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Canonical Wg/Wnt pathway regulates Wolbachia intracellular density in Drosophila

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Thesis

CANONICAL WG/WNT PATHWAY REGULATES WOLBACHIA INTRACELLULAR DENSITY IN DROSOPHILA

by

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CANONICAL WG/WNT PATHWAY REGULATES WOLBACHIA
INTRACELLULAR DENSITY IN DROSOPHILA

HSIN-YI HSIA

ABSTRACT

Wolbachia are widely spread, maternally transmitted insect endosymbiotic intracellular bacteria. They have been implicated in the control of several insect transmitted diseases, including dengue, yellow fever, Zika and malaria. Effective pathogen suppression in the insect host is shown to be proportional to the intracellular levels of bacteria. Therefore, understanding the molecular mechanisms underlying Wolbachia accumulation within organisms is extremely important for future epidemic control and research. Using Drosophila as a model insect, our lab has previously observed Wolbachia tropism to stem cell niches. Current work has identified polar cells as an additional site of Wolbachia tropism and demonstrated that Wg/Wnt signaling is important for Wolbachia intracellular accumulation in these somatic cells. In this thesis, we first observed that the Wg/Wnt pathway protein Armadillo also controls Wolbachia levels in the germline cells, indicating the possibility of having a conserved molecular mechanism controlling Wolbachia. Using RNAi and small molecule inhibitors of Shaggy, another component of the canonical Wg/Wnt pathway, we demonstrate that the canonical Wg/Wnt signaling is essential for Wolbachia intracellular accumulation. Our investigation provides fundamental insights into the mechanisms of Wolbachia intracellular accumulation. Furthermore, it offers novel strategies to modulate Wolbachia in non-model insect species, including various disease transmitting
Anopheles, Culex, and Aedes. These findings potentially will increase the effectiveness of a Wolbachia-based vector transmitted disease suppression.
# TABLE OF CONTENTS

ACKNOWLEDGMENTS ........................................................................................................ iv

ABSTRACT ........................................................................................................................ vi

TABLE OF CONTENTS ...................................................................................................... viii

LIST OF TABLES ........................................................................................................... xii

LIST OF FIGURES .......................................................................................................... xiii

LIST OF ABBREVIATIONS ............................................................................................... xv

CHAPTER 1 Introduction ................................................................................................. 1

1.1 Wolbachia and disease transmission ........................................................................... 1

1.1.1 Wolbachia and virus transmission in insects ......................................................... 1

1.2 Host-symbiont crosstalk ............................................................................................ 2

1.2.1 Wingless/Wnt pathway as a pathway affected by bacteria symbiont ............... 3

1.3 Wingless/Wnt pathway in Drosophila ...................................................................... 3

1.3.1 Canonical Wg/Wnt signaling in Drosophila ......................................................... 4

1.3.2 Non-canonical signal transduction cascade under Wingless ......................... 5

1.4 Drosophila as a model system .................................................................................. 6

1.4.1 Female Drosophila ovary morphology .............................................................. 7

1.4.2 Male Drosophila testis morphology ................................................................. 8

CHAPTER 2 Material and Methods ................................................................................ 14

2.1 Fly stocks, transgenes and genetic crosses .............................................................. 14

2.2 Raising of adult culture .......................................................................................... 14

2.2.1 General GAL4-UAS fly crosses ....................................................................... 14
2.2.2 *tubulin*-GAL80 crosses ................................................................. 14

2.2.3 LiCl treatment ........................................................................ 15

2.3 DNA and RNA analysis ................................................................. 15

2.3.1 Nucleic acid isolation ............................................................... 15

2.3.3 Polymerase chain reaction (PCR) ............................................. 16

2.4 Protein analysis ......................................................................... 18

2.4.1 Protein isolation from adult flies ........................................... 18

2.4.2 Western blot analysis ............................................................... 18

2.5 Immunofluorescence microscopy ................................................. 19

2.5.1. General immunostaining ...................................................... 19

2.5.2. *In situ* hybridization .......................................................... 20

2.6 Image analysis of *Wolbachia* ..................................................... 20

2.6.1 *Wolbachia* quantification in hub cells .................................. 20

2.6.2 *Wolbachia* quantification in germline cells ........................... 21

2.7 GAL4-UAS system ..................................................................... 21

2.8 Statistical Analysis .................................................................... 22

CHAPTER 3 Canonical Wg/Wnt pathway regulates *Wolbachia* intracellular density in *Drosophila* ......................................................................................... 32

3.1 Introduction ............................................................................... 32

3.2 Armadillo affects *Wolbachia* level in germline ......................... 34

3.2.1 Armadillo$^{S10}$ upregulates *Wolbachia* level in germline .......... 35

3.2.2 RNAi against Armadillo downregulates *Wolbachia* level in germline .... 35
3.3 Modulating the activity of other genes in the Wg/Wnt pathway also affects Wolbachia density........................................................................................................................................................................ 36

3.4 LiCl treatment leads to an increase in Wolbachia intracellular levels in Drosophila ........................................................................................................................................................................................................................................... 38

  3.4.1 Preliminary experiments with standard fly food determine that Li⁺ treatment leads to an increased Wolbachia density in whole flies........................................... 38

  3.4.2 LiCl treatment in Nutri-Fly German Food shows elevated Wolbachia level in whole wMel ovaries and stage 8 germline cells ......................................................... 39

  3.4.3. LiCl alters Wolbachia level through Armadillo......................................... 40

3.5 Armadillo does not alter Wolbachia through interaction with Pangolin ........ 42

CHAPTER 4 Summary and Future Perspectives ......................................................................................................................... 55

  4.1 Wolbachia accumulation is regulated under an evolutionarily conserved pathway ................................................................................................................................................................................................. 55

  4.2 Small molecule inhibitors as potential methods to affect disease transmission 56

  4.3 Armadillo affects Wolbachia level through an alternative pathway............ 57

  4.4 Transcriptional profiling is required to identify more downstream effectors... 59

Appendix 1 .............................................................................................................................................................................................................................. 60

Appendix 2 .............................................................................................................................................................................................................................. 62

Appendix 3 .............................................................................................................................................................................................................................. 65

Appendix 4 .............................................................................................................................................................................................................................. 67

Appendix 5 .............................................................................................................................................................................................................................. 69

Appendix 6 .............................................................................................................................................................................................................................. 71
Appendix 7 .................................................................................................................. 74
BIBLIOGRAPHY ........................................................................................................... 77
VITA ............................................................................................................................. 91
LIST OF TABLES

Table 2.1: Sources for *Drosophila* species used for analysis .................................................. 26
Table 2.2: Transgenic fly stocks used for analysis ......................................................................... 27
Table 2.3 PCR primers .................................................................................................................. 28
Table 2.4 Antibodies used for western blotting .......................................................................... 29
Table 2.5 Antibodies used for immunohistochemistry ............................................................... 30
Table 2.6 *In situ* hybridization oligos ...................................................................................... 31
LIST OF FIGURES

Figure 1.1 Canonical and non-canonical Wg/Wnt signaling pathway .............................................. 9
Figure 1.2 The Drosophila female anatomy ................................................................................. 10
Figure 1.3 The Drosophila male anatomy .................................................................................. 12
Figure 2.1 The GAL80-GAL4-UAS system ............................................................................. 23
Figure 2.2 Generic GAL4-UAS cross scheme .......................................................................... 24
Figure 2.3 Generic GAL80-GAL4-UAS cross scheme ............................................................... 25
Figure 3.1 Armadillo$^{S10}$ expression in germline cells results increases Wolbachia level .................................................................................................................................................. 45
Figure 3.2 RNAi mediated knockdown of Armadillo in germline cells results in reduced Wolbachia level .................................................................................................................................. 47
Figure 3.3 RNAi mediated Frizzled knockdown in hub cells does not affect Wolbachia level .......................................................................................................................... 48
Figure 3.4 Wolbachia level in wMel infected whole fly is significantly increased after LiCL treatment .................................................................................................................................. 49
Figure 3.5 Elevation of Wolbachia was masked by arm$^{S10}$ and could not be further upregulated in germline after LiCL treatment .................................................................................. 51
Figure 3.6 Armadillo increases Wolbachia level through a pathway independent from Pangolin .................................................................................................................................. 53
Figure A1.1 Wingless expression is observed in hub cell membranes and somatic stem cells but not in ovaries .................................................................................................................. 61
Figure A2.1 Autophagy proteins Atg8 and Ref(2)P have different expression patterns in *Wolbachia* infected and uninfected testes .......................................................... 64

Figure A3.1 *Wolbachia* decreases the fitness of control flies ................................. 66

Figure A4.1 *Wolbachia* level in carcass increases after LiCl treatment ...................... 68

Figure A5.1 Li$^+$ leads to phosphorylation of Shaggy on Ser$^9$ and increases full-length Armadillo level in female whole flies ......................................................... 70

Figure A6.1 Wg/Wnt responsive gene expressions were analyzed in wMelCS and wMelPop infected newly eclosed ovaries with and without Armadillo$^{S10}$ expression 73

Figure A7.1 Li$^+$ does not elevate *Wolbachia* level in non-melanogaster *Drosophila* species under current treating condition................................................................. 75
LIST OF ABBREVIATIONS

5d    5 days
7d    7 days
Apc   Adenomatous polyposis coli
AR    androgen receptor
Arm   Armadillo
Arr   Arrow
BRG1  brahma-related gene 1
BSA   bovine serum albumin
℃     Celsius
C.    *Caenorhabditis*
CamKII calmodulin-dependent kinase
CBP   CREB binding protein
Ck1   Casein kinase 1
CO₂   carbon dioxide
CREB  cAMP response element-binding protein
CtBP  Carboxyl-terminal binding protein
*D.*  *Drosophila*
DEC   *Drosophila* E-cadherin
DEPC  diethylpyrocarbonate
DNA   Deoxyribonucleic acid
Dsh   Dishevelled
DSHB  Developmental Studies Hybridoma Bank
EtOH  ethanol
FOXO  Forkhead box O
Fz    Frizzled
GSC   germline stem cell
GSCN  germline stem cell niche
GSK3  glycogen synthase kinase 3
HIF1α hypoxia inducible factor 1-alpha
JNK  Jun kinase
KD    knockdown
LRH1  liver receptor homolog 1
LRP   lipoprotein receptor-related protein
LRP5/6 LDL-related receptor protein 5/6
MED12 mediator complex subunit 12
Nos   Nanos
NE    newly eclosed
NF-κB nuclear factor kappa-light-chain-enhancer of activated B cells
NGS   normal goat serum
NGT   Nanos-GAL4-Tubulin
OCT-4 octamer-binding transcription factor 4
Pan   Pangolin
PBANG phosphate buffered saline with Triton-X 100, bovine serum albumin and normal goat serum
PBS   phosphate buffered saline
PBS/BSA phosphate buffered saline with bovine serum albumin
PBT   phosphate buffered saline with Triton-X 100
PCP   Planar Cell Polarity
PCR   polymerase chain reaction
PFA   paraformaldehyde
PC    polar cell
PKC   protein kinase C
PLC   phospholipase C
qPCR  quantitative PCR
RT PCR reverse transcription PCR
RNA   ribonucleic acid
RNAi  RNA interference
rRNA  ribosomal RNA
ROCK  Rho kinase
<table>
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<td>ROS</td>
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<td>RT</td>
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<td>serine</td>
</tr>
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<td>Shaggy</td>
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<td>SOX17</td>
<td>sex determining region Y-box 17</td>
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<tr>
<td>SSC</td>
<td>saline-sodium citrate</td>
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<td>somatic stem cell</td>
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<tr>
<td>SSCN</td>
<td>somatic stem cell niche</td>
</tr>
<tr>
<td>TCF/LEF</td>
<td>T-cell factor/lymphoid enhancer factor</td>
</tr>
<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>UAS</td>
<td>upstream activation sequence</td>
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<tr>
<td>Upd</td>
<td>Unpaired</td>
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<td><em>Wolbachia</em> uninfected <em>Drosophila</em></td>
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CHAPTER 1 Introduction

1.1 *Wolbachia* and disease transmission

*Wolbachia* are obligate intracellular alphaproteobacteria in which infect filarial nematodes and arthropods. They were first described in *Culex pipiens* in 1924 as an bacterial endosymbiont (Hertig and Wolbach 1924), and are now predicated to infect more than 40% of the insect species (Zug and Hammerstein 2012). *Wolbachia* live in host cell cytoplasm and depend largely on host cell resources for replication. They are vertically inherited through the maternal cytoplasm.

