if no fresh *CidA* is provided by egg resident *Wolbachia*.

To reiterate, induction of CI could proceed by multiple mechanisms based on the data in hand. The simplest model is that *CidB* directly deubiquitylates a single key target, possibly Kap-α2. In this model ubiquitylated Kap-α2 is crucial for delivery of some key factor, perhaps P32, Nap1, or histones to the male pronucleus. Alternatively, *CidB* may have multiple direct targets and CI results from the accumulated defects caused by these changes. A more indirect model would posit that *CidB* binds Kap-α2 as a way into the nucleus where its relevant substrates localize. Localization studies will be crucial for determining the precise mechanisms.

In general, Nuclear transport as a target of CI is tantalizing because it suggests divergent selfish reproductive manipulators converge on related embryonic processes. Segregation Distorter (SD) was also linked to nuclear import disruption (Larracuente & Presgraves, 2012; Merrill, 1999). SD is a meiotic driver in natural *D. melanogaster* populations involving two autosomal loci. The Sd driver locus encodes a truncated but catalytically active RanGAP (nuclear transport regulator) that mislocalizes to the nucleus (Kusano et al., 2002) and the responder (Rsp) locus is a large block of satellite DNA. During spermiogenesis, Sd-RanGAP alters the histone-to-protamine transition, culling drive-sensitive spermatids. Phylogenomic analysis of karyopherins in *Drosophila* also suggested frequent gain and loss of Kap-α genes, consistent with selection targeting nuclear transport for host protection against genetic conflicts (Phadnis et al., 2012). Independently, a *Drosophila* testes-specific X-linked Kap-α gene was found to be duplicated and overexpressed in response to a sex-ratio driver (SR) that selectively blocks maturation of Y chromosome-bearing sperm (Pieper et al., 2018). Hence, the molecular features of SD and SR show remarkable parallels

with the processes we have linked to CI, particularly nuclear transport (Kap-α, Moleskin) and the protamine-to-histone transition (P32 and Nap1).

2.5.2 A Further Discussion on Non-specific suppression of synthetic CI by GFP

Cytological studies indicate that CI toxicity may occur at multiple levels and embryos develop various developmental abnormalities. Some embryos die in the first round of the zygotic nuclear division, and remaining show arrested development at advanced developmental stages and die just before hatching (Beckmann et al., 2017; Callaini et al., 1996). One possible reason for this weak non-specific suppression may be that crosses to mothers with a transgene (GFP) driven by the UAS mitigate a later, secondary stage of CI killing by reducing embryonic expression of the transgenic *CidB* toxin in older embryos. It may result from the competitive binding of the Gal4 transcription factor to the insertions of the mother's UAS, which restricts binding to the transgenic UAS-cidB gene. Owing to non-specific suppression, we advise caution about conclusions with suppression of transgenic CI. Suppression results should be backed up by a study of suppression against natural bacteria induced CI, as we did for P32 and Kap-α. We finally concluded that weak suppression by GFP was an artifact of the transgenic CI model.

2.5.3 Host suppression of CI

Multiple *Wolbachia* systems have reported the host suppression of reproductive parasitism involving CI (Cooper et al., 2017) and male killing (Hornett et al., 2006). Theory expects a gradual progression of CI to weaker incompatibilities (Turelli, 1994). However different loci of the suppressor gene were never identified. There are two reasons why genetic CI suppressors are

significant. First, they provide hints toward pathways targeted by CI. Secondly, they might co-evolve as resistance factors to CI. Importantly, suppression of CI in vectors will reduce the effectiveness of global mosquito control efforts harnessing *Wolbachia* and CI. We note that Kap-α and P32 were both robust dosage suppressors of transgenic and natural CI (Figure 2.4 and 2.5) and both are maternally deposited (Emelyanov et al., 2014; Emelyanov & Fyodorov, 2016; Phadnis et al., 2012). Therefore, these proteins could well be important factors in the evolution of host resistance to *Wolbachia*-induced CI."

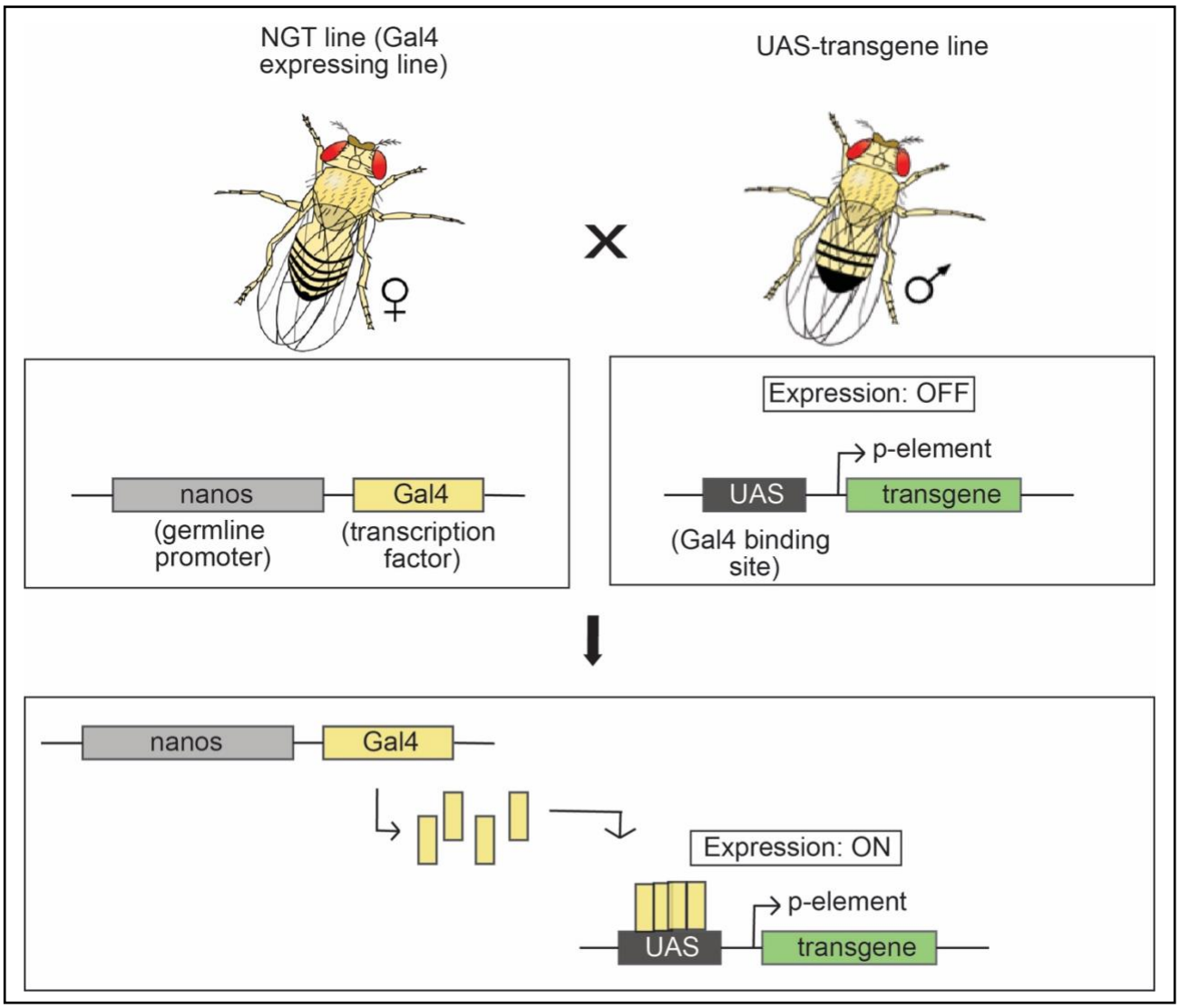

Figure 2.1. Schematic structure of the Gal4/UAS system. Gal4 and UAS are held in separate parental fruit fly lines. Gal4 is a transcription factor from yeast. Gal4 up-regulates transcription of any gene downstream of an Upstream Activating Sequence (UAS). UAS can be inserted 5' (upstream) of any transgene of interest. In fly genotypes that have both components, Gal4 binds to the UAS and turns on transcription of the gene of interest. Resulting F1 progeny will bear the heterozygous genotype of Gal4/UAS-transgene; effectively turning the gene on.

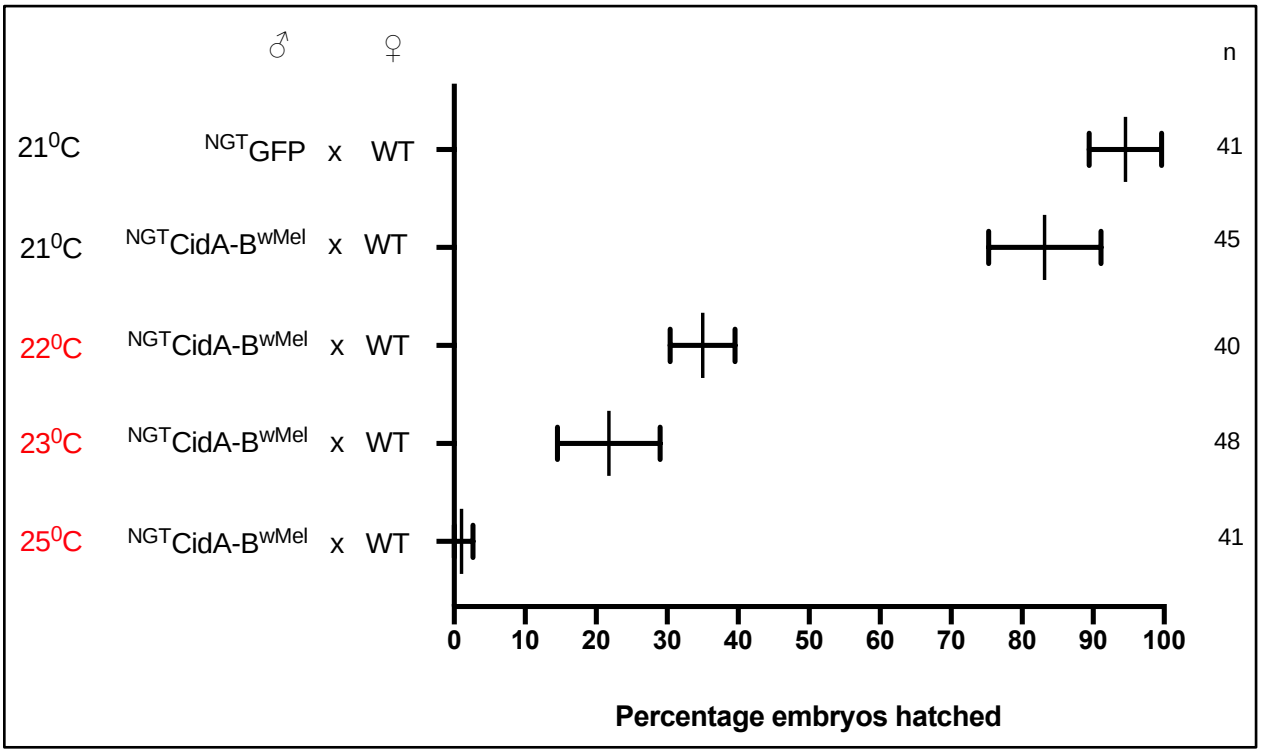

Figure 2.2. Transgenic CI was temperature-sensitive. Maximum hatch rate observed at 21$_0$C and minimum hatch rate observed at 25$_0$C. GFP used as a control (no transgene). CidA-B$_{wmel}$ is the fly with synthetic CI. WT is the Wild Type fly. Error bars represent means ± s.d. 'n' represents the number of crosses done at respective temperatures.

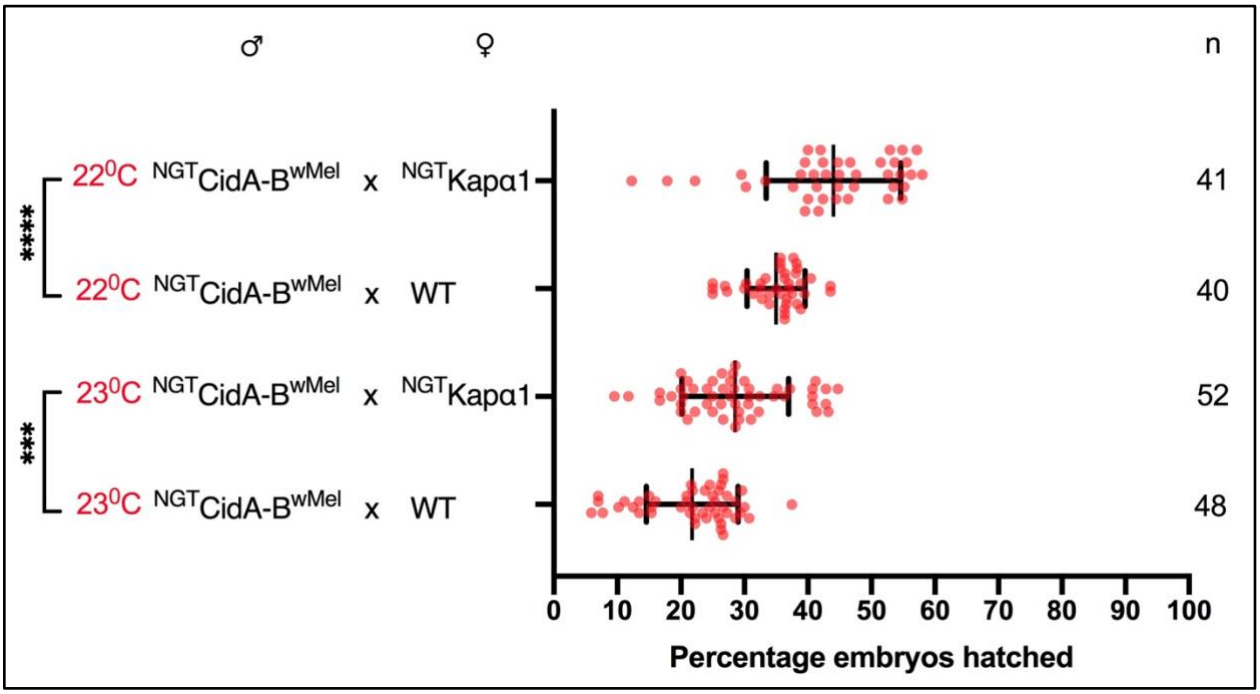

Figure 2.3. Rationale for observing partial suppressive effects on CI at 22₀C. Mean difference in hatch rate at 23₀C from the WT (control) cross was around 6% and at 22₀C was around 9%. CidA-B_{wmel} is the fly with synthetic CI. Kap-α1 is *Drosophila melaongaster*'s karyopherin-α1 WT is the Wild Type fly. Error bars represent means ± s.d. 'n' represents the number of crosses done at respective temperatures. ***p<0.001,****p<0.0001 by by ANOVA with multiple comparison between all groups and Tukey's post-hoc analysis.

