2020

Identification of Gene Expression Changes in Sleep Mutants Associated With Reduced Longevity in Drosophila

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IDENTIFICATION OF GENE EXPRESSION CHANGES IN SLEEP MUTANTS ASSOCIATED WITH REDUCED LONGEVITY IN *DROSOPHILA*

A Thesis Presented to the Faculty of

The Rockefeller University

in Partial Fulfillment of the Requirements for

the degree of Doctor of Philosophy

by

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June 2020

IDENTIFICATION OF GENE EXPRESSION CHANGES IN SLEEP MUTANTS ASSOCIATED WITH REDUCED LONGEVITY IN *DROSOPHILA*

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Sleep deprivation has become a common problem in modern society, yet the physiological consequences of sleep deprivation remain poorly understood. Sleep disruption has been shown to shorten lifespan in multiple animal species. In my thesis study, I investigated the relationship between sleep and longevity using *Drosophila melanogaster*. By analyzing the sleep and longevity profiles from a panel of sleep mutants, I discovered a robust positive correlation between daily sleep time and median longevity. This discovery led to my hypothesis that sleep disruption as a result of genetic mutation would elicit certain gene expression changes that ultimately would lead to shortened lifespan.

To identify the genes that exhibited altered expression, I profiled the transcriptomes of wild type flies, aged wild type flies, and two sleep mutants *insomniac*^{1} (*inc*^{1}) and *wide awake*^{D2} (*wake*^{D2}) with total RNA samples collected every 4 hours for 2 days. Circadian analysis was performed to detect oscillating transcripts in each group, and the results revealed substantial variations in number of oscillating genes. By comparing the experimental groups to the control wild type group, I discovered large scale rhythmicity changes in the experimental groups. Aged wild type flies predominantly showed a gain of rhythmicity, while loss of rhythmicity was more evident in sleep mutants. Furthermore, with the transcriptome data as input, I developed a differential gene expression (DGE) analysis pipeline to select candidate genes that might serve as the connection between sleep disruption and longevity reduction. In the DGE analysis, each experimental group was compared with the control wild type group. Results from each comparison were subsequently intersected to pinpoint genes that were significantly changed in all experimental groups. Ultimately, 15 candidate genes stood out from the analysis.

For the scope of this study, I focused on candidate gene *Neuropeptide-like precursor 3* (*Nplp3*), a putative neuropeptide precursor. RNA sequencing results revealed that expression levels of *Nplp3* were reduced in sleep mutants and aged wild type animals, compared to control flies. These results were validated by quantitative reverse transcription polymerase chain reaction (RT-qPCR). More importantly, I found that *Nplp3* expression was reduced in several sleep mutants in addition to *inc*^{1} and *wake*^{D2}. Decreased *Nplp3* expression resulted in significant shortening in lifespan but did not affect sleep amount. Transgenic fly strains were generated to selectively overexpress either wild type *Nplp3* or *Nplp3* without signal peptide using *Nplp3* specific driver. Overexpression of normal *Nplp3* reduced sleep. Longevity results indicated that signal peptide might be important for *Nplp3* function.

These findings expanded our understandings of the relationship between sleep and longevity and suggested a potential neuropeptide signaling pathway for regulation of longevity by sleep.

To my parents, Jing Wang and Wenxue Wang, for life.

To Manbing, for love.

ACKNOWLEDGMENTS

Although I have never imagined that I would finish my graduate school under such situation, I am extremely grateful for the time I had at Rockefeller. First of all, I would like to thank my mentor, Mike Young, for bringing me to this great lab, for supervising my thesis project, and for setting up a role model of great scientist. I have learned a tremendous deal of scientific aesthetics, independent thinking, and generosity from Mike. I am indebted to him forever for all these years' mentorship.

I want to thank my faculty advisory committee members, Leslie Vosshall and Cori Bargmann, for great feedback and continuous support over the past years. I want to thank my external examiner Mimi Shirasu-Hiza for sharing her insights on my thesis project and my career.

I would like to thank every past and present member in the Young Lab. Wanhe Li is a remarkable scientist, collaborator, and friend, from whom I have learned experimental techniques and dedication to science. I enjoyed the discussions we had on our projects and the gossips we shared. Alina Patke has always been providing helpful inputs for my project from a non-fly geneticist perspective. Sofia Axelrod is a nice colleague and our collaboration on the blood brain barrier project has been wonderful. I very much enjoyed working and drinking with Deniz Top, Daniel Cabrera, and Philip Kidd. Our research assistances, Andrew Nguyen, Irena Feng, Samantha Lincoln, and Jenna O'Neil have been indispensable during my thesis study. Our friendly and capable lab manager Deborah Collins-McGuire has solved my problems for countless times. Late Lino Saez supervised me in the first three months of my graduate study and taught me lots of genetics and biochemistry. I would never have made any scientific discoveries without the support of this excellent group.