1.1.1 *Wolbachia* and virus transmission in insects

*Wolbachia* have been observed to induce protection against microbial pathogens in diverse insect species, such as disease transmitting mosquitos (*Aedes, Anopheles, Culex*) and *Drosophila*. They affect disease transmission ability of those human pathogen vectors, and are thus considered as a potential way to biologically control vector disease transmission. It is reported that *wMel Wolbachia* infection blocks accumulation and transmission ability of dengue (DENV), Chikungunya (CHIKV) and *Plasmodium* in *Aedes aegypti*. (Hoffmann et al. 2011; Walker et al. 2011; Moreira et al. 2009). Recent work has also pointed out the possible anti-Zika property of *wMel* in *Aedes aegypti* (Dutra et al. 2016; Aliota et al. 2016).

However, potential mechanisms underlying *Wolbachia*-mediated antiviral effect are still not fully understood. Some groups have suggested that *Wolbachia* infection activates host innate immune responses. Upregulation of reactive oxygen species (ROS) was observed in *Wolbachia* infected mosquitos and *Drosophila* (Wong, Brownlie, and
Johnson 2015; Wong et al. 2011). It is also suggested that the life-shortening property of *Wolbachia* strain *w*MelPop blocks disease transmission. Viruses require a long time to replicate and travel to the salivary glands which is essential for their transmission to the next host. Thus, reduced lifespan leads to a decrease in the probability of viruses being transmitted without minimizing the population size (Bourtzis et al. 2014). Resource (cholesterol) competition among host, virus and *Wolbachia* might also limit pathogen replication in host (Moreira et al. 2009; Rasgon, Styer, and Scott 2003; Caragata et al. 2013).

Phenotypically, *Wolbachia* infection level has been shown to negatively correlate with arboviral level *in vivo*. The more *Wolbachia*, the less the virus, and also less viral transmission (Lu et al. 2012). Therefore, investigation of the underlying molecular crosstalk within microbes, and, between host and microbes, is important.

### 1.2 Host-symbiont crosstalk

Symbiotic bacteria are known to affect hosts in a broad spectrum, including development (Koropatnick et al. 2004), nutrient production (Baumann 2005), reproduction (Hurst and Jiggins 2000), and immunity (Hurst and Jiggins 2000). Symbionts evolve mechanisms to utilize host resources and to escape from hosts’ innate immune responses. Differences between pathogenic and mutualistic symbiosis are not clear cut. In fact, many of the molecular interactions are very similar (Dale and Moran 2006). Mutualistic symbionts have to survive and replicate within host species, but at the same time have to keep the hosts viable in order to transmit and maintain their level in the general host population. Levels of symbionts have to be tightly
regulated. Thus, it is important to study which host pathways are required for symbionts to maintain at their densities.

1.2.1 Wingless/Wnt pathway as a pathway affected by bacteria symbiont

*Ehrlichia* and *Wolbachia* are closely related genera in *Rickettsia* family. Recently, it has been suggested that *Ehrlichia chaffeensis*, intracellular bacteria that live in early-endosome-like membrane-bound vacuoles in human mononuclear phagocytes, secret a bacterial effector protein to turn on canonical and non-canonical host Wnt signaling pathway, stimulating phagocytosis and promoting its intracellular survival (Luo et al. 2016). This research demonstrates that *Ehrlichia* utilizes several host pathways including Wingless (Wg)/Wnt to maintain intracellular levels. Wg/Wnt may be considered an important candidate pathway for *Wolbachia*-host crosstalk.

1.3 Wingless/Wnt pathway in *Drosophila*

Wg/Wnt signaling pathway represents an evolutionary conserved cascade that plays an important role in embryonic development, adult tissue homeostasis, cell renewal and regeneration. Considerable work has also focused on the pathological potential of the pathway as well (Clevers and Nusse 2012). The variety of Wg/Wnt targets highlights the importance of understanding this pathway.

*Drosophila wingless* is named after the phenotype observed in a hypomorphic mutant, which has a variable transformation of adult wing(s) to thoracic notum (Sharma and Chopra 1976). The segment polarity phenotype of embryonic lethal *wg* null mutant was first described in the Heidelberg screen in 1980 (Nusslein-Volhard and Wieschaus 1980). Wild type embryonic cuticle has a repeating pattern of ventral
denticle and naked belts while wg loss-of-function results in loss of naked cuticle region. The wg gene was cloned in 1987 (Baker 1987) and the sequence was found to be homologous to a mammalian oncogene, integrated or Int-1. Later the mammalian oncogene was renamed “Wnt 1” as in “wingless/Int 1” (Rijsewijk et al. 1987).

1.3.1 Canonical Wg/Wnt signaling in Drosophila

The signaling cascade downstream of Wg has been implicated by seminal screens of mutations, to be affecting the larval cuticle patterning (Wieschaus, Nüsslein-Volhard, and Jürgens 1984). Armadillo/Arm (mammalian ortholog β-catenin) was the first protein identified downstream of Wg by epistatic analysis (Riggleman, Schedl, and Wieschaus 1990b). Subsequent investigations have identified a group of evolutionarily conserved factors involved in this signal transduction pathway.

Activation of the pathway requires binding of extracellular Wg ligand to membrane receptor Frizzled. Frizzled family proteins are G-protein coupled receptor with 7 transmembrane domains. In Drosophila, it is believed that Frizzled and Frizzled2 serve as redundant receptors of Wingless family proteins. Ligand binding initiates clustering with co-receptor Arrow/Arr (mammalian ortholog LRP5/6) (Alexandre, Baena-Lopez, and Vincent 2014; Wehrli et al. 2000). However, Bartscherer et al (Bartscherer et al. 2006) pointed out that Fz and Fz4, but not Fz2, are required to turn on Wingless signal transduction in a non-redundant manner.

The “destruction complex”, composed of Axin, Adenomatous polyposis coli/Apc scaffold and two kinases, Casein kinase 1 (Ck1) and Shaggy/Sgg (also known as Zeste-white 3/Zw3; mammalian ortholog GSK-3), is an important constituent of this
pathway. Shaggy is a key element in the destruction complex. It phosphorylates the N-terminus of Armadillo and targets the protein for ubiquitin ligase mediated degradation. Cytosolic levels of Armadillo are kept low by the destruction complex.

A key element of the destruction complex is the scaffold protein called Axin. Axin stability promotes the formation of the complex. Upon ligand binding, Dishevelled/Dsh binds to an intracellular loop and the cytosolic region of Fz (Wong et al. 2003). It is proposed that this binding event also recruits Axin to the cytosolic domain of Arrow where it is further degraded, thereby inactivating the destruction complex and promoting a high cytosolic Armadillo accumulation (Tolwinski et al. 2003). Upon reaching a particular threshold, Armadillo is translocated into the nucleus where it binds to transcription factor Pangolin and initiates the transcription of Wg/Wnt responsive genes. Details will be further discussed in Chapter 3.1.

The cell signaling cascade described above is referred to as Armadillo-dependent “canonical Wg/Wnt signaling pathway” (Figure 1.1).

1.3.2 Non-canonical signal transduction cascade under Wingless

Apart from the canonical pathway, the proteins of the Wg/Wnt pathway can also be involved in Armadillo independent, evolutionarily conserved “non-canonical Wg/Wnt signaling pathways” such as the Wg/Fz/Planar Cell Polarity (PCP) pathway and Wg-dependent/protein kinase C (PKC)-dependent pathway (Seifert and Mlodzik 2007). These pathways do not regulate cell fate specification during embryonic development but rather control morphogenetic cell movement (Croce et al. 2006). The most studied non-canonical pathway in Drosophila is Wg/Fz/Planar Cell Polarity (PCP)
pathway, which establishes cell polarity and controls convergent extension movements (Seifert and Mlodzik 2007; Croce and McClay 2008; Axelrod et al. 1998). The PCP pathway was first discovered in *Drosophila* where it controls epithelial planar polarity within the eye, wing and thorax (Mlodzik 1999; Maung and Jenny 2011). The pathway shares many components with the canonical pathway, but utilizes different transducing molecules. Like the canonical pathways, it is initiated by Wg binding to Fz membrane receptors and subsequent cytoplasmic Dsh activation. However, in the PCP pathway, the signal is then transduced through small G proteins Rho and Rac and further turns on effectors, Rho-kinase (ROCK) and Jun-kinase (JNK) respectively (Figure 1).

Another non-canonical pathway described in *Drosophila* model is the Wg-protein kinase C (PKC) pathway, which suggests a positive correlation between Wg and PKC activity (Cook et al. 1996). In vertebrate model systems (*Xenopus* and zebrafish), Wnt is shown to activate calcium sensitive factors (PKC, calcineurin and calcium/ CamKII) by activating protein lipase C (PLC) and by promoting the release of intracellular calcium (Sheldahl et al. 1999; Kuhl et al. 2000). However, a relationship between Wg and calcium has not yet been reported in *Drosophila* (Figure 1).

1.4 *Drosophila* as a model system

The common fruit fly, *Drosophila melanogaster*, is a powerful biological system that has been used for many advances in various fields of research. It has been used as a model organism for genetic research from the beginning of 20\textsuperscript{th} century, when Thomas Hunt Morgan and his colleagues started their Nobel Prize awarded work on the mechanisms of heredity. Later on, researchers in *Drosophila* have expanded to
many other fields of interest such as developmental biology, neurobiology, and cancer biology.

*Drosophila* is a model system that is inexpensive and easy to culture. It has considerably short life cycle and a high reproduction rate. Moreover, a large complement of molecular and genetic tools have been developed in this model system. The development of a genetic binary expression system, the GAL4-UAS system, makes it possibly to temporally and tissue-specifically express or knock down specific gene constructs *in vivo*, which will be further discussed later in Materials and Methods, chapter 2.7.

1.4.1 Female *Drosophila* ovary morphology

Anatomically, *Drosophilae* have fairly simple and well-studied gonads. The *Drosophila* ovary is a model system which has been used extensively to study cell death, stem cell and developmental biology. Each female *Drosophila* has two ovaries. Each ovary is composed of 16 strings of serially developing egg chambers called ovarioles. At the very anterior tip of the ovariole is a structure called germarium, which harbors the somatic, and germline stem cells and their respective niches. These are essential for proper egg development. The germline stem cell (GSC) asymmetrically divides into one germline cell and one cystoblast, which further divides 4 times and matures to form an egg chamber composed of one oocyte and 15 nurse cells. The somatic stem cell (SSC) asymmetrically divides into one follicle cell, which undergoes further division and encases the germline cells to create an egg chamber. A small group
of follicle cells differentiate into polar cells and stalk cells required for the separation of adjacent egg chambers (Figure 1.2).

1.4.2 Male *Drosophila* testis morphology

Adult male *Drosophila* have a pair of testes. They are long, coiled bi-ended tubes with one end connected to accessory genital glands. Located at the blind apical end of the testis are 10-15 somatically derived hub cells which function as the niche for both somatic and germline cells. Around 6-8 GSCs flanked by cyst stem cells are physically attached to hub cells (Figure 1.3).
Figure 1.1 Canonical and non-canonical Wg/Wnt signaling pathway

The model of mammalian Wnt signaling pathway. Green boxes indicate the presence of the genes in Drosophila model. Red boxes indicate the members that are modulated in this thesis. (Wnt: fly ortholog Wingless; LRP5/6: fly ortholog Arrow; GSK-3: fly ortholog Shaggy; β-catenin: fly ortholog Armadillo; TCF/LEF: fly ortholog Pangolin. Figure adapted from KEGG.)
Figure 1.2 The *Drosophila* female anatomy

A. Location of the ovaries in female flies. B. The reproductive system of female *Drosophila melanogaster*. Each female fly has two ovaries connecting to the common oviduct. Each ovary is a cluster of strings of developing egg chambers, the ovarioles. C. Diagram of the ovariole. Development proceeds from left to right. The germline cyst exits the germarium and undergoes 14 stages of oogenesis to mature. D. Diagram of a magnified germarium and egg chambers. The germarium is located at the anterior tip of the ovariole, housing the germline and somatic stem cells and their niches. An egg chambers is composed of one oocyte and 15 nurse cells encased by a layer of follicle cells. A small group of follicle cells at the apical end of each egg chamber differentiate into polar cells and stalk cells required for the separation of adjacent egg chambers. Figures are adapted from (Mahowald *et al.*, 1980), (Cohen, 2013), and (Hartenstein, 1993).
Figure 1.3 The *Drosophila* male anatomy

A. Location of the testes in male flies. B. The reproductive system of male *Drosophila melanogaster*. Each male fly has two testes connecting to the accessory glands and ejaculatory duct. Spermatogenesis starts from the apical tip of the testis and progresses through the testis lobe. C. Diagram of the apical tip of the testis. Hub cells function as the niche for both somatic and germline cells. The germline stem cells (GSCs) flanked by cyst stem cells (CyCSc) are physically attached to hub cells. Figures are adapted from (Patterson, 1943) and (de Cuevas *et al.*, 2011)
CHAPTER 2 Material and Methods

2.1 Fly stocks, transgenes and genetic crosses

*Drosophila* stocks were maintained at room temperature on standard cornmeal, molasses, yeast, and agar medium. *D. sechellia* flies were supplemented with reconstituted Noni Fruit (Hawaiian Health Ohana, LLC) (Amlou, Moreteau, and David 1998). Details for *Wolbachia* infection status and sources are listed in Table 2.1. Details for fly lines used for genetic overexpression and knockdown are listed in Table 2.2.