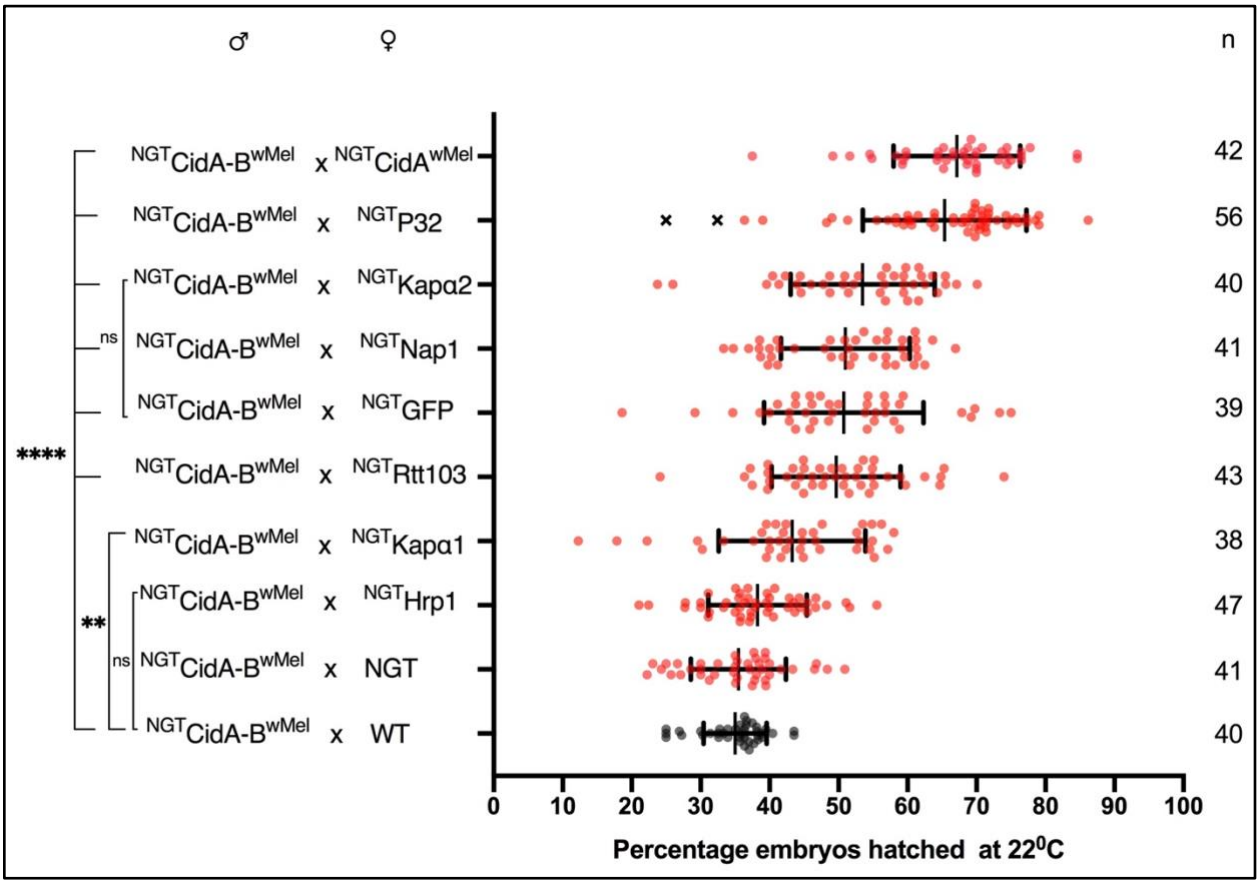

Figure 2.4. At 22₀C overexpression of *D.m*.Kap-α1, S.c.Rtt103, GFP, D.m.Nap1, *D.m*.Kap-α2, *D.m*.P32 and *CidA*$_{wMel}$ suppressed transgenic CI relative to the control. Both D.m.P32 and CidA$_{wMel}$ suppression were still highly significant when compared to the GFP control. Error bars represent means ± s.d. 'n' represents the number of crosses done at 22₀C. ns=p>0.05*p<0.05, **p<0.01, ****p<0.0001 by ANOVA with multiple comparison between all groups and Tukey's post-hoc analysis. Two outliers (x) removed by ROUT analysis.

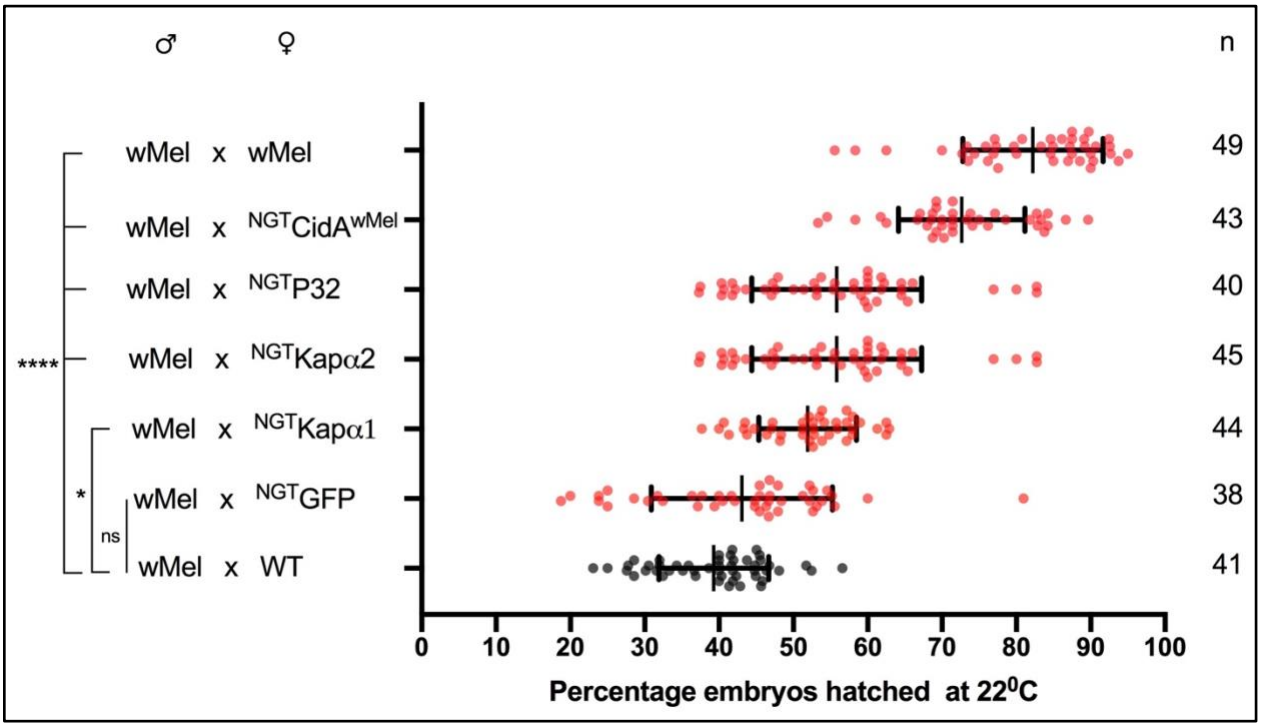

Figure 2.5. Overexpressed *D.m.*Kap-α1, *D.m.*Kap-α2, *D.m.*P32 and CidA$_{wMel}$ suppressed wMel CI relative to the control as well as GFP. Error bars represent means ± s.d. 'n' represents the number of crosses done at 22$_0$C. ns=p>0.05*p<0.05, ****p<0.0001 by ANOVA with multiple comparison between all groups and Tukey's post-hoc analysis.

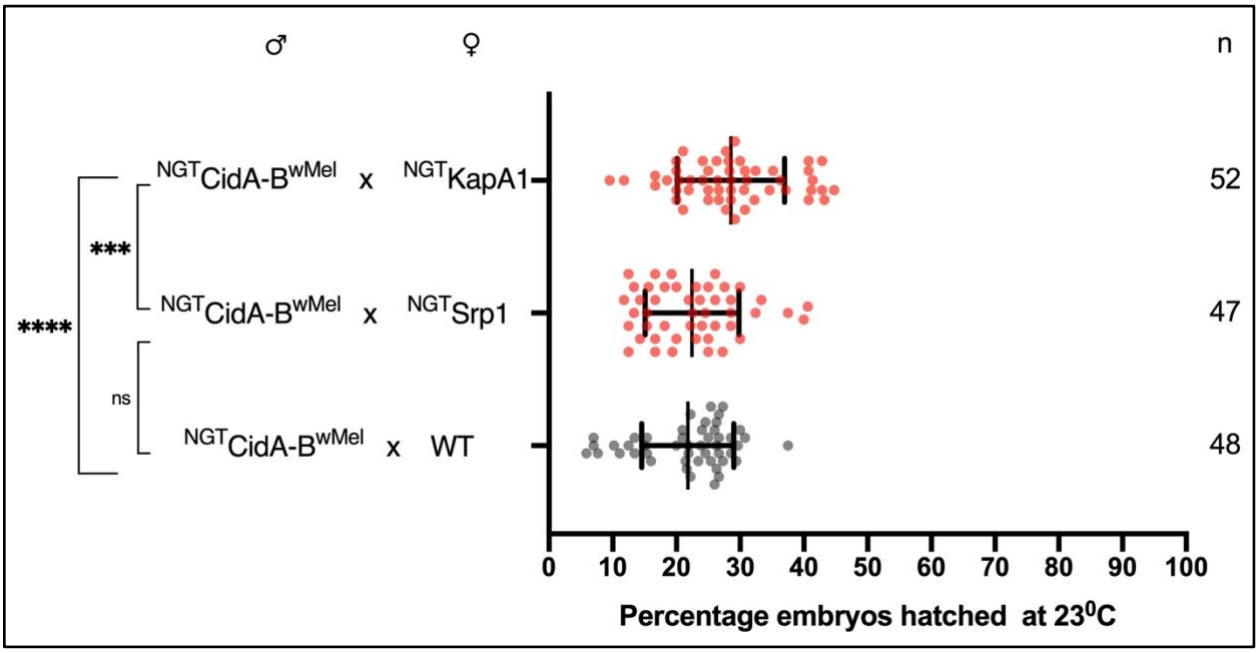

Figure 2.6. CI suppression by Karyopherin's is species specific. *D.m.* Kap-α1 suppressed the synthetic CI whereas yeast Kap-α not able to suppress the CI in *Drosophila melanogaster*. Srp1 is *Saccharomyces cerevisiae* karyopherin and Kap-α is *Drosophila melanogaster* karyopherin. Error bars represent means ± s.d. 'n' represents the number of crosses done at 23$_0$C. ns=p>0.05,***p<0.001, ****p<0.0001 by ANOVA with multiple comparison between all groups and Tukey's post-hoc analysis.

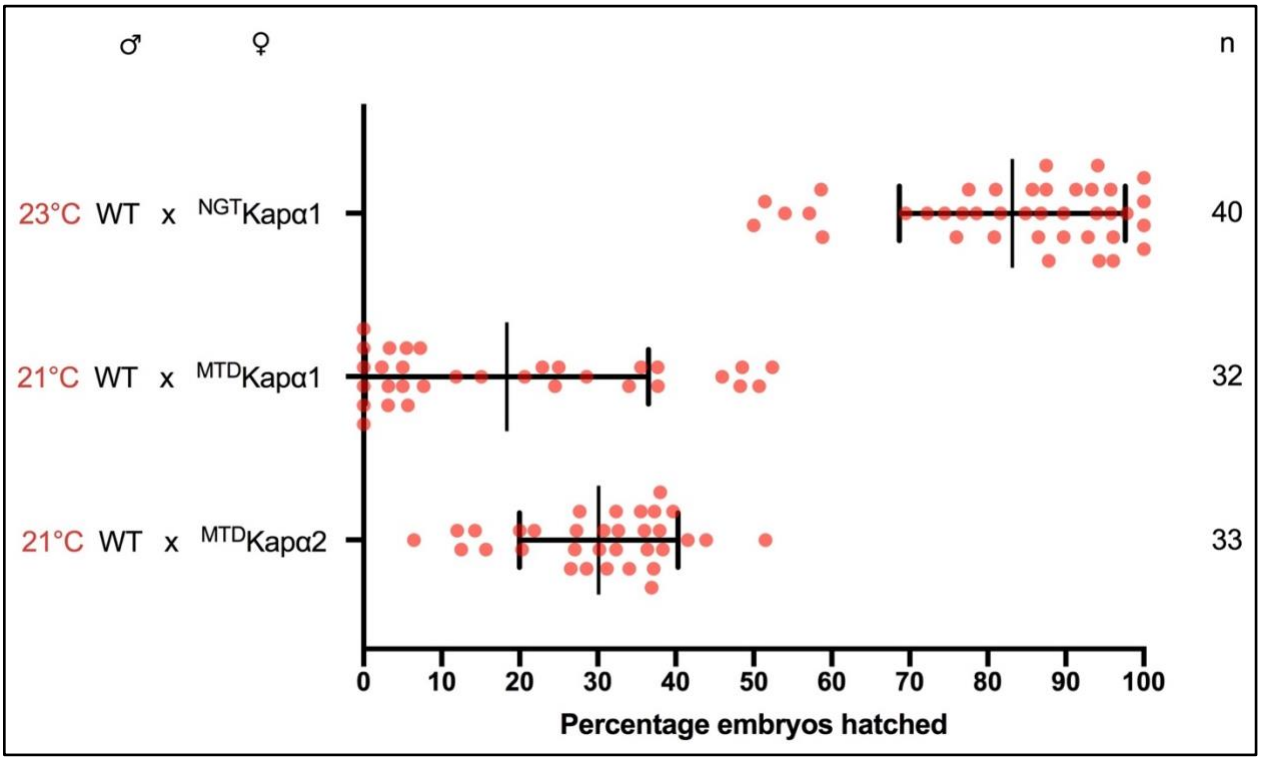

Figure 2.7. Karyopherin's have maternal toxicity. MTD is maternal triple driver and NGT is Nanos-Gal4-Tubulin driver. Kap-α1 and Kap-α2 are the *Drosophila melanogaster* karyopherin genes. WT is wild type. Error bars represent means ± s.d. 'n' represents the number of crosses done at respective temperatures.