I am grateful for the other three groups that I rotated with during the first year of my PhD. Gaby Maimon and Vikram Vijayan guided me to biophysics and computational analysis. The rotations in Jeffrey Friedman's laboratory and Cori Bargmann's laboratory introduced me to behavioral neuroscience in other animal models. The Friedman Lab was also kind to share equipment necessary for my research. These interdisciplinary experiences have contributed to my critical thinking and problem-solving skills.

My thesis project has received considerable support from the Resource Centers at Rockefeller University. Connie Zhao, Hong Duan, and Sophie Huang from Genomics Resource Center helped with RNA sequencing and sequence alignment. Thomas Carroll from Bioinformatics Resource Center assisted me in analyzing the sequencing data.

Many thanks go to the Dean's Office for all the support. Cristian Rosario, Sidney Strickland, Emily Harms, Kristen Cullen, Stephanie Fernandez, and Marta Delgado have ensured that my graduate study progress as smoothly as possible.

Outside of Rockefeller, I thank everyone I came across and spent time with, including Jiajun, Shuting, Dezhong, Yuedan, Xiang, Zeda, Menghan, Shujing, Shaojun, Runxi, Zeyu, Mingfei, Jingfeng, Shi, Ming, Fanying, Ke, Anjin, Zeyu, Shijie, Xiaohan, and many more. It is you that have made New York like home, especially in times like this.

Lastly, I greatly appreciate my parents and family, who have always supported me with their unconditional love. Most importantly, Manbing, my other half, has filled every second of my life with happiness. Our next journey down the road will just be as splendid as our past adventures.

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2D-DIGE	two-dimensional differential in-gel electrophoresis
aa	amino acids
ACh	acetylcholine
ATPsynD	ATP synthase, subunit D
BBB	blood-brain barrier
bp	base pairs
bsh	brain-specific homeobox
CCDC53	Coiled-coil domain containing 53
Cdk1	Cyclin-dependent kinase 1
CDS	coding sequence
Clk	Clock
cpm	count per million
Cpr49Ae	Cuticular protein 49Ae
Cpr76Bc	Cuticular protein 76Bc
cry	cryptochrome
Cul3	Cullin-3
cyc	cycle
CycA	cyclin A
dac	dachshund
DAT	Dopamine transporter
DAVID	Database for Annotation, Visualization, and Integrated Discovery
dbt	double-time
DCTN1-p150	Dynactin 1, p150 subunit
DGE	differential gene expression
ea	easter
EEG	electroencephalogram
Fbxl4	F box and leucine-rich-repeat gene 4
Fmr1	fragile X mental retardation
FOXO	forkhead box O transcription factor
fwd	four wheel drive
GABA	γ-aminobutyric acid
GABAT	GABA transaminase
GLM	generalized linear models
GPCR	G-protein coupled receptor
GPI	Glycosylphosphatidylinositol
Hk	Hyperkinetic
IGF	insulin-like growth factor
Ilp2	insulin-like peptide 2
inc	insomniac
INR	insulin-like receptor
jet	jetlag
kb	kilobase
LC-MS	liquid chromatography–mass spectrometry

LHA	lateral hypothalamus
lLNv	large ventral lateral neurons
LRT	likelihood ratio test
MAPK	mitogen-activated protein kinase
mth	methuselah
mthl8	methuselah-like 8
mthl10	methuselah-like 10
mya	million years ago
Naam	Nicotinamide amidase
naz	nazgul
Nkt	Noktochor
Nplp3	Neuropeptide-like precursor 3
NREM	non-rapid eye movement
nur	nemuri
OAMB	Octopamine receptor in mushroom bodies
p38b	p38b MAP kinase
PCA	principle components analysis
pdf	pigment dispersing factor
pen-2	presenilin enhancer
per	period
PI	pars intercerebralis
PK1-R	Pyrokinin 1 receptor
PKA	Protein Kinase A
Rca1	regulator of cyclin A1
Rcd4	Reduction in Cnn dots 4
Rdl	Resistant to dieldrin
REM	rapid eye movement
RNAi	RNA interference
RNA-seq	RNA sequencing
RPKM	Reads Per Kilobase of transcript, per Million mapped reads
RT-qPCR	quantitative reverse transcription polymerase chain reaction
rye	redeye
scaf	scarface
SCN	suprachaismatic nucleus
se	sepia
SEM	standard error of the mean
Sgt1	suppressor-of-G2-allele-of-skp1
Sh	Shaker
sLNv	small ventral lateral neuron
snf	sans fille
sNPF	short neuropeptide F
sss	sleepless
Sun	Stunted
tara	taranis
TBC1d7	TBC1 domain family member 7
tim	timeless