2.2 Raising of adult culture

2.2.1 General GAL4-UAS fly crosses

Virgin females were collected from stocks maintained in 18°C and crosses with males maintained in room temperature. Crosses were kept in a 25°C incubator with 60% humidity until proper age of analysis.

2.2.2 *tubulin*-GAL80 crosses

2.2.2.1 RNAi mediated Armadillo RNAi cross

Virgin; *NGT40*-GAL4; *nos*-GAL4 females were crossed with balanced; *tub*-GAL80ts/Cyo; UAS-arm RNAi/Tm6B males in 18°C. Progenies were shifted to 29°C incubator with 60% humidity every 4 days to maximally turn on RNAi expression and avoid embryonic lethality. Both NE and 7-day old flies were collected for dissection and subsequent analysis.

2.2.2.2. *arm*<sup>S10</sup>/pangolinAN cross
Virgin; *upd*-GAL4; *tub*-GAL80ts females were crossed with balanced; UAS-*arm*<sup>S10</sup>/CyO; UAS-*pan*(dTCF)ΔN/MKR males in 18°C. Progenies were collected every 2 days and sifted to 29°C incubator with 60% humidity for 5 days until dissection to turn on *arm*<sup>S10</sup> and *panΔN* expression.

### 2.2.3 LiCl treatment

#### 2.2.3.1 LiCl treatment in standard fly food

Stock 8M LiCl (Sigma) were added into standard cornmeal, molasses, yeast, and agar medium during regular preparation protocol. Final LiCl concentration used in the experiment are 30mM and 100mM as suggested by Sofola et al (Sofola et al. 2010). Equivalent volumes of vehicle (H<sub>2</sub>O) were supplemented to the medium to compensate for dilution.

#### 2.2.3.2. LiCl treatment in Nutri-Fly German Food “Sick Fly” Formulation

Nutri-Fly German Food “Sick Fly” Formulation (Genesee scientific), as a powered *Drosophila* media, is easier to precisely control LiCl concentration during preparation. Food was prepared according to the protocol provided by the manufacturer. Stock 8M LiCl was supplemented before dispensation.

### 2.3 DNA and RNA analysis

#### 2.3.1 Nucleic acid isolation

For whole fly analysis, flies were collected and homogenized in tissue lysis buffer directly. For adult ovaries and testes analysis, tissues were dissected in Grace’s Insect Media (Lonza) and then moved to tissue lysis buffer.

#### 2.3.1.1 DNA isolation from adult fly samples
DNeasy Blood and Tissue Kit (Qiagen) was used for DNA isolation according to the protocol provided by the manufacturer.

2.3.1.2 RNA isolation from adult fly samples

RNeasy Mini Kit (Qiagen) was used for RNA isolation according to the protocol provided by the manufacturer. However, QIAzol Lysis Reagent (Qiagen) and chloroform (Fisher Scientific) were used to substitute RLT lysis buffer as described in miRNeasy Mini Handbook (http://asmlab.org/public/files/miRNeasy-Mini-Kit-EN.pdf).

2.3.3 Polymerase chain reaction (PCR)

2.3.3.1 Traditional PCR and subsequent agarose gel electrophoresis

This assay was used to verify the specificity and validity of primers against housekeeping genes. PCR Reactions were performed using T100 Thermal Cycler (Bio-Rad) with cycle conditions: denaturation step at 95°C for 3 minutes, followed by 40 cycles of denaturation at 95°C for 30 seconds, primer annealing at 60°C for 30 seconds and extension 72°C for 1 minutes. Amplification was done using GoTaq Green Master Mix (Promega). All primer sequences are provided in Table 2.3.

DNA fragments amplified by PCR were then separated via 2% agarose gel electrophoresis. Gels were prepared by dissolving agarose powder (Fisher Scientific) in TAE buffer. To visualize DNA, EtBr (Fisher Bioreagent) was supplied at 0.5µg/mL. Electrophoresis was performed at 100V. DNA was detected using UV light and the size of DNA was determined using TriDye 100bp DNA Ladder (NEW ENGLAND BioLabs).
2.3.3.2 Quantitative real-time PCR (qPCR)

Expression level of *Wolbachia* gene *wsp* and the six Wg/Wnt responsive genes was analyzed by qPCR. All qPCR reactions were performed with 5ng DNA using 7900HT Sequence Detection System (Applied Biosystems) with cycle conditions: denaturation step at 95°C for 2 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds, primer annealing and extension at 60°C for 1 minutes. Amplifications were done using either PowerUp SYBR Green Master Mix (Thermo Fisher Scientific; for *nos>arm*\(^{S10}\), *nos>arm* RNAi and non-*melanogaster* species analysis) or SYBR Select Master Mix (Applied Biosystems; for all the other analyses). Reactions were run in triplicates in three independent experiments. Housekeeping gene *14-3-3* was used for *D. melanogaster*; *gapdh1* was used for *D. yakuba*; 28SrRNA was used for *D. teissieri*, *D. tropicalis*, *D. simulans*; as internal control. To control the variability during sample dilution, expression data were normalized to the mean of housekeeping gene. All primer sequences are provided in Table 2.3.

2.3.3.3 One step real-time reverse-transcription PCR (one step RT-PCR)

Total RNA extracted from the samples was reverse transcribed by SuperScript VILO Master Mix (Thermo Fisher Scientific) and then amplified by PowerUp SYBR Green Master Mix (Thermo Fisher Scientific). Reactions were performed with 5ng RNA using 7900HT Sequence Detection System (Applied Biosystems) with cycle condition: 50°C 5 minutes for reverse transcription, denaturation step at 95°C for 2 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds, primer annealing and extension at 60°C for 1 minutes. Samples were run in triplicates. To
control the variability during sample dilution, expression data were normalized to the mean of housekeeping gene, rpl32. All primer sequences are provided in Table 2.3.

2.4 Protein analysis

2.4.1 Protein isolation from adult flies

Fly samples were homogenized and incubated for 20 minutes in ELB buffer (150mM NaCl, 50mM Hepes pH7, 5mM EDTA, 0.1% NP-40) containing protease inhibitor, 1mM PMSF and 1mM DTT. Supernatant containing proteins was collected after centrifugation at 12000g for 10min.

2.4.2 Western blot analysis

Protein extracts, separated by SDS-PAGE and transferred onto nitrocellulose membrane according to manufacturer’s protocol (Bio-Rad), were blocked with condensed milk powder in TBS (10 mM Tris, pH 8.0, 150 mM NaCl) overnight in 4°C and then probed with primary antibodies diluted in TBST (TBS with 0.5% Tween 20) for an hour. After removal of antibodies, the membrane was washed three times with TBST and incubated in HRP conjugated secondary antibody for 30 minutes at room temperature. Detailed antibody dilutions and sources are listed in Table 2.4.

Subsequent to staining, membrane was washed in TBST for three times and developed with Western Lightening Plus-ECL (PerkinElmer) according to the manufacturer’s protocol.
2.5 Immunofluorescence microscopy

2.5.1. General immunostaining

Adult fly tissues were dissected in Grace’s Insect Media (Lonza) and fixed for 20 minutes in 4% paraformaldehyde (PFA, EM grade), 0.2% Triton X-100 dissolved in Grace’s Insect Media. Fix were removed by three washes in PBS containing 0.2% Triton X-100 (PBT) and then stored in PBT containing 0.2% bovine serum albumin (BSA) and 0.005% sodium azide (PBT/BSA) at 4°C.

Tissue was blocked in PBT/BSA containing 5% normal goat serum (PBANG) for 30 minutes before incubation with primary antibody diluted in PBANG. Details for antibody sources and dilutions are listed in Table 2.5. After primary antibody incubation either 2-4 hours in room temperature or overnight at 4°C, tissue was applied to three quick washes with PBT and three 40-minute washes with PBT/BSA. The tissue was further blocked with PBANG for 30 minutes. Subsequent to blocking, tissue was incubated with secondary antibody diluted in PBANG, nutating in the dark, room temperature. Followed by the removal of antibody after two hours staining, tissue was quickly washed three times with PBT and then applied to a 2 hour washing in PBT/BSA. Next, to label nuclei, tissue was incubated in 10µg/mL Hoechst diluted in PBT nutating at room temperature, dark. Following incubation, Hoechst was removed and tissue was quickly washed two times in PBT/BSA and two times in PBS. The tissue was then mounted in Prolong Gold (Life Technology) and the slide was sealed with nail polish after mounting media polymerized.
2.5.2. **In situ hybridization**

Protocol was adapted from (Moreira et al.).

Adult fly tissues were dissected in Grace’s Insect Media (Lonza) and fixed for 20 minutes in 4% paraformaldehyde (PFA, EM grade) dissolved in Grace’s Insect Media. Hybridization was performed at 37°C in 50% Formamide (v/v), 5x SSC, 250 mg/l Salmon sperm DNA, 0.5x Denhardt’s solution, 20mM Tris-HCl, and 0.1% SDS (w/v) diluted in DEPC treated water. After a 30 min blocking in hybridization buffer, tissue was incubated for 3 hours in 100ng oligonucleotide probe against *Wolbachia* 16SrRNA diluted in hybridization buffer. Probe sequences and sources was listed in Table 2.6. Tissue was then washed two times in a 1x SSC washing buffer containing 0.1% SDS and 20 mM Tris-HCl for 15 minutes at 56°C. And then two times in a 0.5x SSC washing buffer containing 0.1% SDS, 20 mM Tris-HCl and 10 µg/mL Hoechst for 15 minutes at 56°C. After the removal of washing buffer, tissue was quickly washed twice in PBT and then mounted in Prolong Gold (Life Technology). Slide was sealed with nail polish after mounting media polymerized.

**2.6 Image analysis of Wolbachia**

**2.6.1 Wolbachia quantification in hub cells**

Z stacks of representative images were analyzed for *Wolbachia* density in the hub and surrounding tissue using immuno-markers to label the hub and MatLab software. Manual masks were drawn around the hub and surrounding soma and germline to obtain measurements of the relative *Wolbachia* level between the two regions.
2.6.2 Wolbachia quantification in germline cells

Z stacks of representative images were analyzed for Wolbachia staining intensity relative to Hoechst staining intensity in stage 8 germline cells using MatLab software. Manual masks were drawn around the entire germline cells, excluding follicle cells, to obtain measurements of the intensity of the two stainings within the area.

2.7 GAL4-UAS system

In Drosophila model system, GAL4-UAS system is a commonly used yeast-derived tool to express genetic constructs (Brand and Perrimon 1993). GAL4, an 881 amino acid big protein, turns on transcription of downstream genes among binding to upstream activation sequence (UAS). Therefore, by fusing tissue specific promotors to GAL4 sequence, expression of UAS-fused particular genetic construct of interest could be precisely controlled in a tissue specific manner. Genetic construct could be either a wildtype gene sequence, mutated gene sequence or RNAi against specific gene.