Primer	Sequence	Product length, bp
AC009_F	CTGGTAAAGGTGTTGGTTTTG	202
AC010_R	GCAGTTGCAGTATCAATCAATG	
JFB 646_F	GGCCGCTCGCAAGAGTG	540
JFB 647_R	CATAACCGCCGAGCTCTG	

F- forward primer, R- reverse primer

Table 1.1. Primers used in this study for PCR or for *Wolbachia* infection checks in transgenic flies used in the experiment (Figure 2.8)

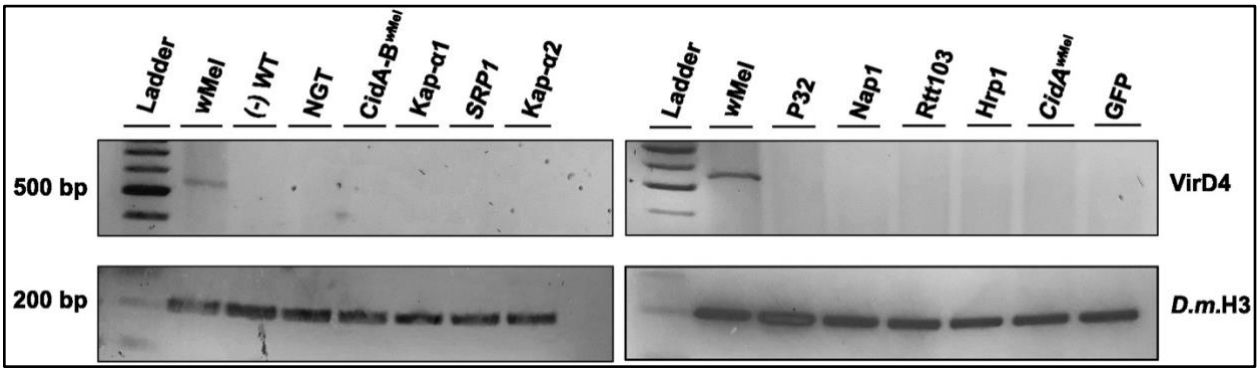

Figure 2.8. PCR analysis demonstrates that transgenic flies used in this study are not infected with *Wolbachia*. VirD4 is a conserved *Wolbachia* gene. Amplification of the *Drosophila melanogaster* Histone three gene served as a positive control. NGT is the Nanos-Gal4-Tubulin driver (three replicates).

CHAPTER 3

FUNCTIONAL GENETIC ANALYSIS OF KAPA2 AND P32 GENES IN *DROSOPHILA* AND THEIR ROLE IN SUPPRESSION OF *WOLBACHIA*-MEDIATED CYTOPLASMIC INCOMPATIBILITY

3.1 Abstract

Wolbachia is an endosymbiotic bacterium inherited maternally that infects nearly half of all arthropod species. A *Wolbachia* deubiquitylating enzyme *CidB* is central to CI induction. Our hypothesis is that CI is induced *via* the interactions of *CidB* with insect karyopherins and protamine-histone exchange factors, like P32. Overexpression of Kap-α or P32 in female *Drosophila* suppressed CI induced by wild bacteria as well as an artificial transgenic CI system. Our next question was focused on determining whether *CidB* cleaves ubiquitin directly off Kap-α or P32 to induce CI. Here, we explore functional mutations and genetic modifications in these two genes to test and fully evaluate the role of these two genes in the suppression and induction of CI in *Drosophila*. One mutation that eliminates Kap-α2's ability to bind NLS sequences eliminated its ability to suppress CI in flies. The results show that suppression of CI by Kap-α2 depends on its nuclear importing ability. We also evaluate an uncleavable Ubiquitin-fusion to Kap-α2 as well as to P32. The Ub-Kap-α2 and Ub-P32 constructs suppressed CI even better than wild Kap-α2 and P32 respectively. These data suggest that *CidB* cleaves Ubiquitin from Kap-α2 and P32 to induce CI. In addition, we build and evaluate fusions of 3XFLAG-P32 and 3XFLAG-Kap-α2 as tools for future immunoprecipitation studies.

3.2 Introduction

Maternally inherited intracellular bacteria *Wolbachia* infect arthropods and filarial nematodes (Ferri et al., 2011; Zug & Hammerstein, 2012). The bacterium alters the host reproduction in several ways, which favors their spread (Werren et al., 2008) by hijacking the host cellular machinery; of which the most common reproduction alteration is Cytoplasmic Incompatibility (CI) (Serbus et al., 2008). CI is defined as the embryonic lethality that occurs when the sperm modified by *Wolbachia* fail to fertilize eggs that are not infected with *Wolbachia* (Larracuente & Presgraves, 2012). Contrary to this, the cross between an infected female with *Wolbachia* infected or uninfected male will produce viable offspring (Poinsot et al., 2003b). Recently, *Wolbachia*-induced CI has been widely exploited as biocontrol tool for the control of vector-borne diseases (especially those spread by mosquitoes) such as Dengue, Zika, West Nile and Yellow fever (Dorigatti et al., 2018; Dutra et al., 2016b; Slatko et al., 2014). However, the mechanism behind this *Wolbachia*-induced CI is still not fully known yet. *Wolbachia CidA* and *CidB* proteins were recently found to be central to CI (Beckmann et al., 2017, 2019a; Beckmann et al., 2019). *CidB* is a deubiquitylating enzyme (DUB) and its cognate partner is an antidote, *CidA* (Beckmann et al., 2017). Our best hypothesis is that *CidB* induces CI by cleaving ubiquitin from an unknown substrate/s (Beckmann et al., 2017; Beckmann et al., 2019). Ubiquitin is a 76 amino acid eukaryotic protein and is transcribed as a fusion protein N-terminally attached to other proteins of high expression, such as ribosomal subunits (Welchman et al., 2005). From there, ubiquitin is cleaved by cellular DUBs from the polyprotein and becomes available as a cellular tag that can be attached to substrates. Ubiquitin post-translationally modifies many proteins and

commonly regulates substrate stability and localization (Ronau et al., 2016). Isopeptide bond is formed between the C-terminal glycine carboxyl group and the lysine of the substrate (Pickart, 2001; Weissman, 2001). *CidB* is most efficient at cleaving ubiquitin chains linked at Lys63 (Beckmann et al., 2017). In our last study, we proposed that *CidB* targets karyopherin-α, a nuclear-import receptor and P32, protamine-histone exchange factor (Beckmann et al., 2019). Evidence in support of this hypothesis is listed below.

Overexpression of Kap-α suppressed the *CidB*-derived toxicity in yeast as well as wild CI in flies and artificial CI induced by transgenic CI operons in *Drosophila* (Beckmann et al., 2019). Insect karyopherins were also found to co-precipitate in *CidB* purifications. Kap-α2 (karyopherin-α/importin-α) is a highly conserved protein among eukaryotes (Goldfarb et al., 2004). Small proteins can enter the nucleus on their own, whereas most of the proteins need transport factors (carriers), which can escort them into the nucleus (Bayliss et al., 2000). The Nucleus is guarded by nuclear pore complexes (NPC's), to enter the nucleus, proteins require a component that can enable to cross the barriers (Beck & Hurt, 2017). Kap-α binds to only those proteins which bear Nuclear Localization Signals (NLS's) (Kalderon et al., 1984; Leung et al., 2003). Kap-α binds Kap-β (karyopherin-β /importin-β) after recruiting a substrate/s and then the complex imports the cargo into the nucleus through NPC (Chook, 2001). This nucleo-cytoplasmic traffic is driven by a nuclear Ran-GTP gradient (Ryan et al., 2003). Ran-GTP dissociates this complex in the nucleus, after this, Kap-α will again be transported back to the cytoplasm through a nuclear export signal (Goldfarb et al., 2004). Kap-α performs alternative functions other than nuclear import. It is

important for the execution of mitosis and inhibiting co-translational protein degradation, etc. (Chen & Madura, 2014; Loeb et al., 1995).

Another factor of interest is P32, the protamine-histone exchange factor. This protein was another the other suppressor identified, which suppressed wild-type and as well as transgenic CI when overexpressed in female transgenic *Drosophila* (Beckmann et al., 2019). P32 also binds *CidB* in pull-downs (Beckmann et al., 2019). One function of P32 relates to zygotic chromosome structures. Sperm chromatin inside the male gametes is compacted by protamines. Protamines are small, positively charged arginine, and cysteine-rich proteins (Balhorn, 2007). After fertilization, protamines are replaced with histones, and P32 is one core protein required for the removal of protamines (Emelyanov et al., 2014). It is suggested that CI impacts chromosome structure in the male pronucleus (Landmann et al., 2009)

In this study, we address one possible explanation for the physical interactions of *CidB* binding to Kap-α and P32. Different models can be proposed based on the ability of these overexpressed proteins (Kap-α and P32) to suppress *CidB* toxicity. Here in this study, we tested two hypotheses. One hypothesis was that *CidB* might cleave ubiquitin directly from Kap-α2 as well as P32 to cause CI. To test this hypothesis, we made uncleavable Ubiquitin fusion proteins together with P32 and Karyopherin-α2. Our rationale was that if *CidB* induces CI by cleaving ubiquitin from one of these substrates, we would be able to prevent CI by overexpression of an uncleavable ubiquitin fusion form. Cellular DUBs rely on a C-terminal glycine motif to cleave ubiquitin (Pickart & Cohen, 2004). To prevent cleavage, we mutated this motif from glycine to alanine, which would eliminate a DUBs ability to cleave it (Lacombe et al., 2009). We then

measure CI penetrance when incompatible males are crossed to female flies overexpressing the uncleavable ubiquitin fusion proteins. Our data suggest that Ub-Kap-α2 and Ub-P32 fusions suppress CI even more than wild Kap-α2 and P32 respectively. From these data we suggest that *CidB* might directly cleave Ubiquitin from Kap-α2 and P32 to induce CI. Another functional analysis we perform is a mutagenic analysis of Karyopherin-α2. A mutation (S98F) which inhibits nuclear localization signal binding also inhibits this proteins ability to suppress CI. These data clarify that the CI suppressing functions of Karyopherin-α2 in flies are linked to nuclear import.

3.3 Materials and methods

3.3.1 Fly strains and transgene constructs

For the *D.m*.P32 and *D.m*.Kap-α2 transgene constructs, P32 was cloned from cDNA clone purchased from GenScript (Piscataway, NJ), and Kap-α2 transgene was cloned from Kap-α2 *D.melanogaster* transgenic flies (Beckmann et al., 2019; Mason et al., 2002) respectively by designing specific oligonucleotides (primers used in this study are described in table 2.1) and using PCR. Both genes were firstly cloned into a smaller plasmid, i.e., pBsk+ to genetically manipulate the sequence before sub-cloning them into the destination vector. 5' *Not1* and 3' *BamH1* sites were added to facilitate cloning. Internal *BamH1* site was mutated by Quikchange mutagenesis because downstream, we need to use *BamHI* for cloning into the destination vector. Three transgenic constructs of P32 and four transgenic constructs of Kap-α2 (Figure 3.1) were prepared by us to investigate further whether they are the actual targets of *cidB* or not. Ubiquitin was cloned from the wild type *D. melanogaster*. We made a Ub^{G76A} mutation to make uncleavable Ub-P32 and Ub-

KapA2 fusion constructs in order to test if *CidB* cleaves ubiquitin off the substrates (Lacombe et al., 2009). We made another construct with Kap-α2^{S98F} point mutation (≈Srp1^{S116F} in yeast) to see if suppression is dependent on NLS binding in *Drosophila* (Beckmann et al., 2019; Chen & Madura, 2014). 3xFlag-transgene constructs were prepared by adding 3xflag sequence in forward primer. These constructs were designed to do the pull-down assays to test if they are ubiquitylated or not. As a negative control, we mutated the first methionine (start codon) to stop codon of P32 and Kap-α2 transgenes in another two constructs. Point mutations were introduced by Quikchange mutagenesis. All the constructs designed above were fully sequenced and verified by sanger sequencing to lack any spurious mutations.

DNA for the CidA-T2A-CidB$_{wMel}$ operon (Beckmann et al., 2017) was codon-optimized for *Drosophila* by Genscript. Transgenes were then sub-cloned from pbsk+ to destination vector, i.e., pUASp-attb (Rørth, 1998; Takeo et al., 2012) by PCR and restriction digest. Final constructs were again fully sequenced by sanger sequencing and also verified by restriction enzyme digests (Figure 3.2). Transgene constructs were sent to BestGene, Inc. (Chino Hills, CA), for embryo microinjection of *D. melanogaster*. Fly background #9744 (attP site on chromosome three) was chosen for ΦC31 integrase-mediated transgene insertion. At least 200 *D. melanogaster* embryos per gene were injected by BestGene and based on *w+* eye color; transformants were selected.

3.3.2 Fly maintenance

Flies were reared at room temperature on standard cornmeal, molasses, and yeast (CMY) media. For the analysis of CI, F_0 crosses were initiated by crossing homozygous Gal4 driver females (NGT- Nanos-Gal4-Tubulin) to UAS-transgene males (CidA-T2A-CidB$_{wMel}$). All the F_0 crosses were kept at 22_0C to control for any temperature-dependent maternal effects (Beckmann

et al., 2019). The temperature was only *temporarily* lowered to 18^0C for overnight virgin collection and was stored at room temperature after collections. All flies were kept under 12-h:12-h light: dark cycle.

3.3.3 Transgenic *Drosophila* CI Crosses and hatch rate assays

CidA-T2A-$CidB_{wMel}$ operon-transformed male flies were crossed with females from strain #4442 carrying the nanos-Gal4-tubulin 3' untranslated region (NGT) driver and wild type induced CI (Figure 3.4). This served as a phenotypic confirmation of transgene expression and agrees with prior results (Beckmann et al., 2017; Beckmann et al., 2019; LePage et al., 2017). F_1 flies, which were heterozygous for both the NGT driver and the Gal4-UAS-transgene, were aged 3–4 days at 22^0C and crossed one to one, male and female, in a 5-ounce, round bottom, Dixie cups. An apple juice-agar solution with a small amount of yeast paste smeared in the center of plate; was poured in the lids of the cups and were affixed. Cups were placed in an incubator at 22^0C. After 36 hr., original lids with apple juice were discarded and replaced with lid having freshly yeasted apple juice. Flies were allowed flies to oviposit for 24 hr. before removing the plate. Eggs were given 36 hr. to hatch while being incubated at the same temperature. Hatch rates were evaluated by microscopy and by counting hatched and unhatched egg totals. Any crosses with less than ten total embryos were excluded out from the hatch rate assay. Flies used in this study were white Canton-S (wCS; WT); nanos-Gal4-tubulin, #4442, UASp-P32, and UASp-Kap-α2, #25400 (Mason et al., 2002). Fly lines were created by us or obtained from the Bloomington Stock Center. We verified that the transgenic flies used in the experiment are uninfected with *Wolbachia* using PCR and primers, recognizing a conserved region in the *Wolbachia* VirD4 gene. As a positive control, we amplified the ~500bp *D. melanogaster* histone H3 gene (Figure 3.6).