TOR	target of rapamycin
Tsp2A	Tetraspanin 2A
UTR	untranslated region
VLPO	ventrolateral preoptic
wake	wide awake
WT	wild type
ZT	Zeitgeber time

CHAPTER 1. INTRODUCTION

Why do we sleep? Humans spend approximately one-third of their lifetime sleeping, while the consequences of sleep deprivation remain poorly understood. Sleep deprivation has become a wide-spread problem in modern society (Liu et al., 2016). Studies in rats demonstrated that total sleep deprivation is lethal (Everson et al., 1989). However, how sleep deprivation affects aging or longevity has not been well characterized. It is my belief that the relationship between sleep and longevity could be illustrated using model organism *Drosophila melanogaster*, commonly known as fruit flies. Considering the evolutionarily conserved mechanisms for sleep regulation and longevity regulation from insects to mammals, the findings in *D. melanogaster* will shed light on our understandings about the effects of sleep loss on lifespan in humans.

In this chapter, I first review sleep as a conserved behavior throughout evolution. Next, I review the epidemiology of sleep deprivation and previous studies regarding the effects of sleep deprivation. Furthermore, I discuss why *D. melanogaster* serves as an ideal model organism for studying the relationship between sleep and longevity. Lastly, I define and explain the specific aims of this study.