This system can also be used to temporally control gene expression. GAL80, also a yeast derived protein, represses activation of GAL4 by interacting with the activation domain of GAL4. By introducing a temperature sensitive GAL80\textsuperscript{ts} construct to fly cross, GAL4 activity could be turned off at 18°C but reversibly turned on at 29°C or higher. Detailed molecular interactions are illustrated in Figure 2.1.

In Drosophila, the GAL4 drivers and the UAS element are maintained in separate fly lines. UAS element downstream genes become activated in the progeny when the two lines are mated together (Duffy, 2002). The generic fly crosses utilized in the
majority of the GAL4-UAS studies are demonstrated in Figure 2.2. Generic fly crosses utilized GAL80 construct are demonstrated in Figure 2.3.

2.8 Statistical Analysis

All statistical tests were performed by Microsoft Excel. Experiments were analyzed by two-tailed student t-test assumed with equal sample variance, unless otherwise indicated.
Figure 2.1 The GAL80-GAL4-UAS system

General scheme of the GAL80-GAL4-UAS system. A. At 18 °C, tubulin drives universal GAL80\textsuperscript{ts} expression, which binds and inactivates GAL4 protein driven by tissue specific promoter. B. At 29°C, GAL80\textsuperscript{ts} degrades allowing the GAL4 protein to bind to upstream activation sequence (UAS) and activate the transcription of gene of interest.
Figure 2.2 Generic GAL4-UAS cross scheme

General generic cross scheme for RNAi construct on the 3rd chromosome.

Semicolons separate the genetic markers and constructs on each chromosome (sex chromosomes, autosomes 2 and autosomes 3, from left to right). Curly O (CyO), Scutoid (Sco), TM6B, and MKRS are balancer chromosomes. A similar scheme was used if RNAi construct was on the 2nd chromosome.
Figure 2.3 Generic GAL80-GAL4-UAS cross scheme

A. Genetic cross theme for GAL80 regulated RNAi expression in germline cells.

tub: tubulin; nos: nanos; NGT:nanos-GAL4-tubulin. B. Genetic cross theme for
GAL80 regulated Pangolin dominant-negative and Armadillo^{S10} expression in hub
and polar cells. upd: unpaired.
Table 2.1: Sources for *Drosophila* species used for analysis

<table>
<thead>
<tr>
<th><em>Drosophila</em> Species</th>
<th>Wolbachia Strain</th>
<th>Source</th>
<th>Frydman Stock#</th>
<th>Stock Center #/#/Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>D. melanogaster</em></td>
<td>wMel</td>
<td>Frydman Lab</td>
<td>200</td>
<td></td>
</tr>
<tr>
<td><em>D. melanogaster</em></td>
<td>wMelCS</td>
<td>Sullivan Lab</td>
<td>201</td>
<td></td>
</tr>
<tr>
<td><em>D. melanogaster</em></td>
<td>wMelPop</td>
<td>Frydman Lab</td>
<td>482</td>
<td></td>
</tr>
<tr>
<td><em>D. simulans</em></td>
<td>wRi</td>
<td>San Diego Stock Center</td>
<td>42</td>
<td>14021-0251.169</td>
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<tr>
<td><em>D. simulans</em></td>
<td>wNo</td>
<td>San Diego Stock Center</td>
<td>33</td>
<td>14021-0251.198</td>
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<tr>
<td><em>D. sechellia</em></td>
<td>wSh</td>
<td>San Diego Stock Center</td>
<td>41</td>
<td>14021-0248.08</td>
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<tr>
<td><em>D. teissieri</em></td>
<td>wTei</td>
<td>San Diego Stock Center</td>
<td>37</td>
<td>14021-0257.00</td>
</tr>
<tr>
<td><em>D. yakuba</em></td>
<td>wYak</td>
<td>Virginie June2008</td>
<td>39</td>
<td></td>
</tr>
<tr>
<td><em>D. tropicalis</em></td>
<td>wWil</td>
<td>San Diego Stock Center</td>
<td>45</td>
<td>14030-0801.01</td>
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</table>
Table 2.2: Transgenic fly stocks used for analysis

*Wolbachia* strains originated from Frydman stocks #200 (W-), #201 (wMel), and #202 (wMelCS).

<table>
<thead>
<tr>
<th>Shorthand Name</th>
<th>Genotype</th>
<th>Frydman Stock #</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>upd</em>-GAL4 Driver bal on II</td>
<td>upd – GAL4 <code>CyO</code> +</td>
<td>224 W-225 wMel 226 wMelCS</td>
<td>Frydman Lab</td>
</tr>
<tr>
<td><em>nos</em>-GAL4 Driver</td>
<td>+ <code>NGT40 – GAL4 nos – GAL4</code> +</td>
<td>418 wMel 419 wMelCS</td>
<td>Frydman Lab</td>
</tr>
<tr>
<td>GAL4-GAL80 Driver</td>
<td>upd – GAL4 <code>tub – GAL80ts</code> +</td>
<td>430 wMel 431 wMelCS</td>
<td>Frydman Lab</td>
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<tr>
<td>tub-GAL80 Driver bal on III</td>
<td>+ <code>tub – GAL80ts</code> <code>TM2</code> +</td>
<td>477 W-487 wMel 488 wMelCS</td>
<td>Bloomington Stock Center BL#7108</td>
</tr>
<tr>
<td>Double Balancer</td>
<td>+ <code>CyO</code> <code>TM6B</code> + <code>Sco</code> <code>MKRS</code></td>
<td></td>
<td>McCall Lab/ Frydman Lab</td>
</tr>
<tr>
<td>UAS-Armadillo-o-RNAi</td>
<td>+ + <code>UAS – RNAi</code> + <code>UAS – RNAi</code></td>
<td>363</td>
<td>McCall Lab BL#31304</td>
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<tr>
<td>UAS-<em>arm</em>_{S10}</td>
<td>+ <code>UAS – arms10</code> + <code>UAS – arms10</code></td>
<td>382</td>
<td>Perrimon Lab</td>
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<tr>
<td>UAS-<em>pan</em>_{DN} bal on II</td>
<td><code>CyO</code> <code>UAS – pan – dTCFΔN</code> <code>TM6B</code> + <code>Sco</code> <code>MKRS</code></td>
<td>483</td>
<td>Bloomington Stock Center #4784</td>
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<tr>
<td>UAS-<em>pan</em>_{DN} bal on III</td>
<td><code>UAS – pan – dTCFΔN</code> <code>TM6B</code> <code>CyO</code> <code>MKRS</code></td>
<td>484</td>
<td>Bloomington Stock Center #4785</td>
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Table 2.3 PCR primers

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<tr>
<th>Gene</th>
<th>Product Size</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Exon Spanning</th>
</tr>
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<tr>
<td>Wolbachia surface protein (wsp)</td>
<td>~600</td>
<td>GTCCAATARSTGATGARGAAAC</td>
<td>CYGCACCAAYAGYRCTRTAAA</td>
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<td>14-3-3</td>
<td>139</td>
<td>CATGAACGATCTGCCACCAAC</td>
<td>CTCTCGCTCAATGATATCAAC</td>
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<tr>
<td>Ribosomal protein L32 (rpl32)</td>
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<td>ATGCTAAGCTGTCGCACAAATG</td>
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<td>28S rRNA</td>
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<td>Glyceraldehyde 3 phosphate dehydrogenase 1 (gapdh1)</td>
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<td>GACTACGGGTCTTCAAGG</td>
<td>CACCACATACTCGGCTCA</td>
<td></td>
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<td>Armadillo (arm)</td>
<td>101</td>
<td>GTGGACGATATGAAAGCAG</td>
<td>GTGGAGGAATCTCAATGCCC</td>
<td>No</td>
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<tr>
<td>Naked cuticle</td>
<td>75</td>
<td>ATGGCGGGTAACATTGTCAAA</td>
<td>GCATTCCTGGACTGAGAATTGT</td>
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<tr>
<td>Fratzled3 (fz3)</td>
<td>82</td>
<td>CGAGACGGTTAGGGGCGAG</td>
<td>TCCGTCAGTTAAGGCAACG</td>
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<tr>
<td>Distal-less (dll)</td>
<td>114</td>
<td>CGCTTCCAGCTACCAATAA</td>
<td>GTACTTGAGCGTCGGTCT</td>
<td>Yes</td>
</tr>
<tr>
<td>Ovo (svb)</td>
<td>67</td>
<td>CGCAGACCGATCTAGGTACG</td>
<td>GATAGTGACCTCCGCT</td>
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<tr>
<td>Decapentaplegic (dpp)</td>
<td>98</td>
<td>TGGCGACTTTTCAAGCATTTG</td>
<td>CAGCGGAATATGACCGGCAA</td>
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Table 2.4 Antibodies used for western blotting

<table>
<thead>
<tr>
<th>Name</th>
<th>dilution</th>
<th>host</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary antibodies</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Armadillo</td>
<td>1:100</td>
<td>Mouse</td>
<td>DSHB (N27A1)</td>
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<tr>
<td>Tubulin</td>
<td>1:10000</td>
<td>Mouse</td>
<td>DSHB (E7)</td>
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<tr>
<td>Shaggy</td>
<td>1:250</td>
<td>Mouse</td>
<td></td>
</tr>
<tr>
<td>phospho-Shaggy</td>
<td>1:250</td>
<td>Rabbit</td>
<td>Cell Signaling (9331)</td>
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<td>Secondary antibodies</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>anti-mouse, HRP conjugated</td>
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<td>Goat</td>
<td>PerkinElmer</td>
</tr>
<tr>
<td>anti-rabbit, HRP conjugated</td>
<td>1:10000</td>
<td>Goat</td>
<td>PerkinElmer</td>
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</table>
Table 2.5 Antibodies used for immunohistochemistry

<table>
<thead>
<tr>
<th>Name</th>
<th>dilution</th>
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<th>Source</th>
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</thead>
<tbody>
<tr>
<td>Primary antibodies</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hsp60</td>
<td>1:100</td>
<td>Mouse</td>
<td>Lk2, Sigma</td>
</tr>
<tr>
<td>Vasa</td>
<td>1:500</td>
<td>Rat</td>
<td>Paul Lasko</td>
</tr>
<tr>
<td>Vasa</td>
<td>1:1000</td>
<td>Rabbit</td>
<td>Ruth Lehmann</td>
</tr>
<tr>
<td>Lamin C28</td>
<td>1:20</td>
<td>Mouse</td>
<td>DSHB (LC28.26)</td>
</tr>
<tr>
<td>Armadillo</td>
<td>1:100</td>
<td>Rabbit</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>DE-Cadherin</td>
<td>1:50</td>
<td>Rat</td>
<td>DSHB (DCAD2)</td>
</tr>
<tr>
<td>DE-Cadherin</td>
<td>1:100</td>
<td>Rabbit</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>DN-Cadherin</td>
<td>1:20</td>
<td>Rat</td>
<td>DSHB (DN Ex #8)</td>
</tr>
<tr>
<td>Wingless</td>
<td>1:50</td>
<td>Mouse</td>
<td>DSHB (4D4)</td>
</tr>
<tr>
<td>Secondary antibodies</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>anti-mouse, alexa 546</td>
<td>1:500</td>
<td>goat</td>
<td>Life Technologies</td>
</tr>
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<td>anti-mouse, alexa 647</td>
<td>1:500</td>
<td>goat</td>
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</tr>
<tr>
<td>anti-rabbit, alexa 546</td>
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<td>goat</td>
<td>Life Technologies</td>
</tr>
<tr>
<td>anti-rabbit alexa 633</td>
<td>1:500</td>
<td>goat</td>
<td>Life Technologies</td>
</tr>
<tr>
<td>anti-rat, alexa 568</td>
<td>1:500</td>
<td>goat</td>
<td>Life Technologies</td>
</tr>
<tr>
<td>anti-rat, alexa 647</td>
<td>1:500</td>
<td>goat</td>
<td>Life Technologies</td>
</tr>
<tr>
<td>anti-mouse, alexa 488</td>
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<td>goat</td>
<td>Life Technologies</td>
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Table 2.6 *In situ* hybridization oligos

<table>
<thead>
<tr>
<th>Probe name</th>
<th>Sequence</th>
<th>Gene target</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wpan16S887 - Cy3</td>
<td>5'Cy3 - ATCTTGCGACCGTAGTCC - 3'</td>
<td>ribosomal RNA</td>
</tr>
<tr>
<td>Wpan16S450 - Cy3</td>
<td>5'Cy3 - CTTCTGTGAGTACCGTCATTAC - 3'</td>
<td>ribosomal RNA</td>
</tr>
</tbody>
</table>
CHAPTER 3 Canonical Wg/Wnt pathway regulates \textit{Wolbachia} intracellular density in \textit{Drosophila}

3.1 Introduction

Armadillo consist of 12 Arm repeats flanking by N- and C-termini sequence. It is known to be involved in two main cellular processes, adherens junctions and Wingless(Wg)/Wnt signaling pathway. In cadherin-based cell adhesion system, Armadillo binds to transmembrane protein Shotgun (DE-cad)/E-cadherin with the 12 Arm repeats and regulates actin filament assembly upon the calcium-dependent homophilic interactions between the extracellular domains of cadherin (Tepass et al. 1996). This process is also regulated by α-Catenin, which physically interacts with N-terminal domain of Armadillo, and adherens junction protein p120, which interacts with cadherin juxtamembrane domain (Anastasiadis and Reynolds 2000). Adherens junction activity is important for anchoring both germline and somatic stem cell to their niches in \textit{Drosophila} ovary (Song and Xie 2002).