3.3.4 Protein alignment

Kap-α is a highly conserved protein amongst eukaryotic organisms (Goldfarb et al., 2004). The Multiple Sequence Alignment (MSA) of homologs of Kap-α2 protein from different species presented in Fig. 3.3 (a) was generated by using the Clustal Omega alignment tool (Sievers et al., 2011). Fig. 3.3 (b) shows the S98F mutation created in the *D.m* Kap-α2 gene similar to S116F mutation created in Srp1 (Kap-α2 homolog), which unable to suppress the *CidB* toxicity in yeast (Beckmann et al., 2019).

3.3.5 Statistical analysis

All statistical analyses of hatch rates were performed using GraphPad Prism 8.4.2 software. One-way analysis of variance (ANOVA) with multiple comparisons followed by Dunn's test of multiple corrections was performed on all the crosses used in the experiment. Outliers were eliminated by using the ROUT method. All *P* values are reported.

3.4 Results

3.4.1 Mutating start codons to stop codons in Kap-α2 and P32 overexpression transgenes eliminates suppression

Kap-α2 and P32, when overexpressed in female transgenic *Drosophila* suppressed synthetic as well as wild CI (Beckmann et al., 2019). However, alternative explanations for this suppression might be that the DNA sequence of these transgenes and/or impacts on Gal4/UAS system might account for suppressive effects. To develop controls to clarify whether

overproduction of the actual Kap-α2 and P32 proteins was the direct cause of suppression we mutated the start codons of these transgenes to stop codons. Our hope for this control was that the artificially psuedogenized transgenes would no longer suppress CI. We inserted these new transgenes into flies (as described above) and repeated the same crosses. As expected, we observed that the psuedogenized transgenes ($_{Stop}$P32 and $_{Stop}$Kap-α2) no longer suppressed wild and artificial CI (Figure 3.4 and 3.5). The results were not statistically different from the wild CI cross. We conclude that suppression of CI by Kap-α2 and P32 is directly attributed to their protein products. We also used this data as an opportunity to reproduce our former data.

3.4.2 CI suppression by Kap-α2 in fruit flies is dependent on its nuclear importing functions

Kap-α is encoded by Srp1 in yeast, which is a homolog of Kap-α2 (Figure 3.3a). Srp1 performs various biological functions which depend upon its ability to bind nuclear import signals (Chen & Madura, 2014). We previously evaluated Srp1 mutations in the yeast context. Endogenous Srp1 suppressed *cidB* toxicity in yeast; similarly, Kap-α2 also suppressed CI in *Drosophila*. An S116F mutation (*srp1-31* allele) that interrupts the binding of Srp1 and substrate NLS elements, reduced Srp1's ability to suppress the *cidB* toxicity in overexpression plasmids; similarly, but reciprocally, a chromosomal S116F mutation of Srp1 on the haploid yeasts only copy was synthetic with overexpression of *CidB*; the mutation sensitized yeast to *CidB* toxicity did not suppress the *cidB* toxicity (Beckmann et al., 2019). Based on these data, we wanted to evaluate a similar mutation in transgenic flies. We constructed a fly with *D.melanogaster* analogous mutation (S98F; see alignment Figure 3.3b). When mothers overexpressing, this transgene were crossed to incompatible males the mutated karyopherin no longer suppressed CI.

(Figure 3.4 and 3.5). In sum, we conclude that CI suppression by karyopherins is dependent on NLS binding ability and nuclear import. These data add new fly data, which bolsters prior discoveries made in yeast. There are various explanations for why suppression by Kap-α2 depends on its ability to bind NLS sequences. One possible hypothesis is that Kap-α2 brings something into the nucleus that counteracts toxic effects of *CidB*.

3.4.3 Uncleavable Ubiquitin P32 and Kap-α2 fusions suppress CI more than wild-type P32 and Kap-α2 respectively

DUB's cleave ubiquitin at a C-terminal glycine linked to a lysine of the substrate (Pickart, 2001; Weissman, 2001). Ubiquitin elongate in chaining linkages growing from lysine residues within Ubiquitin itself. *CidB* most efficiently cleaves Lys63 linked Ub chains (Beckmann et al., 2017). DUBs recognize and cleave ubiquitin at the C-terminal glycine. A mutation in that C-terminus (G76A; UbG76A) leads to the formation of non-cleavable ubiquitin-substrate fusion (Lacombe et al., 2009). Based on the hypothesis that *CidB* might cleaves ubiquitin off from the Kap-α2 and P32 to induce CI, we made a fly with uncleavable ubiquitin-Kap-α2 and ubiquitin-P32 fusions. If our hypothesis were correct, mating females overexpressing Ub^{G76A}-Kap-α2 and Ub^{G76A}-P32 crossed to incompatible males with synthetic or wild CI should lead to reduced incompatibility and higher hatch rates; or in layman's terms, CI should no-longer work. To test this hypothesis, we crossed, females, overexpressing Ub^{G76A}-Kap-α2 and Ub^{G76A}-P32 respectively to CidA-T2A-$CidB_{wmel}$ males, and we observed significantly higher hatch rates ~15% in case of Ub^{G76A}-Kap-α2 and ~10% in case of Ub^{G76A}-P32 (Figure 3.4). When crossed to incompatible males infected with wild bacteria, the Ub^{G76A}-Kap-α2 and Ub^{G76A}-P32 fusions were even more

effective at suppressing CI and increased hatch rates by an effect size of about ~20% and ~15% respectively when compared to the suppressive capability of wild Kap-α2 and P32 (Figure 3.5). These results support the hypothesis that *CidB* induces CI by cleaving ubiquitin from Kap-α2 and P32; however, these are insufficient data to irrefutably conclude as much.

3.4.4 Inserting N terminus 3xFlag tags doesn't affect the suppressive functions of Kap-α2 and P32.

To facilitated future immunoprecipitation studies of hypothesized *CidB* substrates we made N-terminus 3xFLAG tagged fusion constructs to Kap-α2 and P32. However, for these experiments to be valid, the functional capabilities of Kap-α2 and P32 would need to remain intact, despite the addition of the N-terminal 3xFLAG tags. Tags are known to effect protein functions (Beckmann et al., 2019), so we also validated that these tags did not disrupt the suppressive functions of these particular candidates. Therefore, we crossed females overexpressing these fusion constructs with incompatible males, and the hatch rate was not statistically different from the crosses with females overexpressing Kap-α2 and P32 alone (Figure 3.4 and 3.5). In sum, we conclude that 3xFLAG fusions behave in a similar way to wild alleles.

3.5 Discussion

Previous studies identified the *Wolbachia* proteins (*CidA* and *CidB*) known to cause CI in insects (Beckmann et al., 2017, 2019a, 2019b; Beckmann & Fallon, 2013). CidB is a deubiquitylase (DUB), which means it induces CI by cleaving ubiquitin from the substrate/s (Beckmann et al., 2017). In our last study, we discovered that Kap-α2, nuclear-protein import

factor, and P32, protamine-histone exchange factor might be the targets of *Wolbachia's CidB* as overexpressed Kap-α and P32 in female insect germlines suppressed natural as well as transgenic CI (Beckmann et al., 2019). Previous findings led to an observation that the Kap-α NLS-binding site is required for suppression of CI in yeast. Specifically, in yeast, suppression of *CidB* toxicity by Kap-α was weakened by an S116F mutation which disrupts the binding of Kap-α and substrate NLS elements (Beckmann et al., 2019). Prior to my work, this particular mutation and experiment had not been tested within *Drosophila*. A final hypothesis was that *CidB* might induce CI by cleaving ubiquitin from Kap-α2 or P32.

Here we test the above hypothesis, by showing that the Kap-α NLS-binding site is required for suppression of CI in *Drosophila*. Kap-α is highly conserved gene among eukaryotes (Goldfarb et al., 2004). Therefore, we identified the analogous serine amino acid by alignment and constructed an analogous mutant in *D. melanogaster* Kap-α2. The Multiple Sequence Alignment (MSA) of Kap-α2 protein from *Aedes* to *Drosophila* shows the same (Figure 3.3a). As expected, when we tested the same mutation in transgenic flies, we observed the same effect, mutated Kap-α2 no longer suppressed the transgenic as well as wild CI. So, we conclude that the Kap-α2 NLS-binding site is required for CI suppression in fruit flies.

Our study also supports a hypothesis that *CidB* induces CI by cleaving ubiquitin from Kap-α2 as well as P32. Srp1 (homolog of Kap-α2) as well as histones H2A and H2B are known to be ubiquitylated (Swaney et al., 2013; Wu et al., 2017). Based on these data, *CidB* might somehow reduce the functionality of these proteins by cleaving ubiquitin from them. To test, we made uncleavable ubiquitin fusions to these putative substrates. In support of our hypothesis,

uncleavable Kap-α2 as well as P32 suppressed CI even better than wild Kap-α2 as well as P32 respectively in both our transgenic and *wMel* model CI systems. This was not wholly what we had expected. One favored hypothesis was that CI is induced by direct cleavage of ubiquitin from a single target. If one of these proteins was the main target of *CidB*; then we would have expected the two results to be divergent, i.e., perhaps one substrate would show enhanced suppression and the other no effect. This was not the case as both showed enhanced suppression. Due to the positive results derived from both Ub-fused substrates, this leads us to propose two scenarios. The first scenario is that CI is induced by cleavage of Ub from multiple substrates, included both Kap-α2 as well as P32, and possibly even others. Another scenario is that our experiment is flawed, and it can be interpreted via a *CidB* sink mechanism, whereby toxic *CidB* is sequestered and chelated by the overexpression of an uncleavable Ub-fusion. Therefore, in this model, uncleavable Ub fused to literally any protein might produce these same results if overexpressed. One future experiment required to eliminate this second model would be a better negative control testing overexpression of an uncleavable Ub-GFP fusion. If this substrate also suppresses, we would be forced to conclude that the experiments were flawed, and these data reflected non-specific experimental artifacts rather than bona-fide CI mechanism observations.

However, as the data stand now, these are insufficient data to irrefutably conclude either proposed model. Another experimental system that will allow us to draw more robust conclusions, would be to explore immuno precipitation and western blotting to measure ubiquitylation status of wild P32 and Kap-α2 in fruit flies. I have built the reagents required for these experiments. In the future, my N-terminus 3xFLAG tagged clones would be used to directly pull-down Kap-α2 and

P32 from the fruit fly and immunoblot for ubiquitylation status. Studies might then be performed under contexts with and without CI induction to directly determine if ubiquitylation status changes in response. We also need ubiquitin-GFP control to validate that it is not a non-specific effect.

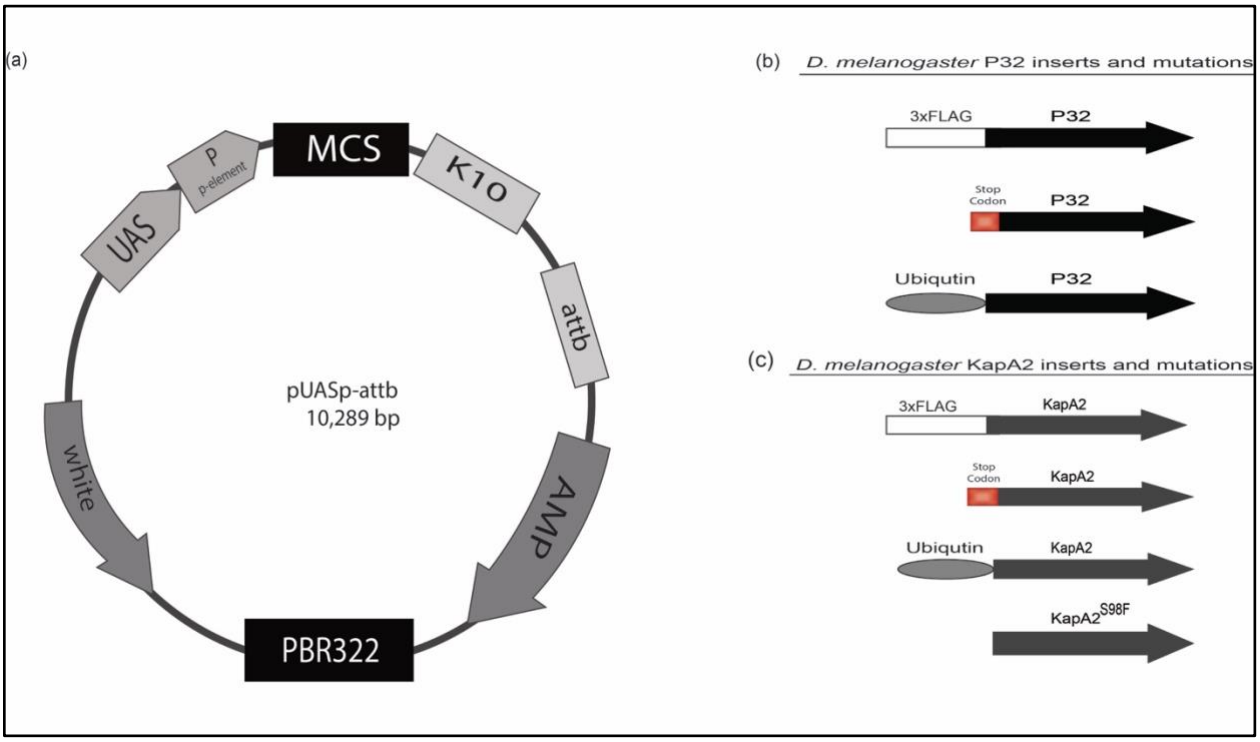

Figure 3.1. Creation of transgenic *D. melanogaster* strains and transgene constructs (a) pUASp-attb vector (Rørth, 1998; Takeo et al., 2012). Various components of the pUASp-attb vector are: 1) UAS which is the upstream activating sequence for GAL4 2) P-element which is the basal germline promoter. 3) MCS is the multi-cloning site. 4) K10 which has 3′ untranslated region from the K10 terminator that localizes transcripts to *Drosophila* oocyte (Serano & Cohen, 1995). 5) attb is the recombination site for ΦC31 integrase. (**b)** Three transgenic constructs of P32 and (**c)** four transgenic constructs of KapA2 were created by heterologous gene insertion into pUASp-attb respectively on the N-terminus.