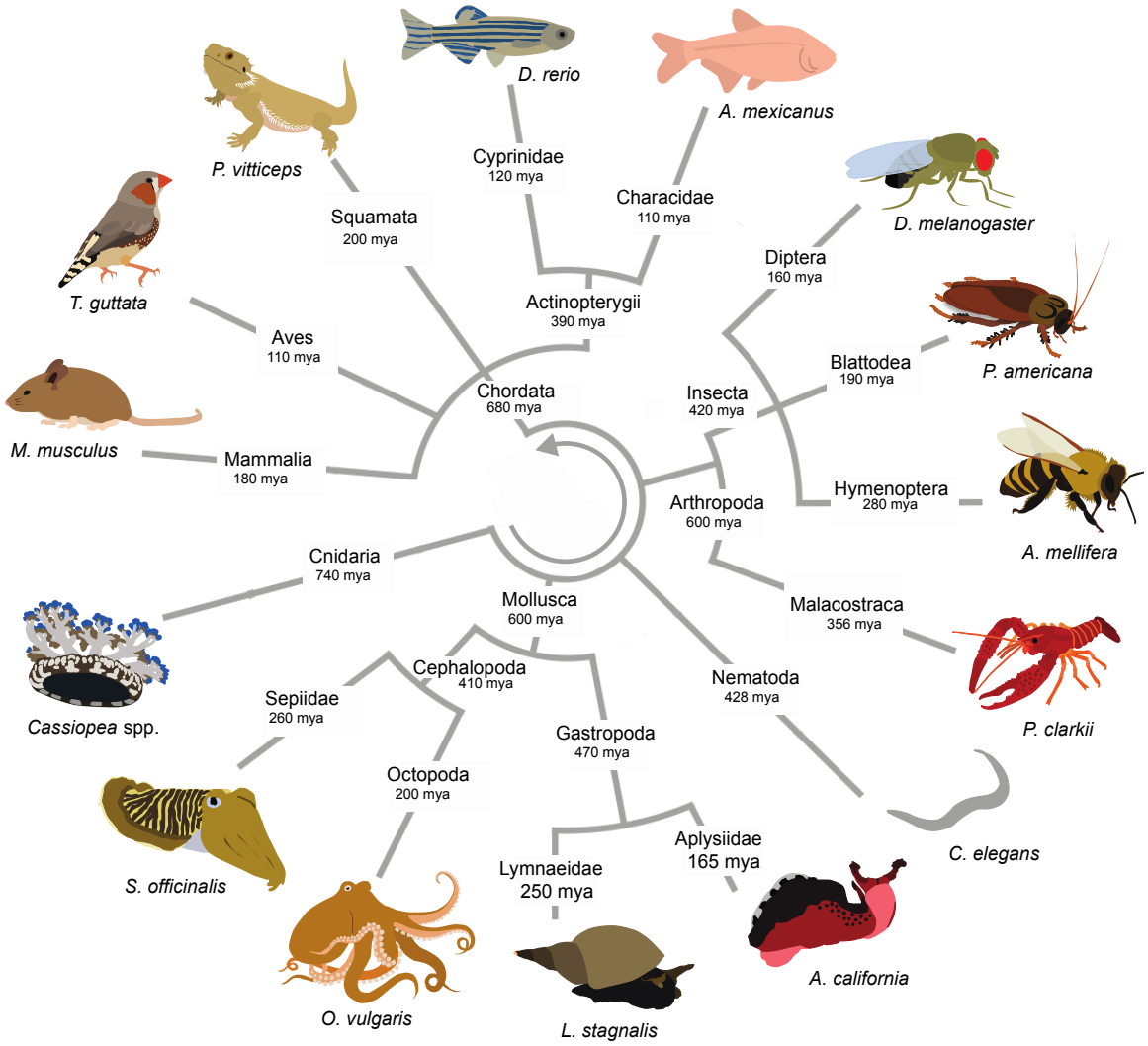
1.1 Sleep behavior throughout evolution

Until about two decades ago, the field of sleep research has been dominated by the use of electrographic measurements, thus mostly confined within the mammalian and avian orders (Campbell and Tobler, 1984; Hendricks et al., 2000b). Electroencephalogram (EEG) recordings of brain activity show that there are three major states in human activity: wake, rapid eye movement (REM) sleep and non-rapid eye movement (NREM) sleep. The EEG definition of sleep excludes animals without a cortex, like invertebrates. It has been documented, however, that there is a sleep-like state in at least some invertebrate species, such as forager bees, cockroach, octopus, and fruit flies (Campbell and Tobler, 1984; Hendricks et al., 2000a; Shaw et al., 2000).

The limitations of electrophysiological recordings highlight the need for a behavioral definition of sleep. Sleep researchers proposed a set of behavioral criteria to define sleep-like state as: 1) prolonged behavioral quiescence with species-specific posture; 2) reversible upon sensory stimulation; 3) accompanied by an increase in arousal threshold; 4) spontaneous, controlled by a circadian clock; and 5) regulated by a homeostatic system that ensures adequate levels of the state (Campbell and Tobler, 1984; Hendricks et al., 2000b; Keene and Duboue, 2018; Sehgal and Mignot, 2011). The establishment of a behavioral definition of sleep has expanded sleep research into a number of small animal models not practical for EEG measurements (Keene and Duboue, 2018) (**Figure 1.1**). The investigation of sleep in small animal models, particularly worms, fruit flies, and zebrafish, provides a comparative perspective, further demonstrating that sleep is a conserved behavior throughout evolution (Hendricks et al., 2000a; Raizen et al., 2008; Shaw et al., 2000; Zhdanova et al., 2001).

Figure 1.1. Small animal models used for sleep research.
Branches show broad relationships between species, but do not represent actual evolutionary distance. mya, million years ago. Modified from Keene and Duboue (2018).

Figure 1.1


D. rerio
A. mexicanus
P. vitticeps
Cyprinidae
120 mya
Characidae
110 mya
D. melanogaster
Squamata
200 mya
Diptera
160 mya
T. guttata
Actinopterygii
390 mya
P. americana
Aves
110 mya
Blattodea
190 mya
Chordata
680 mya
Insecta
420 mya
M. musculus
Mammalia
180 mya
Hymenoptera
280 mya
A. mellifera
Arthropoda
600 mya
Cnidaria
740 mya
Malacostraca
356 mya
Mollusca
600 mya
Cassiopea spp.
Cephalopoda
410 mya
Nematoda
428 mya
P. clarkii
Sepiidae
260 mya
Gastropoda
470 mya
Octopoda
200 mya
C. elegans
S. officinalis
Aplysiidae
165 mya
Lymnaeidae
250 mya
A. california
O. vulgaris
L. stagnalis

1.2 Sleep deprivation and its effects

Considering the potential dangers animals face while asleep, it is well accepted that sleep serves important physiological functions so that it is preserved throughout evolution. Yet it remains an open question how much sleep is necessary for its normal function. It is recommended that human young adults should receive at least 8 hours of sleep per night (Van Dongen et al., 2003). Chronic restriction of sleep to less than 6 hours per night results in significant vigilance performance defects within 2 weeks, the effects of which are comparable to up to 2 nights of total sleep deprivation (Van Dongen et al., 2003).