Secondly, upon activation of Wg/Wnt signaling pathway, Armadillo becomes stabilized and is translocated into the nucleus to turn on target gene transcription. A key transcription factor, Pangolin, is activated by direct binding to 3-10 Arm repeat and removing of transcriptional co-repressor Groucho/TLE and Carboxyl-terminal binding protein (CtBP) (Brembeck, Rosário, and Birchmeier 2006). Heterodimerization of Armadillo and Pangolin is followed by the recruitment of nuclear factors contributing to transcriptional activation. These transcriptional co-activators bind to either N- or C- terminal activating domain, providing nuclear
anchoring (Legless, Pygopus), histone acetyltransferase (CBP/p300), and chromatin remodeling (Brahma/Brg1), histone methyltransferase (Trithorax, SET1) and transcriptional initiation and elongation (MED12, Hyrax) activities (Städeli, Hoffmans, and Basler 2006; Thompson 2004; Mosimann, Hausmann, and Basler 2006).

Previous research in our lab has demonstrated that, *Wolbachia* tropism in gonad was observed in *Drosophila* hub and somatic stem cell niches (Toomey and Frydman 2014). Several pathways are shared among these tissues, including JAK-STAT, Hedgehog signaling, E-cadherin mediated adherens junction and Wg/Wnt signaling. Among the screening of RNAi and overexpression constructs targeting the pathway, we identified Armadillo as an important factor on *Wolbachia* titers. Tissue specific expression of a constitutively active form of *armadillo* (*arm<sup>S10</sup>*) in male hub and female polar cells led to an increase in *Wolbachia* levels.

*Arm<sup>S10</sup>* is a constitutively active form of Armadillo which contains a 54 amino acid deletion in the N-terminal domain preventing it from being phosphorylated by Shaggy at S33, S37 and T41. *Arm<sup>S10</sup>* activity is independent of Wg/Wnt signal and endogenous Armadillo (Pai et al. 1997). On the other hand, it is also shown that RNAi against armadillo significantly down-regulated *Wolbachia* level in hub and polar cells (Ajit Kamath and Michelle Toomey, unpublished data). These results strongly suggested that Armadillo is potentially modulating *Wolbachia* intracellular levels in *Drosophila melanogaster*.
3.2 Armadillo affects *Wolbachia* level in germline

*Wolbachia* are maternally transmitted among generations. The levels of *Wolbachia* in the female germline are important for transmission to the next generation. Previous research in our lab have implicated the role of Wg/Wnt signaling in regulating *Wolbachia* levels in somatic cell types of *Drosophila* gonads. We hypothesized that similar intracellular pathways would affect *Wolbachia* levels in the germline.

To further determine whether *armadillo* could also affect *Wolbachia* in the female germline, we modulated its level in female germline by using *nanos*-GAL4-tubulin(NGT)-GAL4; *nanos(nos*)-GAL4 driver. *Nanos* expresses in the posterior pole of egg cells during embryogenesis. It is one of the two crucial maternal factors responsible for segmental pattern in early (stage 1-3) *Drosophila* embryos (Wang and Lehmann 1991). During oogenesis, *nanos* is required for egg chamber production. Expression is detected in nurse cells and oocytes after stage 5 (Wang, Dickinson, and Lehmann 1994). A *Drosophila* egg chamber is composed of 16 germ-line cells, including one oocyte and 15 nurse cells, and a thin layer of somatic follicle cells. Germline cells occupy large proportion of an egg chamber; therefore, the effects of tissue specific gene alternation are easy to observe and detect through immunostaining. Note that although the effects of follicle cells cannot be eliminated, qPCR analysis of entire ovary largely reflects the chromatin status of highly polypoid nurse cells.
3.2.1 Armadillo<sup>S10</sup> upregulates *Wolbachia* level in germline

By using the GAL4-UAS system, we expressed *arm<sup>S10</sup>* construct in the germline. For both *w*MelCS and *w*MelPop flies, *Wolbachia* levels in newly eclosed (NE) ovaries were quantified by qPCR of *Wolbachia* gene *wsp* and compared with sibling control fly ovaries. There was a 1.4-fold increase in *w*MelCS and 1.6-fold increase in *w*MelPop observed (Figure 3.1A-B; t-test: $P_{w\text{MelCS}}=0.0007$, $P_{w\text{MelPop}}=0.0225$). For *w*Mel, *Wolbachia* level remained the same between control and experimental groups in NE ovaries (Figure 3.1C; t-test: $P=0.1311$). However, significant differences were observed in 7 day old ovaries, in which we observed both higher expression levels of *wsp* gene (Figure 3.1D; t-test: $P=0.0219$) and higher ratio of *Wolbachia*(Hsp60)/DAPI immunostaining in germline cells (Figure 3.1E-F; t-test: $P=0.00048$). We proposed that levels of *Wolbachia* in NE *w*Mel ovaries is probably too low, so the effect of Arm<sup>S10</sup> could not be observed at a detectable level. These results suggested that expression of a constitutively active form of Armadillo is sufficient to drive higher levels of *Wolbachia* in the female germline.

3.2.2 RNAi against Armadillo downregulates *Wolbachia* level in germline

To further confirm *armadillo*’s role in *Wolbachia* accumulation, we carried out a knockdown experiment by expressing RNAi against armadillo in germline (Bloomington #31304, VALIUM1, long hairpin RNA). Since *armadillo* knockdown in early embryos affect normal patterning within embryonic segments and is partially embryonic lethal (Riggleman, Schedl, and Wieschaus 1990a), we carried out fly crosses using a *tubulin*-GAL80 construct as discussed in Materials and Methods. qPCR
of 7-day old wMel ovaries demonstrated that RNAi against armadillo significantly reduced Wolbachia levels in the tissue. Wolbachia level had a 40% reduction in NE ovaries (Figure 3.2A, P=0.044) and 25% reduction in 7d old ovaries (Figure 3.2B, P=0.026). Similar experiments in wMelCS and wMelPop are still in progress.

Together, these results emphasize the importance of Armadillo in Wolbachia accumulation. It is able to promote Wolbachia tropism upon overexpression and decrease Wolbachia level upon RNAi mediated knockdown. This observation along with previous works done by Michelle Toomey and Ajit Kamath in the hub and the polar cells demonstrate the importance of Armadillo in regulating Wolbachia levels in the Drosophila gonads.

3.3 Modulating the activity of other genes in the Wg/Wnt pathway also affects Wolbachia density

It is hypothesized that Armadillo regulates Wolbachia level in hub and polar cells through one of the two Armadillo mediated processes. Adherens junction or Wg/Wnt signaling pathway. However, previous work in our lab points out that RNAi mediated Shotgun (DE-cadherin) knockdown did not have an effect on Wolbachia tropism in the hub (Toomey 2014). These results weaken the possible relationship between cadherin-based cell adhesion and Wolbachia, but on the other hand, implies transcriptional Wg/Wnt singling as an import mechanism regulating Wolbachia level in our model.

Next, to verify whether Armadillo is regulated by canonical Wg/Wnt signaling, multiple RNAi against molecules within Wg/Wnt signaling were crossed to the
unpaired(upd)-GAL4 driver to knockdown gene expression in hub and polar cells. 

unpaired is highly expressed in male hub cells to activate JAK-STAT signaling in adjacent stem cells (de Cuevas and Matunis 2011) and also in female polar cells to affect follicle cell differentiation (McGregor, Xi, and Harrison 2002). Wolbachia tropism for these two cell types was discovered previously in our lab (Ajit Kamath, unpublished data and (Toomey et al. 2013)). They are considered as good models for Wolbachia investigation.

Among all the molecules in Wg/Wnt signaling pathway, we carried out knockdown with RNAi against frizzled (fz), shaggy(sgg) and disheveled(dsh). These three factors are upstream to armadillo in the canonical Wg/Wnt signaling pathway. Wolbachia density in hub and polar cells were quantified by the staining intensity in the tissue relative to surrounding area as discussed in materials and methods. Results showed that tissue specific fz knockdown driven by upd-GAL4 driver did not affect Wolbachia level in 7-day-old male hub cells (Figure 3.3A-B; t-test: P=0.214), which was possibly due to the redundant nature of the receptor (Alexandre, Baena-Lopez, and Vincent 2014). However, altering two other Wg/Wnt factors showed expected results. RNAi against destruction protein, sgg, increased Wolbachia level; whereas RNAi against Wg cytosolic effector, dsh, decreased Wolbachia in hub and polar cells comparing to sibling control (Yu Ouyoung, unpublished data). These results strengthen the idea that Wolbachia is affected by canonical Wg/Wnt signaling in Drosophila gonad.
3.4 LiCl treatment leads to an increase in *Wolbachia* intracellular levels in *Drosophila*

In addition to using genetic tools, we also manipulated the Wg/Wnt signaling pathway by chemicals.

Lithium ion is a common Shaggy/GSK-3 inhibitor which prevents Armadillo degradation and thus leads to an upregulation of Wg/Wnt signaling pathway. This chemical reagent inhibits Shaggy activity by competing with Magnesium ions (Ryves and Harwood 2001) and by activating serine/threonine kinase AKT-1 and phosphorylating Shaggy at Ser$^9$ and Ser$^{21}$ (Chalecka-Franszdek and Chuang 1999). Shaggy degradation stabilizes Armadillo and leads to overall up-regulation of Wg/Wnt signaling pathway.

Other than Shaggy inhibition, Li$^+$ is reported to up-regulate mammalian cell autophagy, inhibiting inositol monophosphatase (Sarkar et al. 2005). However, LiCl is widely used in *Drosophila* model as a reliable Shaggy inhibitor (Pai et al. 1997; Jope 2003; Sofola et al. 2010).

### 3.4.1 Preliminary experiments with standard fly food determine that Li$^+$ treatment leads to an increased *Wolbachia* density in whole flies

To determine an optimal concentration of Li$^+$, we conducted preliminary experiments with *D.mel* infected with *wMel*. We collected 0-2 days old flies and aged them in standard fly food (as described in Materials and Methods) supplemented with 0mM, 30mM and 100mM Lithium Chloride (LiCl) respectively for 5 days. qPCR for *wsp* gene in whole flies showed elevated *Wolbachia* level in *wMel* strain correlating
with Li\(^+\) concentration. Under 100mM Li\(^+\) treatment, 2-fold upregulation was observed in males, and 2.5-fold was shown in females (Figure 3.4A). But no trend was observed in wMelCS (Figure 3.4B) or wMelPop (Figure 3.4C).

wMelCS-like Wolbachia strains, wMelCS and wMelPop, tend to target tissues at a significantly higher frequency and density than wMel-like strains, wMel, (Toomey and Frydman 2014). We propose that Wolbachia levels in 5-7 days old wMelCS and wMelPop have already reached a very high level, and therefore further increases could be modest and undetectable by qPCRs. However, in situ staining of wMelCS and wMelPop ovary both showed increased Wolbachia level in early stage egg chamber. In stage 5 egg chamber, Wolbachia usually surrounds oocyte in a “crescent moon shape”; but under LiCl treatment, Wolbachia level goes up and eventually encircle the entire oocyte. (Figure 3.4E)

From these preliminary results, we observed the trend of Li\(^+\) elevating Wolbachia. However, during food preparation, we found that normal fly food was sticky, which made it hard to precisely control Li\(^+\) concentration. Also, Li\(^+\) might not distribute unevenly. Thus, we want to repeat this experiment in a medium that is more liquid during preparation process.