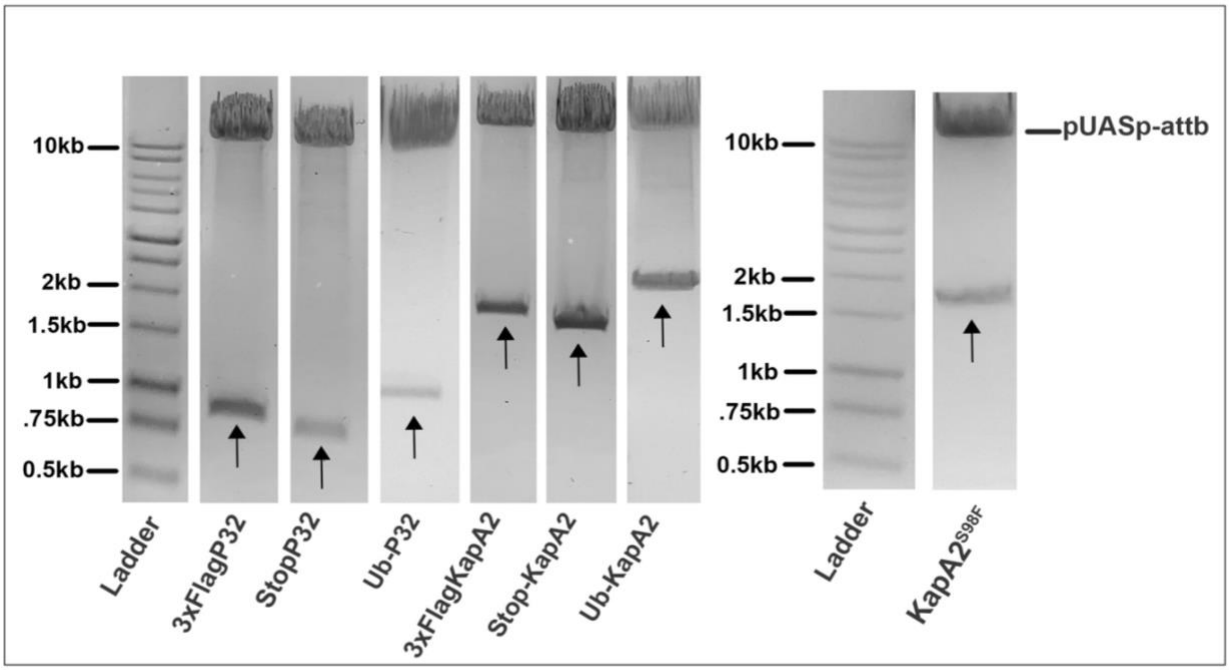

Figure 3.2. Restriction enzyme digest confirms the presence of insert. Presence of transgenes in the final constructs were verified by restriction enzyme digest. Done overnight at 37₀C with Not1 and BamH1 restriction enzymes. Ran on 0.8% Agarose gel.

Primers	Sequence (5'→3')	Description
GD001	TTTTGCGGCCGCATGAACAGCCTAAGGACAGC	Forward and reverse primers for amplification of P32 gene from Hi1 (GenScript) clone to clone into pbsK+ vector
GD002	TTTTCTCGAGGGATCCTTACTTGCCACCGGCAGTG	
GD003	CGGAGGTTCCGATCATGTAACCGTGTTGAATCTCC	Quikchange forward and reverse primers for mutating internal BamH1 site in P32 gene
GD004	CGGAACCTCCGGCTGTCACCGGCTTTTTGGTCATG	
GD009	TTTTGCGGCCGCATGAGTAAGGCGGATTCTAAC	Forward and reverse primers for amplification of KapA2 gene from KapA2 *D.melanogaster* flies
GD033	TTTTGGATCCTTAGAACGTGTAGC	
GD013	CACAAAGGACCCGCGTGCCATCAAGGTGGTGCAGACG	Quikchange forward and reverse primers for mutating internal BamH1 site in KapA2gene
GD025	GCACGCGGGTCCTTTGTGTCCAGCAAATCGATAAAAG	
GD023	TTTTGCGGCCGCTAGAACAGCCTAAGGACAGCAAC	Forward and R primers for mutating start codon to stop codon in P32 gene
GD024	TTTTGGATCCCTTACTTGCCACCGGCAGTG	
GD017	TTTTGCGGCCGCATGTTTTATATTGCTTTCAGTGACG	Forward and reverse primers for amplification of ubiquting from WT *D.melaongaster* flies with UB76A mutation in reverse primer
GD018	TTTTACTAGTACCACCACGCAGGCGGAGC	
GD026	TTTTGCGGCCGCTAGAGTAAGGCGGATTCTAACTCAC	Forward and Reverse primers for mutating start codon to stop codon in KapA2 gene
GD027	TTTTGGATCCTTAGAACGTGTAGCCACCTTC	
GD041	CTCTTTCGGGAGCGCAATCCACCCATC	Quikchange forward and reverse primers for inducing S98F mutation in KapA2 gene
GD042	GGGTGGATTGCGCTCCCGAAAGAGCATCTTGC	
GD028	TTTGCGGCCGCATGGACTACAAAGACCATGACGGT GATTATAAAGATCATGACATCGACTACAAGGATGAC GATGACAAGATGAACAGCCTAAGGACAGC	Forward primer for adding 5' 3XFlagTag to P32 gene
GD029	TTGCGGCCGCATGGACTACAAAGACCATGACGGTG ATTATAAAGATCATGACATCGACTACAAGGATGACG ATGACAAGATGAGTAAGGCGGATTCTAAC	Forward primer for adding 5' 3XFlagTag to KapA2 gene

Table 2.1. Primers used in this study

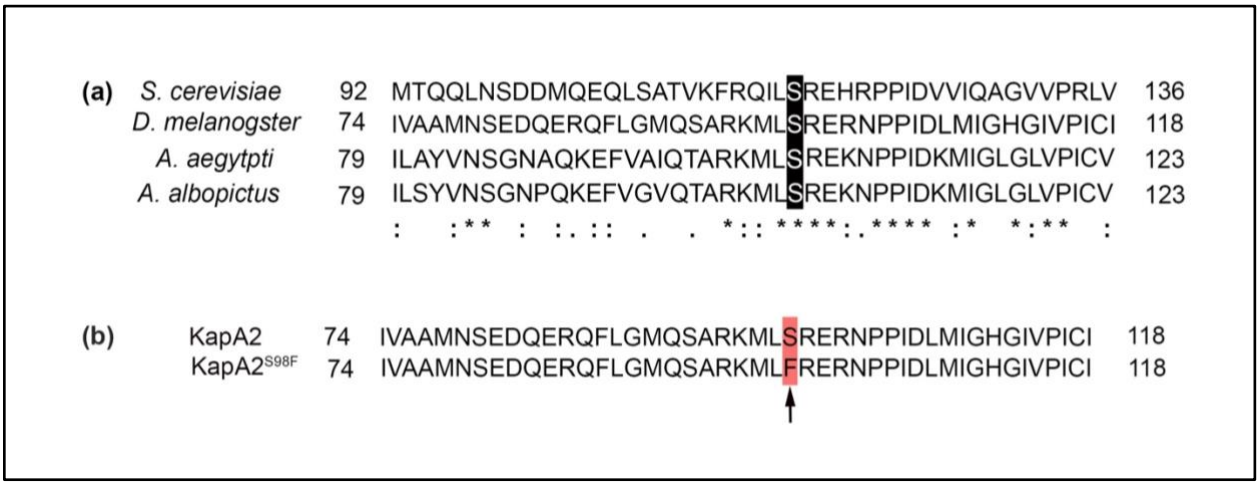

Figure 3.3. KapA is a conserved gene (a) Multiple-sequence alignment (MSA) of *D.m.* Kapα-2 protein and its homologs **(b)** KapA$_{S98F}$ mutation in *D. melanogaster* highlighted with red color. Black highlight indicates the NLS binding site. Asterisks indicates perfect alignment; colon indicates a site belonging to group showing strong similarity and a dot indicates a site belonging to a group showing weak similarity.

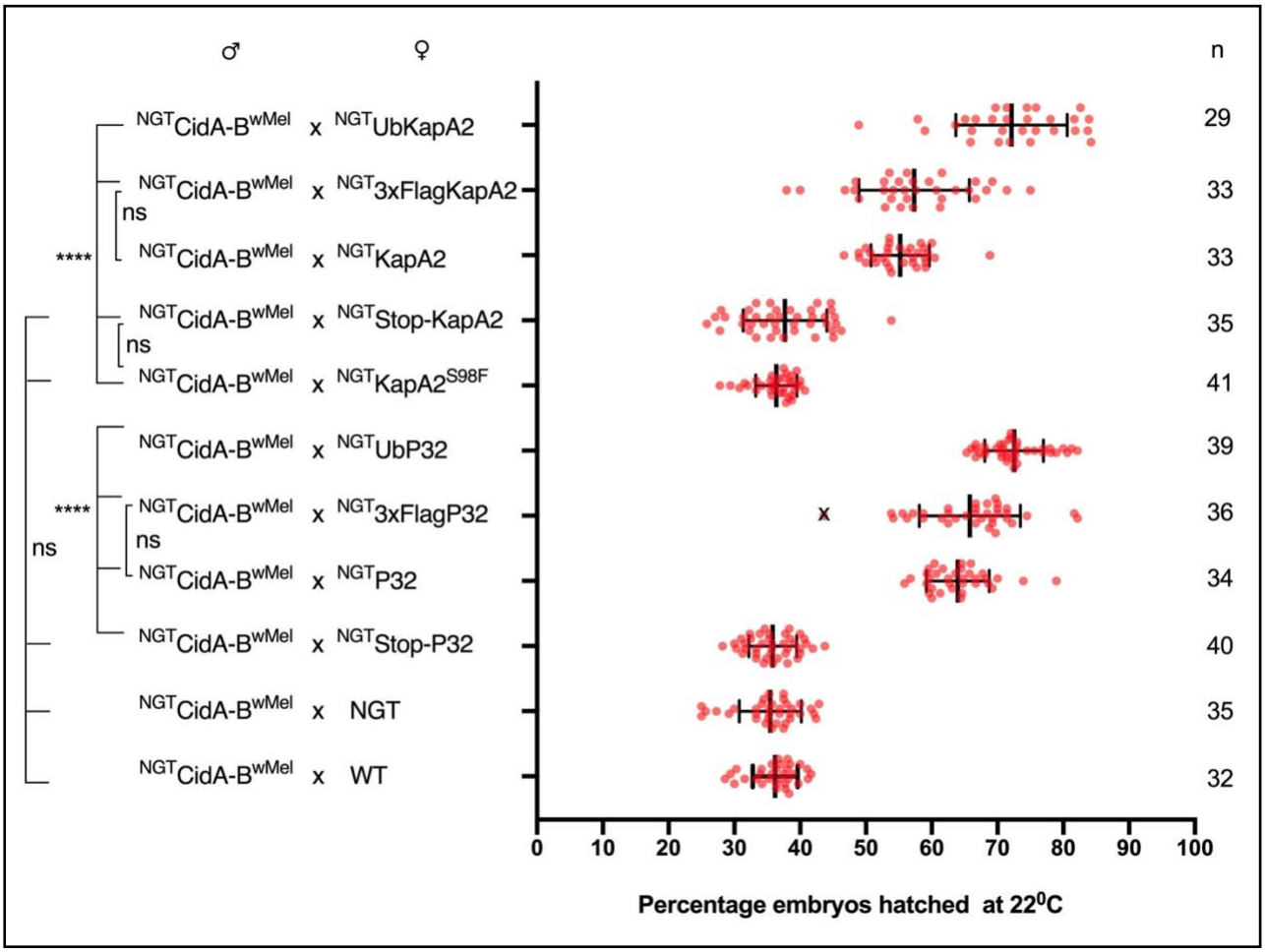

Figure 3.4. At 22₀C overexpression of *D.m.*Kap-α2, *D.m.*P32, 3xflag-Kap-α2, 3xflagP32, *D.m.*Ub-P32 suppressed transgenic CI relative to the control. Kap-α2^{S98F} failed to suppress transgenic CI. Error bars represent means ± s.d. 'n' represents the number of crosses done at 22₀C. ns=p>0.05*p<0.05, **p<0.01, ****p<0.0001 by ANOVA with multiple comparison between all groups and Tukey's post-hoc analysis. Two outliers (x) removed by ROUT analysis.