However, sleep deprivation has become a wide-spread problem in modern society. In the United States, more than a third of adults report that they sleep less than 7 hours (Liu et al., 2016). A poll conducted among college students found that 25% of the students regularly obtained less than 6.5 hours of sleep per night and 70% obtained less than 8 hours of sleep per night (Lund et al., 2010). Studies have shown that multiple factors contribute to sleep deprivation, including geographic location, caffeine use, school start time, etc. (Grandner et al., 2015; Owens et al., 2014). The consequences of chronic sleep deprivation have been linked to multiple health issues, including impaired cognition, cardiovascular diseases, and metabolic disorders (Killgore, 2010; Tobaldini et al., 2017).

Sleep researchers have been using animal models to study the effects of acute or chronic sleep deprivation. Defects in learning and memory following sleep deprivation have been demonstrated in both flies and rodents (Zhao et al., 2017). Memory consolidation for hippocampus-dependent contextual fear conditioning is impaired if mice are deprived from sleep for the first 5 hours immediately after training, while hippocampus-independent cued fear conditioning is not affected (Graves et al., 2003). Mice subjected to 12 hours of sleep deprivation prior to learning tasks show impaired spatial working memory due to defects in hippocampal glutamate signaling pathway (Hagewoud et al., 2010). Flies exposed to acute sleep deprivation during the first day after eclosion have reduced dopamine signaling, which lead to impairments in short-term memory in early adulthood (Seugnet et al., 2011).

Change in lifespan is another major consequence of sleep deprivation. For example, in the studies of Everson et al. (1989) and Rechtschaffen et al. (1983), rats enduring total sleep deprivation died in about a month's time. In these studies, symptoms before death included poor appearance, skin lesions, increased food intake, weight loss, increased energy expenditure, decreased body temperature, increased plasma norepinephrine, and decreased plasma thyroxine (Rechtschaffen et al., 1989). Yet it is intriguing how animals die from these non-life-threatening symptoms. Flies carrying mutation in circadian clock gene *cycle* (cyc^{01}) show mortality after as little as 10 hours of sleep deprivation, which can be rescued by activating stress response genes (Shaw et al., 2002). The actual cause of longevity problems remains unknown.

The similarities in the consequences of sleep deprivation discovered in flies and rodents not only support that sleep regulation and function are conserved from invertebrates to vertebrates, but also set up the stage for investigating the relationship between sleep and longevity using *D. melanogaster*.

1.3 Model organisms in sleep and longevity research

The most widely used model organisms for both sleep and longevity studies include worms, flies, mice, and rats. Each model organism has its advantages in answering certain scientific questions. For example, mice and rats are generally considered more suitable for studying effects of acute sleep deprivation because the methodology of sleep deprivation is better established and more reliable (Zhao et al., 2017). Thanks to its short lifespan, worms have enabled the identification of multiple longevity regulation pathways, which later are proved to be conserved in insects and mammals (Fontana et al., 2010). However, it is my belief that *D. melanogaster*, commonly known as fruit fly, serves as the ideal model organism for dissecting the connection between sleep and longevity.

There are certain features that are needed for the study of both sleep and longevity. These features can be classified into three categories: general features, features for sleep study, and features for longevity study (**Table 1.1**).

Desired general features include short life cycle, low cost, and powerful genetic tools. Short life cycle and low cost are important for quickly generating large number of animals and for maintaining these animals in proper laboratory setting, respectively. Rodents do not carry these features because sexual maturity takes 3-5 weeks and it is expensive to keep many cages of animals. Powerful genetic tools are available for all three model organisms, allowing sophisticated and precise manipulations including genetic screen, genome editing, neuronal activity perturbation, etc.

The five behavior criteria for sleep-like state definition discussed above capture the required features for sleep research in a model organism (Sehgal and Mignot, 2011). Reversible behavior quiescence is observed in all three animal models. Increased arousal threshold and homeostatic regulation of sleep have also been demonstrated. However, sleep in nematode *Caenorhabditis elegans* is characterized during lethargus, a 2- to 3-hour long developmental stage, which doesn't occur every day (Raizen et al., 2008). Although the timing of lethargus is shown to be controlled by *lin-42*, a *C. elegans* ortholog of circadian clock gene *period* (*per*), it remains an open debate whether a robust circadian clock is present in *C. elegans* (Goya et al., 2016; Olmedo et al., 2017).