3.4.2 LiCl treatment in Nutri-Fly German Food shows elevated Wolbachia level in whole wMel ovaries and stage 8 germline cells

In order to precisely control Li\(^+\) concentration in the experiment, we shifted to commercial Nutri-Fly German Food (Nutri-Fly GF, as described in Materials and Methods) rather than standard fly food used in trial experiments. After a 5 days
treatment of Li⁺, levels of Wolbachia in whole flies infected with wMel were significantly elevated. We observed a 1.33-fold increase in males and 1.51-fold increase in females as shown in Figure 3.4D (t-test: P_{males}=0.0179, P_{females}=0.0469). To corroborate these results, we immunostained ovaries with antibodies against Wolbachia Hsp60 and quantified stage 8 egg chambers’ germlines Wolbachia level using MatLab. A 2.7-fold increase in Wolbachia after LiCl treatment was observed. (Figure 3.4F-G; t-test: P=1.07047×10⁻¹⁰).

Together, these results demonstrate that by manipulating the Wg/Wnt pathway in flies using drugs, we can modulate the intracellular Wolbachia density.

3.4.3. LiCl alters Wolbachia level through Armadillo

Theoretically, Li⁺ ions compete with Magnesium ions and activate AKT-1 kinase to promote Shaggy phosphorylation and eventual degradation. It is reasonable to assume that effects of Li⁺ might not be limited to canonical Wg/Wnt signaling pathway. For example, AKT-1 is reported to regulate multiple biological pathways including, but not limited to, promoting cell growth/size (Potter, Pedraza, and Xu 2002), survival (Scanga et al. 2000), glucose metabolism (Scanga et al. 2000) and adaptive response to amino acid starvation (Wei and Lilly 2014). Considering lithium’s role as a Shaggy/GSK-3 inhibitor, it can possibly promote AP-1 dependent neuronal development (Franciscovich et al. 2008), affect Notch signaling pathway (Jordan et al. 2006) and even inhibit autophagy (Érdi et al. 2012).

In our model, we observed Li⁺ inhibiting oogenesis. Female flies have visually smaller ovaries lacking late stage egg chambers after treatment. The number of eggs
laid is also much less than that in control flies. Additionally, if larvae are allowed to develop on Li\textsuperscript{+} food, they tend to be L1 larval lethal which is expected as the Wnt/Wg signaling is extremely important for early larval development. These observations, together with the literature, suggested that Li\textsuperscript{+} might have multiple other affects toward other cascades rather than solely affecting canonical Wg/Wnt signaling pathway. Thus, to answer whether Li\textsuperscript{+}'s effect toward *Wolbachia* level is through the canonical Wg/Wnt signaling, we designed an experiment to modulate multiple genes in the pathway to study the effects of their interaction on *Wolbachia*.

Epistasis refers to a form of gene interaction, in which one gene masks or modifies the phenotypic effect due to the change in expression of another (Michels 2002). To determine the specificity of Li\textsuperscript{+}'s effects previously observed in whole flies and in ovaries, we treated flies expressing germline specific arm\textsuperscript{S10} construct with Li\textsuperscript{+} and quantified the effects of both these manipulations using both qPCR and immunostaining. As mentioned previously, ovary size shrinks after Li\textsuperscript{+} treatment. So for ovary DNA extraction and subsequent q-PCR analysis, ovary samples without Li\textsuperscript{+} was manually cut, removing late stage egg chambers to mimic the ovary size in samples with Li\textsuperscript{+} and to eliminate stage-dependent effects.

If the effect of Li\textsuperscript{+} on *Wolbachia* levels is independent of Arm\textsuperscript{S10} (canonical Wg/Wnt signaling), the effects of both the treatments on *Wolbachia* would be additive. Whereas if the Li\textsuperscript{+} and Arm\textsuperscript{S10} act on *Wolbachia* via the same pathway (canonical Wg/Wnt signaling), the combined effect of both these treatments would not be more than each of these treatments alone (Figure 3.5A-B). Expression of Arm\textsuperscript{S10} or
treatment with Li\(^+\) by themselves increase *Wolbachia* levels in whole ovaries (as expected). However, upon a combined treatment, there was no further upregulation of *Wolbachia* level (Figure 3.5C-E) (t-test: \(P_{qPCR}=0.3504\), \(P_{IHC}=0.1244\)). This indicates that Li\(^+\)’s effect on *Wolbachia* is upstream of Armadillo. We corroborated the results by immunostaining and image quantification. Together, all the above results suggest that the increased *Wolbachia* level observed in whole flies and ovaries after Li\(^+\) treatment was through canonical Wg/Wnt signaling pathway.

### 3.5 Armadillo does not alter Wolbachia through interaction with Pangolin

As we have previously demonstrated, the canonical Wg/Wnt signaling seems to be important in *Wolbachia* intracellular accumulation. Therefore, we next want to answer what factors downstream to Armadillo affect *Wolbachia* levels. In the accepted canonical Wg/Wnt signaling model, Armadillo is translocated to the nucleus and heterodimerizes with Pangolin to activate the expression of Wg/Wnt pathway target genes (Schweizer, Nellen, and Basler 2003). Based on our previous observations, it seems that certain downstream genes activated by this interaction might be affecting *Wolbachia* density.

To address this question, we first wanted to determine whether the Armadillo-Pangolin interaction is important for *Wolbachia* accumulation. We introduced a dominant negative variant of the Pangolin protein (UAS-pan-dTCF\(\Delta N\), see Materials and Methods) to turn off transcriptional Wg/Wnt pathway. This dominant negative mutation construct has a truncation on the N-terminal, which deletes its Armadillo binding domain. Without Arm, mutated Pangolin serves as a
transcription repressor. \textit{pan\textDelta N} was shown to work in \textit{Drosophila} wings under Act88F-GAL4 (Morris et al. 2008) and MS1096-GAL4 driver (Shukla et al. 2014). Here we carried out another epistasis experiment. If Armadillo’s effect is independent of Pangolin, dominant-negative construct would not show any effect (Figure 3.6A). Whereas if Armadillo affects \textit{Wolbachia} via Pangolin, the dominant-negative construct would reduce \textit{Wolbachia} level (Figure 3.6B). Using GAL4-UAS system, we expressed the construct in wMel and wMelCS hub and polar cells by upd-GAL4 driver and quantified fold change of \textit{Wolbachia} density in hub cells relative to surrounding area by image analysis. It was previously described in our lab that \textit{pan\textDelta N} alone is not able to affect \textit{Wolbachia} level in hub/polar cells (Ajit Kamath, unpublished data). One explanation for this could be that, in native tissue, the levels of Wg/Wnt signaling might be low and expressing the \textit{pan\textDelta N} construct might not be able to show a detectable decrease.

Upon expression of \textit{pan\textDelta N} by itself did not have any effect on \textit{Wolbachia} levels as reported previously (t-test: \(P_{\text{wMel}}=0.057, P_{\text{wMelCS}}=0.254\)). Whereas \textit{Arm}\textsuperscript{S10} expression increased \textit{Wolbachia} level by 8.4-fold in wMel (Figure 3.6C and E; t-test: \(P=0.0323\)) and 3.5-fold increase in wMelCS (Figure 3.6D and F; t-test: \(P=0.0002\)). However, upon the expression of both construct together, there was no significant difference observed comparing to the \textit{Arm}\textsuperscript{S10} expressing group (t-test: \(P_{\text{wMel}}=0.405, P_{\text{wMelCS}}=0.243\)). Similar trend was also observed in polar cells (Ajit Kamath, unpublished data).

Together, these results imply that upregulation of \textit{Wolbachia} caused by \textit{arm}\textsuperscript{S10} is
independent from the conventional transcriptional activation of Wg/Wnt pathway target genes.
Figure 3.1 ArmadilloS10 expression in germline cells results increases Wolbachia level

A-C. Level of Wolbachia quantified in NE ovaries by the expression level of Wolbachia gene wsp in control (nos-GAL4/+>CYO/+ and OE (nos-GAL4/+>UAS-armS10/+ fly ovaries infected with wMelCS (A, 4 biological replicates, each has 5-10 ovaries), wMelPop (B, 4 biological replicates, each has 5-10 ovaries) and wMel (C, 3 biological replicates, each has 5-10 ovaries). D. Level of Wolbachia quantified by the expression level of Wolbachia gene wsp in control and OE 7d old fly ovaries infected with wMel, 7 biological replicates, each has 5-10 ovaries. E. Representative images for Wolbachia staining in control (E’) and OE (E’’) stage 8 egg chamber infected with wMel. [green: Wolbachia(Hsp60); blue: DAPI]. F. Quantification for Wolbachia/DAPI staining intensity in germline was done by MatLab as defined in Materials and Methods. Higher Wolbachia level in armadillo OE flies was observed in all three Wolbachia infected strains. (*P<0.05, ***P<0.001).
**A** Wolbachia level in NE wMelCS infected ovaries with and without arm<sup>510</sup> expression

**B** Wolbachia level in NE wMelPop infected ovaries with and without arm<sup>510</sup> expression

**C** Wolbachia level in NE wMel infected ovaries with and without arm<sup>510</sup> expression

**D** Wolbachia level in 7d old wMel infected ovaries with and without arm<sup>510</sup> expression

**E′** nos>

**E″** nos>arm<sup>510</sup>

**F** Relative intensity of Wolbachia staining in wMel infected ovaries
Figure 3.2 RNAi mediated knockdown of Armadillo in germline cells results in reduced *Wolbachia* level

A-B. Level of *Wolbachia* quantified by the expression level of *Wolbachia* gene *wsp* in control (*nos-GAL4/+*>MKRS/+) and OE (*nos-GAL4/+*>UAS-armRNAi/+) fly ovaries infected with wMel in newly eclosed (A, 2 biological replicates, each has 5-10 ovaries) and 7d old (B, 3 biological replicates, each has 5-10 ovaries) ovaries. (*P<0.05*).

![Graph A](image1.png)  ![Graph B](image2.png)
Figure 3.3 RNAi mediated Frizzled knockdown in hub cells does not affect *Wolbachia* level

A. Representative images for *Wolbachia* staining in control (upd-GAL4/+>MKRS/) (A’) and fz KD (upd-GAL4>fz RNAi/) (A’’) hub. 22 testes were analyzed in each group. [green: *Wolbachia*(Hsp60); red: hub marker(Armadillo); blue: DAPI]. B. Quantification for *Wolbachia* pixel density in hub was done by MatLab as defined in Materials and Methods. Similar *Wolbachia* level in control and fz KD flies was observed.
Figure 3.4 *Wolbachia* level in *w*Mel infected whole fly is significantly increased after LiCl treatment.

A-C. Level of *Wolbachia* in whole flies infected with *w*Mel (A), *w*MelCS (B) and *w*MelPop (C) after 0mM, 30mM and 100mM LiCl treatment in standard fly food for 5 days. Experiment has only been done once with 3-5 flies. D. Level of *Wolbachia* in whole flies after 0mM and 100mM LiCl treatment in Nutri-Fly German Food for 5 days. *Wolbachia* is quantified by the expression level of *Wolbachia* gene *wsp*. Experiment includes 6 biological replicates, each has 5-10 flies. E-F. Representative images for *Wolbachia* staining in stage 5 (E) and stage 8 (F) control (E’ and F’) and LiCl treated (E’’ and F’’) egg chambers after LiCl treatment [green: *Wolbachia*(Hsp60); blue: DAPI]. G. Quantification for *Wolbachia*/DAPI staining intensity in germline was done by MatLab as defined in Materials and Methods. 19 egg chambers were analyzed in each group. *Wolbachia* level in whole fly and in germline are significantly increased after LiCl treatment. (*P*<0.05, ***P*<0.001)
Figure 3.5 Elevation of *Wolbachia* was masked by *arm*<sup>Sl10</sup> and could not be further upregulated in germline after LiCl treatment.