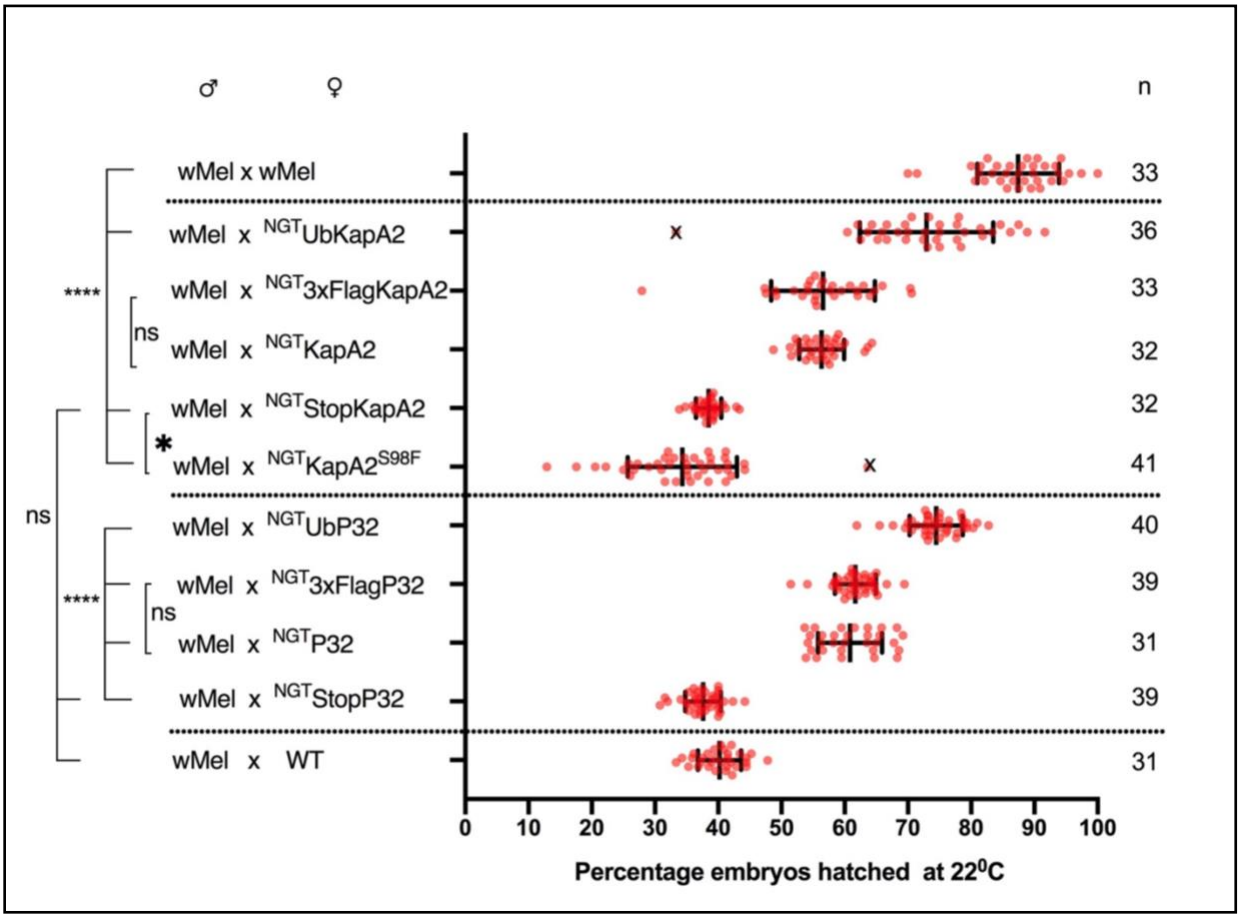

Figure 3.5. At 22₀C overexpression of *D.m.*Kap-α2, *D.m.*P32, 3xflag-Kap-α2, 3xflagP32, *D.m.*Ub-P32, *D.m.*Ub-kapA2 suppressed wild CI relative to the control. Kap-α2^{S98F} failed to suppress wild CI. Error bars represent means ± s.d. 'n' represents the number of crosses done at 22₀C. ns=p>0.05*p<0.05, **p<0.01, ****p<0.0001 by ANOVA with multiple comparison between all groups and Tukey's post-hoc analysis. Two outliers (x) removed by ROUT analysis.

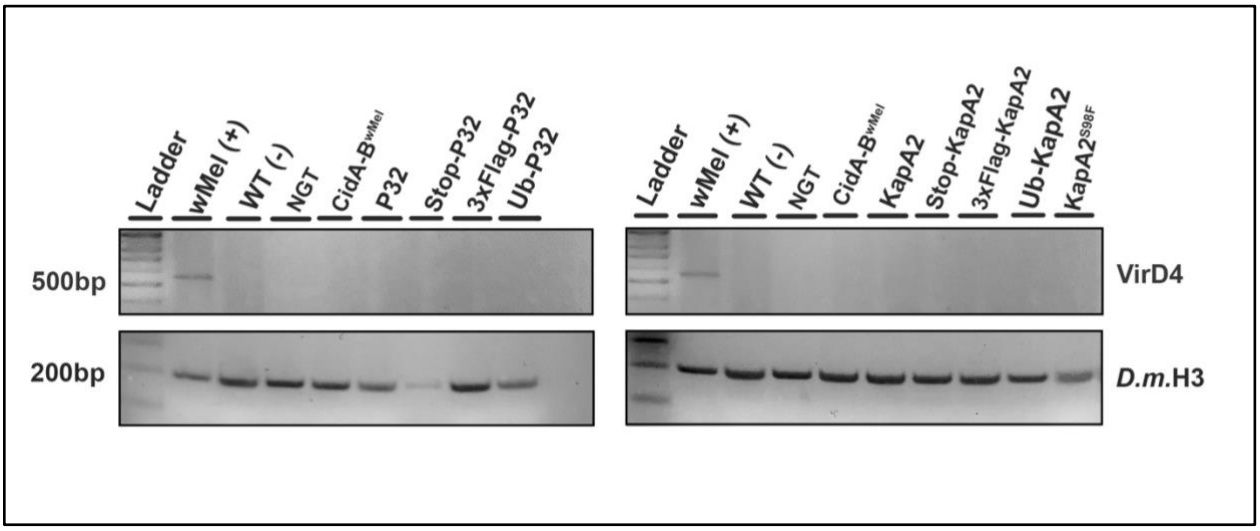

Figure 3.6. PCR analysis demonstrates that transgenic flies used in this study are not infected with *Wolbachia*. VirD4 is a conserved *Wolbachia* gene. Amplification of the *Drosophila melanogaster* Histone three gene served as a positive control. NGT is the Nanos-Gal4-Tubulin driver.

References:

Aliota, M. T., Walker, E. C., Uribe Yepes, A., Dario Velez, I., Christensen, B. M., & Osorio, J. E. (2016). The wMel Strain of Wolbachia Reduces Transmission of Chikungunya Virus in Aedes aegypti. *PLOS Neglected Tropical Diseases*, *10*(4), e0004677. https://doi.org/10.1371/journal.pntd.0004677

Arakaki, N., Miyoshi, T., & Noda, H. (2001). *Wolbachia*– mediated parthenogenesis in the predatory thrips *Franklinothrips vespiformis* (Thysanoptera: Insecta). *Proceedings of the Royal Society of London. Series B: Biological Sciences*, *268*(1471), 1011–1016. https://doi.org/10.1098/rspb.2001.1628

Balhorn, R. (2007). The protamine family of sperm nuclear proteins. *Genome Biology*, *8*(9), 227. https://doi.org/10.1186/gb-2007-8-9-227

Batra, L. R., & Buchner, P. (1968). Endosymbiosis of Animals with Plant Microorganisms. *Mycologia*, *60*(2), 466. https://doi.org/10.2307/3757184

Bayliss, R., Corbett, A. H., & Stewart, M. (2000). The Molecular Mechanism of Transport of Macromolecules Through Nuclear Pore Complexes: Transport Through Nuclear Pore Complexes. *Traffic*, *1*(6), 448–456. https://doi.org/10.1034/j.1600-0854.2000.010602.x

Beck, M., & Hurt, E. (2017). The nuclear pore complex: Understanding its function through structural insight. *Nature Reviews Molecular Cell Biology*, *18*(2), 73–89. https://doi.org/10.1038/nrm.2016.147

Beckmann, John F., Bonneau, M., Chen, H., Hochstrasser, M., Poinsot, D., Merçot, H., Weill, M., Sicard, M., & Charlat, S. (2019a). The Toxin–Antidote Model of Cytoplasmic Incompatibility: Genetics and Evolutionary Implications. *Trends in Genetics*, *35*(3), 175–185. https://doi.org/10.1016/j.tig.2018.12.004

Beckmann, John F., Bonneau, M., Chen, H., Hochstrasser, M., Poinsot, D., Merçot, H., Weill, M., Sicard, M., & Charlat, S. (2019b). Caution Does Not Preclude Predictive and Testable Models of Cytoplasmic Incompatibility: A Reply to Shropshire et al. *Trends in Genetics*, *35*(6), 399–400. https://doi.org/10.1016/j.tig.2019.03.002

Beckmann, John F., & Fallon, A. M. (2013). Detection of the Wolbachia protein WPIP0282 in mosquito spermathecae: Implications for cytoplasmic incompatibility. *Insect Biochemistry and Molecular Biology*, *43*(9), 867–878. https://doi.org/10.1016/j.ibmb.2013.07.002

Beckmann, John F., Ronau, J. A., & Hochstrasser, M. (2017). A Wolbachia deubiquitylating enzyme induces cytoplasmic incompatibility. *Nature Microbiology*, *2*(5), 17007. https://doi.org/10.1038/nmicrobiol.2017.7

Beckmann, John Frederick, Sharma, G. D., Mendez, L., Chen, H., & Hochstrasser, M. (2019). The Wolbachia cytoplasmic incompatibility enzyme CidB targets nuclear import and protamine-histone exchange factors. *ELife*, *8*, e50026. https://doi.org/10.7554/eLife.50026

Bordenstein, S. R., Uy, J. J., & Werren, J. H. (n.d.). *Host Genotype Determines Cytoplasmic Incompatibility Type in the Haplodiploid Genus Nasonia*. 12.

Brand, A. H., & Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development*, *118*(2), 401.

Breeuwer, J. A. J., & Werren, J. H. (1990). Microorganisms associated with chromosome destruction and reproductive isolation between two insect species. *Nature*, *346*(6284), 558–560. https://doi.org/10.1038/346558a0

Brennan, L. J., Haukedal, J. A., Earle, J. C., Keddie, B., & Harris, H. L. (2012). Disruption of redox homeostasis leads to oxidative DNA damage in spermatocytes of Wolbachia-infected Drosophila simulans: Wolbachia induces DNA damage. *Insect Molecular Biology*, *21*(5), 510–520. https://doi.org/10.1111/j.1365-2583.2012.01155.x

Callaini, G., Dallai, R., & Riparbelli, M. G. (n.d.). *Wolbachia-induced delay of paternal chromatin condensation does not prevent maternal chromosomes from entering anaphase in incompatible crosses of Drosophila simulans*. 10.

Callaini, G., Riparbelli, M. G., Giordano, R., & Dallai, R. (1996). Mitotic Defects Associated with Cytoplasmic Incompatibility inDrosophila simulans. *Journal of Invertebrate Pathology*, *67*(1), 55–64. https://doi.org/10.1006/jipa.1996.0009

Chapman, A.D. (2006). *Numbers of living species in Australia and the World*.

Chen, H., Ronau, J. A., Beckmann, J. F., & Hochstrasser, M. (2019). A *Wolbachia* nuclease and its binding partner provide a distinct mechanism for cytoplasmic incompatibility. *Proceedings of the National Academy of Sciences*, *116*(44), 22314–22321. https://doi.org/10.1073/pnas.1914571116

Chen, L., & Madura, K. (2014). Yeast Importin-α (Srp1) Performs Distinct Roles in the Import of Nuclear Proteins and in Targeting Proteasomes to the Nucleus. *Journal of Biological Chemistry*, *289*(46), 32339–32352. https://doi.org/10.1074/jbc.M114.582023

Chook, Y. (2001). Karyopherins and nuclear import. *Current Opinion in Structural Biology*, *11*(6), 703–715. https://doi.org/10.1016/S0959-440X(01)00264-0

Cooper, B. S., Ginsberg, P. S., Turelli, M., & Matute, D. R. (2017). Wolbachia in the *Drosophila yakuba* Complex: Pervasive Frequency Variation and Weak Cytoplasmic Incompatibility, but No Apparent Effect on Reproductive Isolation. *Genetics*, *205*(1), 333–351. https://doi.org/10.1534/genetics.116.196238

Dobson, S. L., Bourtzis, K., Braig, H. R., Jones, B. F., Zhou, W., Rousset, F., & O'Neill, S. L. (1999). Wolbachia infections are distributed throughout insect somatic and germ line tissues. *Insect Biochemistry and Molecular Biology*, *29*(2), 153–160. https://doi.org/10.1016/S0965-1748(98)00119-2

Dorigatti, I., McCormack, C., Nedjati-Gilani, G., & Ferguson, N. M. (2018). Using Wolbachia for Dengue Control: Insights from Modelling. *Trends in Parasitology*, *34*(2), 102–113. https://doi.org/10.1016/j.pt.2017.11.002

Duffy, J. B. (2002). GAL4 system indrosophila: A fly geneticist's swiss army knife. *Genesis*, *34*(1–2), 1–15. https://doi.org/10.1002/gene.10150

Duplouy, A., Hurst, G. D. D., O'Neill, S. L., & Charlat, S. (2010). Rapid spread of male killing *Wolbachia* in the butterfly *Hypolimnas bolina*. *Journal of Evolutionary Biology*, *23*(1), 231–235. https://doi.org/10.1111/j.1420-9101.2009.01891.x

Duron, O., Boureux, A., Echaubard, P., Berthomieu, A., Berticat, C., Fort, P., & Weill, M. (2007). Variability and Expression of Ankyrin Domain Genes in Wolbachia Variants Infecting the Mosquito Culex pipiens. *Journal of Bacteriology*, *189*(12), 4442–4448. https://doi.org/10.1128/JB.00142-07

Dutra, H. L. C., Rocha, M. N., Dias, F. B. S., Mansur, S. B., Caragata, E. P., & Moreira, L. A. (2016a). Wolbachia Blocks Currently Circulating Zika Virus Isolates in Brazilian Aedes aegypti Mosquitoes. *Cell Host & Microbe*, *19*(6), 771–774. https://doi.org/10.1016/j.chom.2016.04.021

Dutra, H. L. C., Rocha, M. N., Dias, F. B. S., Mansur, S. B., Caragata, E. P., & Moreira, L. A. (2016b). Wolbachia Blocks Currently Circulating Zika Virus Isolates in Brazilian Aedes aegypti Mosquitoes. *Cell Host & Microbe*, *19*(6), 771–774. https://doi.org/10.1016/j.chom.2016.04.021

Dyer, K. A., & Jaenike, J. (2004). Evolutionarily Stable Infection by a Male-Killing Endosymbiont in *Drosophila innubila*: Molecular Evidence From the Host and Parasite Genomes. *Genetics*, *168*(3), 1443–1455. https://doi.org/10.1534/genetics.104.027854

Elnagdy, S., Messing, S., & Majerus, M. E. N. (2013). Two Strains of Male-Killing Wolbachia in a Ladybird, Coccinella undecimpunctata, from a Hot Climate. *PLoS ONE*, *8*(1), e54218. https://doi.org/10.1371/journal.pone.0054218

Emelyanov, A. V., & Fyodorov, D. V. (2016). Thioredoxin-dependent disulfide bond reduction is required for protamine eviction from sperm chromatin. *Genes & Development*, *30*(24), 2651–2656. https://doi.org/10.1101/gad.290916.116