To conduct longevity experiments in an efficient manner, the model organism used in the study should have relatively short lifespan and it should be easy to generate large population of animals (Piper and Partridge, 2018). Rodents do not carry these properties. Their life expectancy is ~3 years, much longer than worms (~3 weeks) and fruit flies (~3 months). The long lifespan adds an extra burden on the already high cost of maintaining mice or rats for longevity measurements. I believe that invertebrate animal models are suitable for the discovery of molecular pathways regulating longevity, which can be subsequently validated in longer lived vertebrate systems.

Taken together, fruit flies have all the desired features, making them the ideal model organism to study the relationship between sleep and longevity (**Table 1.1**).

Table 1.1. Comparison of model organisms for sleep and longevity research

	Desired features for research	Worm	Fly	Rodent
General	Short life cycle	Yes	Yes	No
	Low cost	Yes	Yes	No
	Powerful genetic tools	Yes	Yes	Yes
Sleep	Reversible behavioral quiescence	Yes	Yes	Yes
	Increased arousal threshold	Yes	Yes	Yes
	Circadian control of sleep onset	No	Yes	Yes
	Homeostatic sleep rebound	Yes	Yes	Yes
Longevity	Short life span	Yes	Yes	No
	Ease of generating large population	Yes	Yes	No

1.4 Conserved sleep and longevity pathways from flies to mammals

Thanks to the advantages discussed above, numerous studies about sleep control and longevity regulation have been conducted using *D. melanogaster* as animal model in the past two decades. More importantly, most of the principles and mechanisms uncovered using fruit flies have been shown to regulate similar biological processes in mammalian systems.

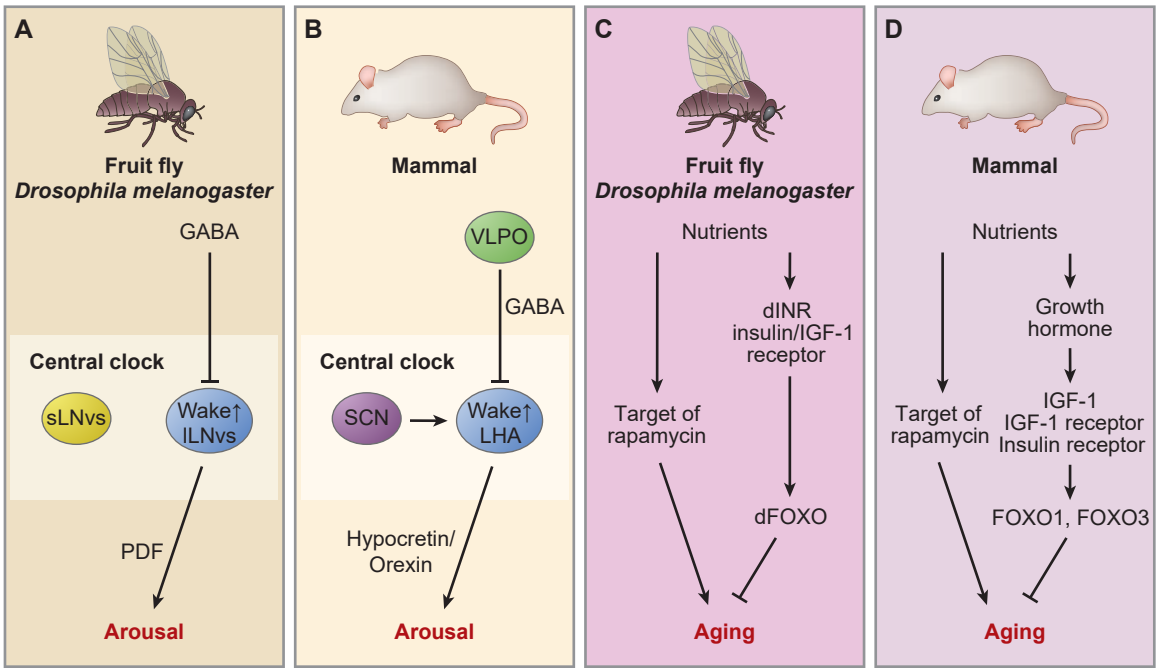
A model for sleep regulation in humans was proposed about 40 years ago, which consists two processes: a circadian process (Process C) and a homeostatic process (Process S) (Borbely, 1982). Sleep studies in model organisms have expanded this two-process model across species.

Figures 1.2A and **1.2B** depict an example of a conserved mechanism regulating sleep (Sehgal and Mignot, 2011). In both mammals and flies, Process C from the central circadian clock controls the timing of sleep onset by regulating the secretion of arousal-promoting molecules (Parisky et al., 2008; Sakurai, 2007). Inputs of Process S from neurotransmitter signaling pathways like γ-aminobutyric acid (GABA) may act on the circadian neurons to promote sleep (Agosto et al., 2008; Andretic et al., 2008a; Gottesmann, 2002).