A-B. Expected *Wolbachia* level when Li<sup>+</sup> affects *Wolbachia* through a pathway independent from Armadillo (A) or through Armadillo (canonical Wg/Wnt pathway) (B). C. Level of *Wolbachia* in control (nos-GAL4/+/CYO/+) and OE (nos-GAL4/+/UAS-arm<sup>Sl10</sup>/+) wMel fly ovaries with and without LiCl treatment, quantified by the expression level of *Wolbachia* gene wsp. Ovaries collected from 0mM groups are manually cut and adjusted to mimic the the size of treated ovaries. 7 biological replicates are carried out, each has 5-10 ovaries. D. Representative images for *Wolbachia* staining in stage 8 egg chambers without (D', D'') and with (D'', D''') germline *arm*<sup>Sl10</sup> expression; without (D', D'') and with (D'', D''') LiCl treatment. [green: *Wolbachia*(Hsp60); blue: DAPI]. E. Quantification for *Wolbachia*/DAPI staining intensity in germline was done by MatLab as defined in Materials and Methods. 19 ovaries were analyzed in each group. *Wolbachia* levels in *arm*<sup>Sl10</sup> overexpressing ovaries and germline cells were not further elevated after 100mM LiCl treatment. (*P<0.05, **P<0.005, ***P<0.001; all compared to nos>, 0mM LiCl group).
Figure 3.6 Armadillo increases *Wolbachia* level through a pathway independent from Pangolin

A-B. Expected *Wolbachia* level when Armadillo affects *Wolbachia* through a pathway independent from Pangolin (A) or through Pangolin (transcriptional Wg/Wnt pathway) (B). C-D. Representative images for *Wolbachia* staining in control (C’ and D’, *upd-GAL4/+* MKRS/+), *arm<sup>S10</sup>* expressing (C’’ and D’’), *upd-GAL4/ arm<sup>S10</sup>/+MKRS/+; ), *panΔN* expressing (C’’’ and D’’’), *upd-GAL4/+* > *panΔN/+*), and *arm<sup>S10</sup> panΔN* co-expressing (C’’’’ and D’’’’), *upd-GAL4/ arm<sup>S10</sup>/ panΔN/+*) hub cells infected with wMel (C) and wMelCS (D). E-F. Quantification for *Wolbachia/DAPI* staining intensity in germline infected with wMel (E, 15 testes were analyzed in each genetic background)) and wMelCS (F, 20 testes were analyzed in genetic background) was done by MatLab as defined in Materials and Methods. Significant differences were observed between group a and group b, but not within each group.
A Expected *Wolbachia* level when Armadillo's effect is independent from Pangolin

B Expected *Wolbachia* level when Armadillo acts through Pangolin

E Level of *Wolbachia* in wMel infected hub

F Level of *Wolbachia* in wMelCS infected hub
CHAPTER 4 Summary and Future Perspectives

In this thesis, we used genetic and chemical approaches to alter canonical Wg/Wnt pathway and successfully demonstrated that in vivo Wolbachia levels are regulated in Drosophila melanogaster through this well-known signaling cascade.

A positive interaction between Armadillo and Wolbachia in germline cells was first identified. The results correspond to previous findings in hub and polar cell system, emphasizing the existence of a conserved mechanism across tissues. Moreover, RNAi mediated knockdown of genes in canonical Wg/Wnt pathway and drug (LiCl) mediated Shaggy inhibition both resulted in drastic alteration in intracellular Wolbachia level; LiCl’s effect toward Wolbachia proved to act directly via Armadillo. However, even with multiple hits on canonical Wg/Wnt cascade, the conventional nuclear receptor of Armadillo, Pangolin, seemed to not affect Wolbachia intracellular density.

In the fruit fly model, Wg/Wnt pathway has been studied for more than 35 years. It has been shown to control embryonic development, adult tissue homeostasis, cell renewal and regeneration. However, this is the first report demonstrating that the host Wg/Wnt affects the accumulation of an intracellular bacteria.

4.1 Wolbachia accumulation is regulated under an evolutionarily conserved pathway

Upon germline specific Armadillo$^{10}$ expression, Wolbachia level in infected germline significantly went up (both in image analysis and ovarian DNA analysis, Figure 3.1). Whereas RNAi against armadillo generated completely opposite outcomes...
(ovarian DNA analysis, Figure 3.2). The molecular pathway underlying the phenomenon was further identified as the canonical Wg/Wnt pathway.

By pointing out the conservation of the cascade in germline, hub and polar cells, we highlighted the possibility to have the same mechanism controlling Wolbachia accumulation among different tissue and in different species.

4.2 Small molecule inhibitors as potential methods to affect disease transmission

Wolbachia are considered as a potential biological tool to prevent arbovirus and malaria transmission. It offers a potential for eliminating a wide variety of devastating human diseases. In our work, we showed that Li\(^+\) as a small molecule which can activate Armadillo and increase Wolbachia titers in Drosophila. It is able to consistently alter in vivo Wolbachia level through Wg/Wnt pathway (Figure 3.4 and Figure 3.5). Our discoveries provide an important pandemic implication showing possibility to control disease transmission by using simple chemicals.

There are a variety of small molecules reported as Wg/Wnt signaling pathway regulators. For example, SB-216713(Shaggy/GSK3 inhibitor) (Coghlan et al.), 2-amino-4-[3,4-(methylenedioxy)benzylamino]-6-(3-methoxyphenyl)pyrimidine (modified pyrimidine)(Kuncwetch et al. 2013), heteroarylpyrimidines (Gilbert et al. 2010), are all reported small molecule agonists which can modulate Wg/Wnt pathway in whole animals.

By showing the importance of the evolutionarily conserved Wg/Wnt pathway in Wolbachia accumulation, we can expect these molecules being able to affect Wolbachia density, which is directly proportional to disease blocking efficiency. The
ultimate goal of investigating small chemicals is to develop a biological based new tool that is cheaper, more environmental friendly and nonetheless more powerful and efficient.

4.3 Armadillo affects Wolbachia level through a novel pathway

Pangolin has been considered as a canonical Armadillo downstream nuclear effector, which turns on multiple downstream genes in various tissues. However, our work surprisingly demonstrated that Armadillo is not affecting Wolbachia through the Pangolin-dependent transcriptional pathway (Figure 3.6). Expression of pangolin\textsuperscript{ΔN} could not decrease the high Wolbachia titers observed in armadillo\textsuperscript{St10} expressing hub cells.

Recently, new transcription factors downstream of β-catenin/Armadillo are being identified in many model systems (Valenta, Hausmann, and Basler 2012). The relationship between TCF and β-catenin is not monogamous. These nuclear receptors refer to as “TCF/LEF-independent β-catenin mediated transcription factors”, including androgen receptor (AR) (Pawlowski et al. 2002), liver receptor homologue 1(LRH1) (Wagner et al. 2010), hypoxia inducible factor 1α (HIF1α) (Kaidi, Williams, and Paraskeva 2007), forkhead box protein O (FOXO), sex determining region Y-box 17 (SOX17)(Archbold et al. 2012), octamer-binding transcription factor 4 (OCT-4)(Kelly et al. 2011) and p50 subunit of nuclear factor kappa-light-chain-enhancer of activated B cells (NF -κB)(Kim et al. 2010). Most of the them have been identified in cancer and stem cell model. But LRH1 and FOXO1 are also found to be cooperating with TCF in C. elegans and are considered to be evolutionarily conserved cross species. LRH1
promotes cell proliferation in multiple cancer cells and is involved in cell fate decision in *C. elegans* somatic gonad precursor cells (Asahina et al. 2006).

On the other hand, under hypoxic condition, HIF1α and FOXO both have been shown to interact with β-catenin to turn on downstream gene transcription. β-catenin/HIF1α association is identified only in cancer and stem cells, whereas FOXO has been shown to be important in *C. elegans* dauer formation and life span (Essers et al. 2005).

Co-activators or co-repressors of Pangolin, such as Legless and Pygopus (Hoffmans, Städeli, and Basler 2005), work inside the nucleus with Armadillo. Furthermore, there is also the possibility of having other unidentified transcription factors or pathways downstream of Armadillo. A deeper understanding to Armadillo, using methods such as a two-hybrid screening for protein-protein/protein-DNA interaction, is definitely required to fully understand the mechanism of *Wolbachia* accumulation *in vivo*.

Cytosolically, Armadillo is known to participate in adherens junction as well. RNAi mediated knockdown against *Drosophila* E-Cadherin, in hub was previously found to have no effect toward *Wolbachia* level (Michelle Toomey, unpublished data). However, we should also carry out a genetic knockdown against other cofactors (α-catenin, and p120-catenin) (Harris 2012) and N-Cadherin to avoid the possibility of redundancy and to completely eliminate adherens junction proteins from the story.

It is also possible that the *pangolinΔN* construct we used does not work in our hub and polar cell model. To rule out this possibility, we should carry out experiments
using other pangolin mutant or RNAi constructs. Wg/Wnt reporters can also be considered as a way to directly confirm the existence of Pangolin-dependent transcription.

4.4 Transcriptional profiling is required to identify more downstream effectors

Wolbachia-host association is a complicated interaction. Being a mutualistic symbiont, Wolbachia have to maintain certain intracellular levels to avoid killing the host but at the same time being able to survive and to transmit enough numbers to the next generation. Multiple mechanisms are definitely required to ensure their viability inside host cell. Proteolysis, autophagy and mTOR signaling pathways are expected to be affected in infected species. These pathways are considered as the potential downstream targets of Wg/Wnt signaling,

Molecular processes such as proteolysis could be required for Wolbachia to obtain energy from host amino acids. However, it also widely hypothesized that this pathway reduces Wolbachia accumulation in certain conditions. Direction of the mechanisms and existence of feedback loops remains largely unknown in the field.

One important future analysis for this field would be the transcriptional profiling of Arm$^{S10}$ expressing cells. Hubs and polar cells are the best model to carry out this experiment due to natural tissue tropism. However, these two cell types are hard to isolate from gonads. Our work pointed out the possibility of carrying out these analyses in the germline as well.
Appendix 1

Immunostaining of Wingless in 7-day old Drosophila gonads

Wg/Wnt pathway is activated upon ligand (members of Wg/Wnt family protein) binding to membrane receptors (Fz/Arr). By far there are seven Wg/Wnt family members identified in Drosophila (Wg, Dwnt-2, Dwnt-4, Dwnt-5, Dwnt-6, Dwnt-8, Dwnt-D, Dwnt-10), but most of the understanding toward Wg/Wnt cascade focus on Wg. To confirm whether the pathway is activated in hub cells, polar cells, and somatic stem cells (SSCs), where natural Wolbachia tropism are observed, we therefore immunostained 7d old Drosophila gonads with antibody against Wg.

Staining in male testes showed multiple positive Wg staining on 6 days old hub cell membrane (Figure A1.1C) and in SSCs (Figure A1.1D), both in Wolbachia infected and uninfected groups. But since the signals are not very clean, we are unable to quantify and compare between the two infection status. We also observed staining in 1.5 days old ovaries supplied with yeast paste, Wg signal was not observed in polar cells (Figure A1.1A) nor SSCs (Figure A1.1B).

The Wg antibody used in this experiment is not ideal for Drosophila gonad, especially in ovaries. I strongly recommend Wg/Wnt reporter assay as an alternative to determine the activation of Wg/Wnt signaling pathway in vivo.
Figure A1.1 Wingless expression is observed in hub cell membranes and somatic stem cells but not in ovaries

A. Representative images for Wingless staining in 1.5 days old mcd8-GFP polar cells uninfected (A’) or infected with wMelCS (A’’). B. Representative images for Wingless staining in mcd8-GFP female somatic stem cells uninfected (B’) or infected with wMelCS (B’’) [red: Wg; green: polar cells (GFP); blue: DAPI]. C. Representative images for Wingless staining in 6 day old polar cells uninfected (C’) or infected with wMel (C’’). D. Representative images for Wingless staining in male somatic stem cells uninfected (D’) or infected with wMel (D’’) [white: germline (Vasa); green: Wg; red: hub (Arm) blue: DAPI]. Females flies were supplied with yeast paste. Positive Wg staining was observed in male tissue but not very clean. No Wg signal was observed in ovaries.
Appendix 2

Immunostaining of autophagy proteins in 7-day old *Drosophila* testes

Autophagy is reported to control intracellular bacteria titers in multiple species (Huang and Brumell 2014). Serbus et al (Serbus et al. 2015) proposed that autophagy increases *Wolbachia* level in *Drosophila*; however, Voronin et al (Voronin et al. 2012) found the opposite in *C. elegans*. The crosstalk between *Wolbachia* and autophagy remains largely unknown. To clarify this question, I first carried out an immunostaining against autophagy proteins in male testes.