Emelyanov, A. V., Rabbani, J., Mehta, M., Vershilova, E., Keogh, M. C., & Fyodorov, D. V. (2014). *Drosophila* TAP/p32 is a core histone chaperone that cooperates with NAP-1, NLP, and nucleophosmin in sperm chromatin remodeling during fertilization. *Genes & Development*, *28*(18), 2027–2040. https://doi.org/10.1101/gad.248583.114

Fast, E. M., Toomey, M. E., Panaram, K., Desjardins, D., Kolaczyk, E. D., & Frydman, H. M. (2011). Wolbachia Enhance Drosophila Stem Cell Proliferation and Target the Germline Stem Cell Niche. *Science*, *334*(6058), 990–992. https://doi.org/10.1126/science.1209609

Feldhaar, H., & Gross, R. (2009). Insects as hosts for mutualistic bacteria. *International Journal of Medical Microbiology*, *299*(1), 1–8. https://doi.org/10.1016/j.ijmm.2008.05.010

Ferree, P. M., & Sullivan, W. (2006). A Genetic Test of the Role of the Maternal Pronucleus in Wolbachia-Induced Cytoplasmic Incompatibility in *Drosophila melanogaster*. *Genetics*, *173*(2), 839–847. https://doi.org/10.1534/genetics.105.053272

Ferri, E., Bain, O., Barbuto, M., Martin, C., Lo, N., Uni, S., Landmann, F., Baccei, S. G., Guerrero, R., de Souza Lima, S., Bandi, C., Wanji, S., Diagne, M., & Casiraghi, M. (2011). New Insights into the Evolution of Wolbachia Infections in Filarial Nematodes Inferred from a Large Range of Screened Species. *PLoS ONE*, *6*(6), e20843. https://doi.org/10.1371/journal.pone.0020843

Fialho, R. F., & Stevens, L. (2000). Male-killing *Wolbachia* in a flour beetle. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, *267*(1451), 1469–1473. https://doi.org/10.1098/rspb.2000.1166

Frentiu, F. D., Zakir, T., Walker, T., Popovici, J., Pyke, A. T., van den Hurk, A., McGraw, E. A., & O'Neill, S. L. (2014). Limited Dengue Virus Replication in Field-Collected Aedes aegypti Mosquitoes Infected with Wolbachia. *PLoS Neglected Tropical Diseases*, *8*(2), e2688. https://doi.org/10.1371/journal.pntd.0002688

Fujii, Y., Kageyama, D., Hoshizaki, S., Ishikawa, H., & Sasaki, T. (2001). Transfection of *Wolbachia* in Lepidoptera: The feminizer of the adzuki bean borer *Ostrinia scapulalis*

causes male killing in the Mediterranean flour moth *Ephestia kuehniella*. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, *268*(1469), 855–859. https://doi.org/10.1098/rspb.2001.1593

Gerth, M., & Bleidorn, C. (2017). Comparative genomics provides a timeframe for Wolbachia evolution and exposes a recent biotin synthesis operon transfer. *Nature Microbiology*, *2*(3), 16241. https://doi.org/10.1038/nmicrobiol.2016.241

Gillespie, J. J., Driscoll, T. P., Verhoeve, V. I., Rahman, M. S., Macaluso, K. R., & Azad, A. F. (2018). A Tangled Web: Origins of Reproductive Parasitism. *Genome Biology and Evolution*, *10*(9), 2292–2309. https://doi.org/10.1093/gbe/evy159

Goldfarb, D. S., Corbett, A. H., Mason, D. A., Harreman, M. T., & Adam, S. A. (2004). Importin α: A multipurpose nuclear-transport receptor. *Trends in Cell Biology*, *14*(9), 505–514. https://doi.org/10.1016/j.tcb.2004.07.016

Gomes, F. M., & Barillas-Mury, C. (2018). Infection of anopheline mosquitoes with Wolbachia: Implications for malaria control. *PLOS Pathogens*, *14*(11), e1007333. https://doi.org/10.1371/journal.ppat.1007333

Harumoto, T., Fukatsu, T., & Lemaitre, B. (2018). Common and unique strategies of male killing evolved in two distinct *Drosophila* symbionts. *Proceedings of the Royal Society B: Biological Sciences*, *285*(1875), 20172167. https://doi.org/10.1098/rspb.2017.2167

Hedges, L. M., Brownlie, J. C., O'Neill, S. L., & Johnson, K. N. (2008). Wolbachia and Virus Protection in Insects. *Science*, *322*(5902), 702–702. https://doi.org/10.1126/science.1162418

Hertig, M. (1936). The Rickettsia, *Wolbachia pipientis* (gen. Et sp.n.) and Associated Inclusions of the Mosquito, *Culex pipiens*. *Parasitology*, *28*(4), 453–486. https://doi.org/10.1017/S0031182000022666

Hertig, Mar. (n.d.). *STUDIES ON RICKETTSIA-LIKE MICRO-ORGANISMS IN INSECTS*. 50.

Hiroki, M., Kato, Y., Kamito, T., & Miura, K. (2002). Feminization of genetic males by a symbiotic bacterium in a butterfly, Eurema hecabe (Lepidoptera: Pieridae). *Naturwissenschaften*, *89*(4), 167–170. https://doi.org/10.1007/s00114-002-0303-5

Hler, M. K., Speck, C., Christiansen, M., Bischoff, F. R., Prehn, S., & Haller, H. (1999). Evidence for Distinct Substrate Specificities of Importin ␣ Family Members in Nuclear Protein Import. *MOL. CELL. BIOL.*, *19*, 10.

Hoffmann, A. A., Montgomery, B. L., Popovici, J., Iturbe-Ormaetxe, I., Johnson, P. H., Muzzi, F., Greenfield, M., Durkan, M., Leong, Y. S., Dong, Y., Cook, H., Axford, J., Callahan, A. G., Kenny, N., Omodei, C., McGraw, E. A., Ryan, P. A., Ritchie, S. A., Turelli, M., & O'Neill, S. L. (2011). Successful establishment of Wolbachia in Aedes populations to suppress dengue transmission. *Nature*, *476*(7361), 454–457. https://doi.org/10.1038/nature10356

Hornett, E. A., Charlat, S., Duplouy, A. M. R., Davies, N., Roderick, G. K., Wedell, N., & Hurst, G. D. D. (2006). Evolution of Male-Killer Suppression in a Natural Population. *PLoS Biology*, *4*(9), e283. https://doi.org/10.1371/journal.pbio.0040283

Hosokawa, T., Koga, R., Kikuchi, Y., Meng, X.-Y., & Fukatsu, T. (2010). Wolbachia as a bacteriocyte-associated nutritional mutualist. *Proceedings of the National Academy of Sciences*, *107*(2), 769–774. https://doi.org/10.1073/pnas.0911476107

Hurst, G. D. D., & Johnson, A. P. (n.d.). *Male-Killing Wolbachia in Drosophila: A Temperature-Sensitive Trait With a Threshold Bacterial Density*. 11.

Jaenike, J. (2007a). SPONTANEOUS EMERGENCE OF A NEW WOLBACHIA PHENOTYPE. *Evolution*, *61*(9), 2244–2252. https://doi.org/10.1111/j.1558-5646.2007.00180.x

Jaenike, J. (2007b). SPONTANEOUS EMERGENCE OF A NEW WOLBACHIA PHENOTYPE. *Evolution*, *61*(9), 2244–2252. https://doi.org/10.1111/j.1558-5646.2007.00180.x

Kalderon, D., Roberts, B. L., Richardson, W. D., & Smith, A. E. (1984). A short amino acid sequence able to specify nuclear location. *Cell*, *39*(3), 499–509. https://doi.org/10.1016/0092-8674(84)90457-4

Kern, P., Cook, J. M., Kageyama, D., & Riegler, M. (2015). Double trouble: Combined action of meiotic drive and *Wolbachia* feminization in *Eurema* butterflies. *Biology Letters*, *11*(5), 20150095. https://doi.org/10.1098/rsbl.2015.0095

Kessler, M. M., Henry, M. F., Shen, E., Zhao, J., Gross, S., Silver, P. A., & Moore, C. L. (1997). Hrp1, a sequence-specific RNA-binding protein that shuttles between the nucleus and the cytoplasm, is required for mRNA 3'-end formation in yeast. *Genes & Development*, *11*(19), 2545–2556. https://doi.org/10.1101/gad.11.19.2545

Kusano, A., Staber, C., & Ganetzky, B. (2002). Segregation distortion induced by wild-type RanGAP in Drosophila. *Proceedings of the National Academy of Sciences*, *99*(10), 6866–6870. https://doi.org/10.1073/pnas.102165099

Lacombe, T., García-Gómez, J. J., De La Cruz, J., Roser, D., Hurt, E., Linder, P., & Kressler, D. (2009). Linear ubiquitin fusion to Rps31 and its subsequent cleavage are required for the efficient production and functional integrity of 40S ribosomal subunits: Role of ubiquitin in ubiquitin-Rps31 fusion protein. *Molecular Microbiology*, *72*(1), 69–84. https://doi.org/10.1111/j.1365-2958.2009.06622.x

Landmann, F., Orsi, G. A., Loppin, B., & Sullivan, W. (2009). Wolbachia-Mediated Cytoplasmic Incompatibility Is Associated with Impaired Histone Deposition in the Male Pronucleus. *PLoS Pathogens*, *5*(3), e1000343. https://doi.org/10.1371/journal.ppat.1000343

Larracuente, A. M., & Presgraves, D. C. (2012). The Selfish *Segregation Distorter* Gene Complex of *Drosophila melanogaster*. *Genetics*, *192*(1), 33–53. https://doi.org/10.1534/genetics.112.141390

LePage, D. P., Metcalf, J. A., Bordenstein, S. R., On, J., Perlmutter, J. I., Shropshire, J. D., Layton, E. M., Funkhouser-Jones, L. J., Beckmann, J. F., & Bordenstein, S. R. (2017). Prophage WO genes recapitulate and enhance Wolbachia-induced cytoplasmic incompatibility. *Nature*, *543*(7644), 243–247. https://doi.org/10.1038/nature21391

Leung, S. W., Harreman, M. T., Hodel, M. R., Hodel, A. E., & Corbett, A. H. (2003). Dissection of the Karyopherin α Nuclear Localization Signal (NLS)-binding Groove: FUNCTIONAL REQUIREMENTS FOR NLS BINDING. *Journal of Biological Chemistry*, *278*(43), 41947–41953. https://doi.org/10.1074/jbc.M307162200

Liu, C., Wang, J.-L., Zheng, Y., Xiong, E.-J., Li, J.-J., Yuan, L.-L., Yu, X.-Q., & Wang, Y.-F. (2014). Wolbachia-induced paternal defect in Drosophila is likely by interaction with the juvenile hormone pathway. *Insect Biochemistry and Molecular Biology*, *49*, 49–58. https://doi.org/10.1016/j.ibmb.2014.03.014

Liu, J., Lin, H., Lopez, J. M., & Wolfner, M. F. (1997). Formation of the Male Pronuclear Lamina inDrosophila melanogaster. *Developmental Biology*, *184*(2), 187–196. https://doi.org/10.1006/dbio.1997.8523

Loeb, J. D., Schlenstedt, G., Pellman, D., Kornitzer, D., Silver, P. A., & Fink, G. R. (1995). The yeast nuclear import receptor is required for mitosis. *Proceedings of the National Academy of Sciences*, *92*(17), 7647–7651. https://doi.org/10.1073/pnas.92.17.7647

Loppin, B., Dubruille, R., & Horard, B. (2015). The intimate genetics of *Drosophila* fertilization. *Open Biology*, *5*(8), 150076. https://doi.org/10.1098/rsob.150076

Mason, D. A., Fleming, R. J., & Goldfarb, D. S. (n.d.). *Drosophila melanogaster Importin ⍺1 and ⍺3 Can Replace Importin ⍺2 During Spermatogenesis but Not Oogenesis*. 14.

McMeniman, C. J., Lane, R. V., Cass, B. N., Fong, A. W. C., Sidhu, M., Wang, Y.-F., & O'Neill, S. L. (2009). Stable Introduction of a Life-Shortening Wolbachia Infection into the Mosquito Aedes aegypti. *Science*, *323*(5910), 141–144. https://doi.org/10.1126/science.1165326

Merrill, C. (1999). Truncated RanGAP Encoded by the Segregation Distorter Locus of Drosophila. *Science*, *283*(5408), 1742–1745. https://doi.org/10.1126/science.283.5408.1742

Mira, A., & Moran, N. A. (2002). Estimating Population Size and Transmission Bottlenecks in Maternally Transmitted Endosymbiotic Bacteria. *Microbial Ecology*, *44*(2), 137–143. https://doi.org/10.1007/s00248-002-0012-9

Narita, S., Kageyama, D., Nomura, M., & Fukatsu, T. (2007). Unexpected Mechanism of Symbiont-Induced Reversal of Insect Sex: Feminizing Wolbachia Continuously Acts on the Butterfly Eurema hecabe during Larval Development. *Applied and Environmental Microbiology*, *73*(13), 4332–4341. https://doi.org/10.1128/AEM.00145-07

Negri, I., Pellecchia, M., Mazzoglio, P. J., Patetta, A., & Alma, A. (2006). Feminizing *Wolbachia* in *Zyginidia pullula* (Insecta, Hemiptera), a leafhopper with an XX/X0 sex-determination system. *Proceedings of the Royal Society B: Biological Sciences*, *273*(1599), 2409–2416. https://doi.org/10.1098/rspb.2006.3592

Nemec, C. M., Yang, F., Gilmore, J. M., Hintermair, C., Ho, Y.-H., Tseng, S. C., Heidemann, M., Zhang, Y., Florens, L., Gasch, A. P., Eick, D., Washburn, M. P., Varani, G., & Ansari, A. Z. (2017). Different phosphoisoforms of RNA polymerase II engage the Rtt103 termination factor in a structurally analogous manner. *Proceedings of the National Academy of Sciences*, *114*(20), E3944–E3953. https://doi.org/10.1073/pnas.1700128114

Novy J.E. (1979). *Screwworm control and eradication in the southern United States of America*.