It is well accepted that the circadian rhythm that controls Process C is conserved from flies to mammals. Since the identification of the first circadian rhythm mutants in *D. melanogaster*, fruit flies have served as the powerhouse for circadian rhythm studies (Konopka and Benzer, 1971). A genetic screen discovered 3 mutations of a single gene that can alter rhythmicity in *D. melanogaster*, arrhythmic per^0, shortened period per^S, and lengthened period per^L. The disrupted rhythm could be restored by transferring a wild type copy of *per* (Bargiello et al., 1984; Zehring et al., 1984). More genes were discovered in the following years by genetic screens using fruit flies, including *timeless* (*tim*), *double-time* (*dbt*), and *cyc* (Kloss et al., 1998; Price et al., 1998; Rutila et al., 1998; Sehgal et al., 1994; Vosshall et al., 1994). The involvement of *Clock* (*Clk*) in circadian rhythm was first revealed in mice and then re-discovered in flies (Allada et al., 1998; Vitaterna et al., 1994). A transcription factor complex consisting of CLK/CYC promotes transcription of *per* and *tim* mRNA. PER and TIM proteins physically associate in the cytoplasm and subsequently translocate into the nucleus, where the complex inhibits CLK/CYC activity. As *per* and *tim* transcription are suppressed and their proteins are degraded, CLK/CYC complexes initiate a new round of transcription activation, closing the transcription-translation feedback loop (Patke et al., 2020; Young, 2018). Photoreceptor protein Cryptochrome (CRY) and E3 ubiquitin ligase Jetlag (JET) degrade TIM protein in a light-dependent manner, thus synchronizing the endogenous clock with the environment (Ceriani et al., 1999; Koh et al., 2006; Peschel et al., 2009). Although there are some variations in the specific representation and function of these clock genes across the animal kingdom, the central features and principles of the molecular clock are largely conserved (Takahashi, 2017).

Figure 1.2. Examples of conserved mechanisms regulating sleep and longevity
A. In *D. melanogaster*, the large ventral lateral neurons (lLNvs) are part of the central clock and they mediate light-driven arousal, at least in part through the release of pigment dispersing factor (PDF). Inhibitory GABAergic inputs to these neurons promote sleep.
B. In mammals, hypocretin/orexin-producing neurons in the lateral hypothalamus (LHA) receive circadian inputs from the central clock in the suprachaismatic nucleus (SCN). They are inhibited by GABAergic inputs from the ventrolateral preoptic (VLPO) area.
C and **D**. An ancient nutrient-sensitive signaling network plays an evolutionarily conserved role in the regulation of aging. Interventions in this pathway can slow aging and extend longevity. FOXO, forkhead box O transcription factor; IGF-1, insulin-like growth factor 1; INR, insulin-like receptor.
A and **B** are modified from Sehgal and Mignot (2011). **C** and **D** are modified from Gems and Partridge (2013).

Figure 1.2


A
Fruit fly
Drosophila melanogaster
GABA
Central clock
sLNvs
Wake↑
lLNvs
PDF
Arousal
B
Mammal
VLPO
GABA
Central clock
SCN
Wake↑
LHA
Hypocretin/
Orexin
Arousal
C
Fruit fly
Drosophila melanogaster
Nutrients
dINR
insulin/IGF-1
receptor
Target of
rapamycin
dFOXO
Aging
D
Mammal
Nutrients
Growth
hormone
Target of
rapamycin
IGF-1
IGF-1 receptor
Insulin receptor
FOXO1, FOXO3
Aging