Autophagy related protein 9/Atg8 is an ubiquitin-like protein required for the formation of autophagosome membranes. On the other hand, Refractory to sigma P/Ref(2)P is a intracellular receptor which selectively binds to ubiquitinated proteins and targets them for degradation. High level of Atg8 usually reflects high autophagic flux, whereas high Ref2P implicates the opposite phenomenon (Mauvezin et al. 2014).

We carried out the staining in 7d old *Drosophila* testes infected with *w*Mel, *w*MelCS and *w*MelPop respectively. Results showed very high Reft(2)P signal in W-cyst cells (Figure A2.1B). However, none of the infected species were observed with this staining pattern but have relatively low Reft(2)P level in cyst cells (Figure A2.1C-D). Interestingly, in testes infected with *w*MelCS (Figure A2.1D), we found Ref(2)P signal only located within the gap between germline cysts.
Interesting phenomenon was also observed in Atg8 channel. Atg8 signal in both uninfected (Figure A2.1B) and infected (Figure A2.1C-D) testes showed a concentration gradient but in opposite direction. Atg8 level was observed high at the tip of uninfected testes but low at the tip of the two infected testes.

Together, these observations indicate that *Wolbachia* is positively related with autophagy level in testes germline cells and negatively related with autophagy in stem cell niches. Significantly different distribution pattern of autophagy proteins in infected and uninfected tissues highlights the importance of this process and emphasizes the needs for future investigation.
Figure A2.1 Autophagy proteins Atg8 and Ref(2)P have different expression patterns in *Wolbachia* infected and uninfected testes

Immunostainings were carried out in 7d old testes. A. Diagram of the *Drosophila* testis. B-D. Representative images for autophagy protein staining in testes uninfected (B) or infected with *wMel* (C), or *wMelCS* (D) [red: Atg8; white: *Wolbachia* (Hsp60); green: Ref(2)P; blue: DAPI]. Different expression patterns were observed between uninfected and infected tissues.
Appendix 3

*Wolbachia* does not rescue the lethality caused by *pangolin* dominant-negative construct but decrease fitness of control flies

Expression of mutated pangolin drive lethality. However, according to previous observations in our lab, *Wolbachia* seems to affect this lethal effect. To confirm, we designed a genomic cross expressing *pangolinΔN* in hub and polar cells. We crossed 20 UAS-*pan ΔN* males with 20 *upd>*GAL4 virgin females in a vail, and flip the vial every 3-4 days. Both number of pupae formed and number/phenotype of adults hatched were recorded.

Results showed that, regardless of genotypes, in total around 40% of the *Wolbachia* uninfected flies hatched, comparing to only 22% in *wMel* infected and 23% in *wMelCS* infected flies (Figure A3.1A). *Wolbachia* infection seems to decrease fitness of control flies, and very possibly decrease fitness of *pangolinΔN* flies as well.

However, among the hatched adults, 96% of the uninfected flies are control flies and only 4% of them carried *pangolinΔN* construct; whereas in *wMel* 15.6%, and in *wMelCS* 14.4% of them are able to carried mutated *pangolin* (Figure A3.1B). Combined with the previous observations, we could conclude that *Wolbachia* is not rescuing the embryonic lethality of *pangolinΔN* but is decreasing the fitness of control flies instead. Whether *Wolbachia* decreases the fitness of *pangolinΔN* flies cannot be concluded from this experiment, but it seems like *Wolbachia* is not affecting *pangolinΔN* flies as much as it does in control flies.
Figure A3.1 *Wolbachia* decreases the fitness of control flies

A. Percentage of pupae hatched in *Wolbachia* infected and uninfected groups.

B. Percentage of flies collected from *Wolbachia* infected and uninfected groups. More experimental flies (*upd*-GAL4/+ > UAS-panΔN/+ ) and less control flies (*upd*-GAL4/+>MKRS/+ ) were collected from W+ groups than W- groups. Both observations have only been carried out once.
Appendix 4

Level of Wolbachia in carcass also increased after LiCl treatment

Other than gonads, Wolbachia has also been reported to colonize many other somatic tissues, including the head, gut Malpighian tubules, thoracic ganglion, fat body and muscles (Osborne et al. 2012). LiCl treatment is considered to have global effects toward the entire Drosophila body; thus, we want to examine whether Li\(^+\) is able to upregulate Wolbachia level in carcass.

A qPCR analysis was carried out in 5-7d old Drosophila infected with wMel. Wolbachia level significantly went up 1.62-fold in female carcasses (Figure A4.1A) and 1.53-fold in male carcasses (Figure A4.1B) after 5 days of LiCl treatment. This experiment has only been carried out once.
Figure A4.1 *Wolbachia* level in carcass increases after LiCl treatment

A-B. Level of *Wolbachia* quantified by the expression level of *Wolbachia* gene *wsp* in *wMel* infected *Drosophila* female (A) and male (B) carcasses. Both group only have 1 biological replicate with 5 flies.
Appendix 5

Western blot analyses were used to verify LiCl effects

One caveat about our LiCl treatment is that we lack direct evidences showing the alteration toward Wg/Wnt signaling pathway. We could not immunostain Armadillo nor other factors within the cascade, since all the commercially available antibodies are not specific enough. Protein analyses would be the best way to address this question.

Theoretically, Li$^+$ promotes the phosphorylation of Shaggy at Ser$^9$ and Ser$^{21}$. Previous literature used antibody against Ser$^9$ to verify the effect of Li$^+$ in *Drosophila* brain (Sofola et al. 2010). Whole fly protein samples were extracted from 5-7d old flies aged in Nutri-Fly GF with and without 100mM LiCl for 5 days. Results showed that phospho-Shaggy level significantly went up 1.8 fold (Figure A5.1B, P=0.017) in female flies after LiCl treatment; whereas Shaggy level remained the same (Figure A5.1C, P=0.128). phospho-Shaggy/total Shaggy (phospho-Shaggy+Shaggy) levels increase 1.3-fold after the treatment (Figure A5.1D, P=0.006). No differences were observed in males. This results strongly showed that Li$^+$ promotes the phosphorylation of Shaggy which lead to further destruction complex degradation.

To showed that Li$^+$ can further affect Armadillo, we also carried out a western blot using antibody against Armadillo. However, this experiment only worked once in our system. A very strong full-length Armadillo band seemed to appear on the gel after LiCl treatment. (Figure A5.1E).
Figure A5.1 Li\(^+\) leads to phosphorylation of Shaggy on Ser\(^9\) and increases full-length Armadillo level in female whole flies

A. Western blot with antibody against Shaggy and phospho-Shaggy as described in Materials and Methods. B-D. Quantification of the staining intensity of Shaggy (B), phospho-Shaggy (C) and phospho-Sgg (phospho-Sgg+Sgg) (D), analyzed by Image J. phospho-Sgg level significantly goes up after LiCl treatment. 4 biological replicates were carried out in Sgg and 3 biological replicates in phospho-Sgg staining. E. Western blot with antibody against Armadillo. Full length Armadillo was observed only in LiCl treated group, only one replicate. LiCl promotes phosphorylation of Shaggy and leads to cytosolic Armadillo accumulation.
Appendix 6

Transcriptional analysis of Wg/Wnt responsive genes

Wg/Wnt pathway is shown to turn on various downstream targets in different tissues. To confirm whether Wg/Wnt pathway was turned on in our system, we carried out a one-step real-time reverse-transcription PCR (one step RT-PCR) to examine the expression level of previously reported Wg/Wnt pathway genes. Expression levels of frizzled 3/fz3 (Sato et al. 1999), distal-less/dll (Campbell and Tomlinson 1998), naked cuticle/nkd (Campbell and Tomlinson 1998), ovo/svb (Yang et al. 2000), decapentaplegic/dpp (Yang et al. 2000) and armadillo/arm are normalized relative to ribosomal protein L32/rpl32 expression. svb are reported being repressed, whereas dpp, dll, nkd, fz3 being increased by Wg/Wnt signaling.

We analyzed Wolbachia infected NE ovaries with and without germline specific Armadillo 510 expression. Most of the Wg/Wnt genes we tested did not show significant change under overexpressing condition. However, fz3 level significantly went down 20% after treatment in wMelCS infected ovaries (FigureA6.1A, P=0.000037). Similar reduction trend, 0.58-fold decrease) was also observed in wMelPop infected ovaries (FigureA6.1A, 2 biological replicates, P=0.077).

Our results suggested that Armadillo 510 expression reduced one gene downstream of Wg/Wnt signaling pathway, but in a direction opposite from the reported literatures. Note that we also did not observe increased Armadillo level in Arm 510 expressing flies although the primer we used does not recognize mutation site of Arm 510.
Wg/Wnt responsive gene expressions have high tissue specificity. Therefore, negative results do not refer to pathway inactivation. Also, the nanos driver expresses low before stage 5 oogenesis, analyses of aged ovary could be more sensitive for RNA level differences.

For future directions, I strongly recommend to repeat this experiment in wMel infected species or in tissue expressing RNAi against armadillo. Also, there are many other Wg/Wnt responsive genes identified in Drosophila, including engrailed (Hooper 1994), stripe (Piepenburg, Vorbruggen, and Jackle 2000) and ultrabithorax/ubx (Riese et al. 1997). Verification for those genes is also important.
Figure A6.1 Wg/Wnt responsive gene expressions were analyzed in wMelCS and wMelPop infected newly eclosed ovaries with and without Armadillo^{S10} expression

A-B. Expression level of each gene in experimental (\textit{nos}>\textit{arm}^{S10}) ovaries is quantified relative to rpl32, and normalized to control (\textit{nos}>) groups. 4 biological replicates were carried out in wMelCS infected ovaries (A) and 2 biological replicates were carried out in wMelPop infected ovaries (B). Each biological replicate has 5-10 NE ovary sampels.
Appendix 7

Wolbachia level in non-melanogaster species after LiCl treatment

LiCl is considered as a potential way to affect Wolbachia level and abolish arbovirus transmission in disease transmitting arthropods due to the evolutionary conservation property of Wg/Wnt signaling pathway. To practically apply LiCl, it is important to show Li$^+$ could also affect intracellular Wolbachia titers in non-model species.

We carried out a preliminary LiCl treatment in Wolbachia infected Drosophila species available in our lab, D. simulans infected with wRi and wNo, D. sechellia infected with wSh, D. teissieri infected with wTei, D. yakuba infected with wYak and D. tropicalis infected with wWil. We are not able to obtain samples from D. sechellia because they are sick and dead soon in LiCl food, whereas female whole fly DNA samples were collected from D. teissieri and D. yakuba samples 3 days after LiCl treatment as well as from D. simulans and D. tropicalis 5 days after treatment. Moreover, different house-keeping genes were used to quantify Wolbachia gene expression in different Drosophila species. 28S rRNA was used in D. teissieri, D. tropicalis and D. simulans and gapdh1 was used in D. yakuba. However, Wolbachia levels were variable after the treatment. No significant difference was observed in this experiment (Figure A6.1A-E). An ovary image analysis is required in the future to verify the qPCR results. From the trial experiment, we can also conclude that Li$^+$ might
have species specific treating condition, which required further investigation to figure out.

**Figure A7.1 Li⁺ does not elevate *Wolbachia* level in non-melanogaster *Drosophila* species under current treating condition**

A-E. Level of *Wolbachia* quantified by the expression level of *Wolbachia* gene *wsp* in *D. simulans* infected with *wRi* (A, 6 biological replicates, each has 5-10 flies) and *wNo* (B, 4 biological replicates, each has 5-10 flies), *D. teissieri* infected with *wTei* (C, 3 biological replicates, each has 5-10 flies), *D. yakuba* infected with *wYak* (D, 3 biological replicates, each has 5-10 flies) and *D. tropicalis* infected with *wWil* (E, 3 biological replicates, each has 5-10 flies).
A Level of *Wolbachia* in female *Drosophila simulans* infected with wRi after LiCl treatment

B Level of *Wolbachia* in female *Drosophila simulans* infected with wNo after LiCl treatment

C Level of *Wolbachia* in female *Drosophila teissieri* infected with wTei after LiCl treatment

D Level of *Wolbachia* in female *Drosophila yakuba* infected with wYak after LiCl treatment

E Level of *Wolbachia* in female *Drosophila tropicalis* infected with wWiI after LiCl treatment
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