Oka, M., & Yoneda, Y. (2018). Importin α: Functions as a nuclear transport factor and beyond. *Proceedings of the Japan Academy, Series B*, *94*(7), 259–274. https://doi.org/10.2183/pjab.94.018

Papafotiou, G., Oehler, S., Savakis, C., & Bourtzis, K. (2011). Regulation of Wolbachia ankyrin domain encoding genes in Drosophila gonads. *Research in Microbiology*, *162*(8), 764–772. https://doi.org/10.1016/j.resmic.2011.06.012

Phadnis, N., Hsieh, E., & Malik, H. S. (2012). Birth, Death, and Replacement of Karyopherins in Drosophila. *Molecular Biology and Evolution*, *29*(5), 1429–1440. https://doi.org/10.1093/molbev/msr306

Pichon, S., Bouchon, D., Liu, C., Chen, L., Garrett, R. A., & Grève, P. (2012). The expression of one ankyrin pk2 allele of the WO prophage is correlated with the Wolbachia feminizing effect in isopods. *BMC Microbiology*, *12*(1), 55. https://doi.org/10.1186/1471-2180-12-55

Pickart, C. M. (2001). Mechanisms Underlying Ubiquitination. *Annual Review of Biochemistry*, *70*(1), 503–533. https://doi.org/10.1146/annurev.biochem.70.1.503

Pickart, C. M., & Cohen, R. E. (2004). Proteasomes and their kin: Proteases in the machine age. *Nature Reviews Molecular Cell Biology*, *5*(3), 177–187. https://doi.org/10.1038/nrm1336

Pieper, K. E., Unckless, R. L., & Dyer, K. A. (2018). A fast-evolving X-linked duplicate of *importin-α2* is overexpressed in *sex-ratio* drive in *Drosophila neotestacea*. *Molecular Ecology*, mec.14928. https://doi.org/10.1111/mec.14928

Poinsot, D., Charlat, S., & Merçot, H. (2003a). On the mechanism of *Wolbachia* -induced cytoplasmic incompatibility: Confronting the models with the facts: Problems and paradigms. *BioEssays*, *25*(3), 259–265. https://doi.org/10.1002/bies.10234

Poinsot, D., Charlat, S., & Merçot, H. (2003b). On the mechanism of *Wolbachia* -induced cytoplasmic incompatibility: Confronting the models with the facts: Problems and paradigms. *BioEssays*, *25*(3), 259–265. https://doi.org/10.1002/bies.10234

Presgraves, D. C. (n.d.). *A Genetic Test of the Mechanism of Wolbachia-Induced Cytoplasmic Incompatibility in Drosophila*. 6.

Rathke, C., Baarends, W. M., Awe, S., & Renkawitz-Pohl, R. (2014). Chromatin dynamics during spermiogenesis. *Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms*, *1839*(3), 155–168. https://doi.org/10.1016/j.bbagrm.2013.08.004

Reed, K. M., & Werren, J. H. (1995). Induction of paternal genome loss by the paternal-sex-ratio chromosome and cytoplasmic incompatibility bacteria (Wolbachia): A comparative study of early embryonic events. *Molecular Reproduction and Development*, *40*(4), 408–418. https://doi.org/10.1002/mrd.1080400404

Ritchie, S. A., Townsend, M., Paton, C. J., Callahan, A. G., & Hoffmann, A. A. (2015). Application of wMelPop Wolbachia Strain to Crash Local Populations of Aedes aegypti. *PLOS Neglected Tropical Diseases*, *9*(7), e0003930. https://doi.org/10.1371/journal.pntd.0003930

Ronau, J. A., Beckmann, J. F., & Hochstrasser, M. (2016). Substrate specificity of the ubiquitin and Ubl proteases. *Cell Research*, *26*(4), 441–456. https://doi.org/10.1038/cr.2016.38

Rørth, P. (1998). Gal4 in the Drosophila female germline. *Mechanisms of Development*, *78*(1–2), 113–118. https://doi.org/10.1016/S0925-4773(98)00157-9

Ryan, K. J., McCaffery, J. M., & Wente, S. R. (2003). The Ran GTPase cycle is required for yeast nuclear pore complex assembly. *Journal of Cell Biology*, *160*(7), 1041–1053. https://doi.org/10.1083/jcb.200209116

Ryan, S. L., & Saul, G. B. (1968). Post-fertilization effect of incompatibility factors in Mormoniella. *Molecular and General Genetics MGG*, *103*(1), 29–36. https://doi.org/10.1007/BF00271154

Sazama, E. J., Ouellette, S. P., & Wesner, J. S. (2019). Bacterial Endosymbionts Are Common Among, but not Necessarily Within, Insect Species. *Environmental Entomology*, *48*(1), 127–133. https://doi.org/10.1093/ee/nvy188

Schmidt, T. L., Barton, N. H., Rašić, G., Turley, A. P., Montgomery, B. L., Iturbe-Ormaetxe, I., Cook, P. E., Ryan, P. A., Ritchie, S. A., Hoffmann, A. A., O'Neill, S. L., & Turelli, M. (2017). Local introduction and heterogeneous spatial spread of dengue-suppressing Wolbachia through an urban population of Aedes aegypti. *PLOS Biology*, *15*(5), e2001894. https://doi.org/10.1371/journal.pbio.2001894

Serano and Cohen, 1995.pdf. (n.d.).

Serano, T. L., & Cohen, R. S. (1995). A small predicted stem-loop structure mediates oocyte localization of Drosophila K10 mRNA. *Development*, *121*(11), 3809.

Serbus, L. R., Casper-Lindley, C., Landmann, F., & Sullivan, W. (2008). The Genetics and Cell Biology of *Wolbachia* -Host Interactions. *Annual Review of Genetics*, *42*(1), 683–707. https://doi.org/10.1146/annurev.genet.41.110306.130354

Shropshire, J. D., & Bordenstein, S. R. (2019). Two-By-One model of cytoplasmic incompatibility: Synthetic recapitulation by transgenic expression of cifA and cifB in Drosophila. *PLOS Genetics*, *15*(6), e1008221. https://doi.org/10.1371/journal.pgen.1008221

Shropshire, J. D., On, J., Layton, E. M., Zhou, H., & Bordenstein, S. R. (2018). One prophage WO gene rescues cytoplasmic incompatibility in *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences*, *115*(19), 4987–4991. https://doi.org/10.1073/pnas.1800650115

Sievers, F., Wilm, A., Dineen, D., Gibson, T. J., Karplus, K., Li, W., Lopez, R., McWilliam, H., Remmert, M., Söding, J., Thompson, J. D., & Higgins, D. G. (2011). Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Molecular Systems Biology*, *7*(1), 539. https://doi.org/10.1038/msb.2011.75

Slatko, B. E., Luck, A. N., Dobson, S. L., & Foster, J. M. (2014). Wolbachia endosymbionts and human disease control. *Molecular and Biochemical Parasitology*, *195*(2), 88–95. https://doi.org/10.1016/j.molbiopara.2014.07.004

Srividya, I., Tirupataiah, S., & Mishra, K. (2012). Yeast Transcription Termination Factor Rtt103 Functions in DNA Damage Response. *PLoS ONE*, *7*(2), e31288. https://doi.org/10.1371/journal.pone.0031288

Stouthamer, R., Breeuwer, J. A. J., & Hurst, G. D. D. (1999). *Wolbachia Pipientis*: Microbial Manipulator of Arthropod Reproduction. *Annual Review of Microbiology*, *53*(1), 71–102. https://doi.org/10.1146/annurev.micro.53.1.71

Stouthamer, Richard, & Kazmer, D. J. (1994). Cytogenetics of microbe-associated parthenogenesis and its consequences for gene flow in Trichogramma wasps. *Heredity*, *73*(3), 317–327. https://doi.org/10.1038/hdy.1994.139

Swaney, D. L., Beltrao, P., Starita, L., Guo, A., Rush, J., Fields, S., Krogan, N. J., & Villén, J. (2013). Global analysis of phosphorylation and ubiquitylation cross-talk in protein degradation. *Nature Methods*, *10*(7), 676–682. https://doi.org/10.1038/nmeth.2519

Takeo, S., Swanson, S. K., Nandanan, K., Nakai, Y., Aigaki, T., Washburn, M. P., Florens, L., & Hawley, R. S. (2012). Shaggy/glycogen synthase kinase 3 and phosphorylation of Sarah/regulator of calcineurin are essential for completion of Drosophila female meiosis. *Proceedings of the National Academy of Sciences*, *109*(17), 6382–6389. https://doi.org/10.1073/pnas.1120367109

Tirmarche, S., Kimura, S., Dubruille, R., Horard, B., & Loppin, B. (2016). Unlocking sperm chromatin at fertilization requires a dedicated egg thioredoxin in Drosophila. *Nature Communications*, *7*(1), 13539. https://doi.org/10.1038/ncomms13539

Tirmarche, S., Kimura, S., Sapey-Triomphe, L., Sullivan, W., Landmann, F., & Loppin, B. (2014). *Drosophila* Protamine-Like Mst35Ba and Mst35Bb Are Required for Proper Sperm Nuclear Morphology but Are Dispensable for Male Fertility. *G3: Genes|Genomes|Genetics*, *4*(11), 2241–2245. https://doi.org/10.1534/g3.114.012724

Tram, U. (2002). Role of Delayed Nuclear Envelope Breakdown and Mitosis in Wolbachia-Induced Cytoplasmic Incompatibility. *Science*, *296*(5570), 1124–1126. https://doi.org/10.1126/science.1070536

Tram, Uyen, Ferree, P. M., & Sullivan, W. (2003). Identification of Wolbachia–host interacting factors through cytological analysis. *Microbes and Infection*, *5*(11), 999–1011. https://doi.org/10.1016/S1286-4579(03)00192-8

Tram, Uyen, Fredrick, K., Werren, J. H., & Sullivan, W. (2006). Paternal chromosome segregation during the first mitotic division determines *Wolbachia* -induced cytoplasmic incompatibility phenotype. *Journal of Cell Science*, *119*(17), 3655–3663. https://doi.org/10.1242/jcs.03095

Turelli, M. (1994). EVOLUTION OF INCOMPATIBILITY-INDUCING MICROBES AND THEIR HOSTS. *Evolution*, *48*(5), 1500–1513. https://doi.org/10.1111/j.1558-5646.1994.tb02192.x

van den Hurk, A. F., Hall-Mendelin, S., Pyke, A. T., Frentiu, F. D., McElroy, K., Day, A., Higgs, S., & O'Neill, S. L. (2012). Impact of Wolbachia on Infection with Chikungunya and Yellow Fever Viruses in the Mosquito Vector Aedes aegypti. *PLoS Neglected Tropical Diseases*, *6*(11), e1892. https://doi.org/10.1371/journal.pntd.0001892

Vandekerckhove, T. T. M., Watteyne, S., Bonne, W., Vanacker, D., Devaere, S., Rumes, B., Maelfait, J.-P., Gillis, M., Swings, J. G., Braig, H. R., & Mertens, J. (2003). *Evolutionary trends in feminization and intersexuality in woodlice (Crustacea, Isopoda) infected with Wolbachia pipientis (α-Proteobacte- ria)*. 9.

Walker, T., Johnson, P. H., Moreira, L. A., Iturbe-Ormaetxe, I., Frentiu, F. D., McMeniman, C. J., Leong, Y. S., Dong, Y., Axford, J., Kriesner, P., Lloyd, A. L., Ritchie, S. A., O'Neill, S. L., & Hoffmann, A. A. (2011). The wMel Wolbachia strain blocks dengue and invades caged Aedes aegypti populations. *Nature*, *476*(7361), 450–453. https://doi.org/10.1038/nature10355

Weeks, A. R., & Breeuwer, J. A. J. (2001). *Wolbachia* –induced parthenogenesis in a genus of phytophagous mites. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, *268*(1482), 2245–2251. https://doi.org/10.1098/rspb.2001.1797

Weinert, L. A., Araujo-Jnr, E. V., Ahmed, M. Z., & Welch, J. J. (2015). The incidence of bacterial endosymbionts in terrestrial arthropods. *Proceedings of the Royal Society B: Biological Sciences*, *282*(1807), 20150249. https://doi.org/10.1098/rspb.2015.0249

Weissman, A. M. (2001). Themes and variations on ubiquitylation. *Nature Reviews Molecular Cell Biology*, *2*(3), 169–178. https://doi.org/10.1038/35056563

Welchman, R. L., Gordon, C., & Mayer, R. J. (2005). Ubiquitin and ubiquitin-like proteins as multifunctional signals. *Nature Reviews Molecular Cell Biology*, *6*(8), 599–609. https://doi.org/10.1038/nrm1700

Wernegreen, J. J. (2002). Genome evolution in bacterial endosymbionts of insects. *Nature Reviews Genetics*, *3*(11), 850–861. https://doi.org/10.1038/nrg931

Werren, J. H. (1997). BIOLOGY OF *WOLBACHIA*. *Annual Review of Entomology*, *42*(1), 587–609. https://doi.org/10.1146/annurev.ento.42.1.587

Werren, J. H., Baldo, L., & Clark, M. E. (2008). Wolbachia: Master manipulators of invertebrate biology. *Nature Reviews Microbiology*, *6*(10), 741–751. https://doi.org/10.1038/nrmicro1969

Wu, M.-Y., Lin, C.-Y., Tseng, H.-Y., Hsu, F.-M., Chen, P.-Y., & Kao, C.-F. (2017). H2B ubiquitylation and the histone chaperone Asf1 cooperatively mediate the formation and maintenance of heterochromatin silencing. *Nucleic Acids Research*, *45*(14), 8225–8238. https://doi.org/10.1093/nar/gkx422

Yamada, R., Floate, K. D., Riegler, M., & O'Neill, S. L. (2007). Male Development Time Influences the Strength of Wolbachia-Induced Cytoplasmic Incompatibility Expression in *Drosophila melanogaster*. *Genetics*, *177*(2), 801–808. https://doi.org/10.1534/genetics.106.068486

Yamaguchi, Y., Park, J.-H., & Inouye, M. (2011). Toxin-Antitoxin Systems in Bacteria and Archaea. *Annual Review of Genetics*, *45*(1), 61–79. https://doi.org/10.1146/annurev-genet-110410-132412

Yen and Barr. (1971). *New Hypothesis of the Cause of Cytoplasmic Incompatibility in Culex pipiens L.*

Zheng, Y., Ren, P.-P., Wang, J.-L., & Wang, Y.-F. (2011). Wolbachia-Induced Cytoplasmic Incompatibility Is Associated with Decreased Hira Expression in Male Drosophila. *PLoS ONE*, *6*(4), e19512. https://doi.org/10.1371/journal.pone.0019512

Zug, R., & Hammerstein, P. (2012). Still a Host of Hosts for Wolbachia: Analysis of Recent Data Suggests That 40% of Terrestrial Arthropod Species Are Infected. *PLoS ONE*, *7*(6), e38544. https://doi.org/10.1371/journal.pone.0038544