Because of the similarities in neurotransmitter signaling pathways between fruit flies and mammals, the conserved mechanisms of Process S in sleep regulation have been extensively studied in neurotransmitter systems. Pharmacological studies showed the abilities of adenosine agonists to induce sleep and adenosine antagonists to decrease sleep (Andretic et al., 2008b; Bjorness and Greene, 2009). The wake promoting effects of caffeine are also conserved, although the molecular mechanism might differ (Bjorness and Greene, 2009; Wu et al., 2009). In both insects and mammals, activation of GABA receptors facilitate sleep, whereas dopamine signals wakefulness in the brain (Agosto et al., 2008; Gottesmann, 2002; Nall and Sehgal, 2014; Oishi and Lazarus, 2017). In flies, GABA is shown to promote sleep by inhibiting *pigment dispersing factor* (*pdf*) expressing neurons through $GABA_A$ receptor *Resistant to dieldrin* (*Rdl*) (Agosto et al., 2008; Chung et al., 2009; Parisky et al., 2008). *wide awake* (*wake*) appears to control sleep onset via its regulation on RDL (Liu et al., 2014). Flies carrying mutation in *Dopamine transporter* (*DAT*), which results in increased level of dopamine in the brain, exhibit reduced daily sleep, decreased arousal threshold and attenuated rebound response (Kume et al., 2005). Norepinephrine and its insect counterpart, octopamine, both have robust wake-promoting effects (Crocker and Sehgal, 2008; Siegel, 2004). Octopamine was shown to regulate sleep through Protein Kinase A (PKA)-dependent mechanisms and the brain area receiving octopamine signal is mapped to the pars intercerebralis (PI) expressing *Octopamine receptor in mushroom bodies* (*OAMB*) (Crocker and Sehgal, 2008; Crocker et al., 2010). Serotonin has been shown to regulate sleep, although its actual effects are receptor dependent (Monti, 2011; Qian et al., 2017; Yuan et al., 2006). *D. melanogaster* serotonin receptor d5-HT1A promotes sleep through mushroom bodies in the brain (Yuan et al., 2006), and receptor d5-HT2B regulates sleep rebound after sleep deprivation (Qian et al., 2017). Regulation of sleep by acetylcholine (ACh) might also be receptor dependent. In mammals, ACh is released during wakefulness (Platt and Riedel, 2011). However, cholinergic neurons in fly mushroom bodies have been shown to promote sleep and mutation of *redeye* (*rye*), a nicotinic acetylcholine receptor α subunit causes sever sleep reduction (Shi et al., 2014; Yi et al., 2013), indicating different sleep regulating pathways of ACh in flies and mammals.

Longevity studies using animal models have identified several biological mechanisms that regulate lifespan in both fruit flies and mammals (**Figures 1.2C** and **1.2D**). Insulin/insulin-like growth factor (IGF-1) signaling pathway was first demonstrated to control aging in *C. elegans* and the mechanisms are evolutionarily conserved (Gems and Partridge, 2013; Kenyon, 2005; Kenyon et al., 1993; Kenyon, 2010; Piper and Partridge, 2018). Dietary restriction also improves longevity in multiple animal models and the effect is partially dependent on inhibition of insulin/IGF-1 signaling pathway (Fontana et al., 2010). Inhibition of the target of rapamycin (TOR) signaling pathway is shown to be required for lifespan extension in response to chronic dietary restriction (Kenyon, 2010; Piper and Partridge, 2018). The necessity of normal circadian rhythm for dietary restriction mediated lifespan extension remains an open question (Katewa et al., 2016; Ulgherait et al., 2016). Deacetylases sirtuins are found to protect animals from aging when overexpressed and it is proposed that sirtuins function as the link between insulin/IGF-1 signaling pathway and TOR signaling pathway (Gems and Partridge, 2013; Kenyon, 2010).

Most interestingly, the insulin-secreting PI neurons are one of the sleep centers in *D. melanogaster*, the activity of which promotes wakefulness in response to octopamine (Crocker et al., 2010). PI neurons have also been reported to be required for dietary restriction induced lifespan extension in flies (Broughton et al., 2010). Lowered insulin signaling and reduced TOR activity can rescue sleep fragmentation in old flies (Metaxakis et al., 2014). Considering the fact that sleep fragmentation is one of the major deficits in sleep mutants and old flies (Bushey et al., 2010; Liu et al., 2014; Stavropoulos and Young, 2011), a possible connection between sleep and longevity could be sleep bout control by insulin-producing neurons.

1.5 Genetics of sleep regulation in *D. melanogaster*

Besides the neurotransmitter pathways discussed above, a series of independent and unbiased genetic screens have identified quite a few genes whose mutation would disrupt sleep in *D. melanogaster*.

It is quite surprising and interesting that three mutations are closely related to potassium channels. Potassium channel *Shaker* (*Sh*) was identified as a sleep-promoting gene in flies (Cirelli et al., 2005a). Point mutation of *Sh* leads to significant reduction of sleep duration and the affected protein sequences are conserved throughout animal kingdoms. These flies, however, appear to have a normal rebound response after sleep deprivation, indicating that *Sh* contributes to setting the baseline for sleep (Cirelli et al., 2005a). Similar phenotypes have been observed in *Hyperkinetic* (*Hk*) mutants, which encodes a modulatory subunit of *Sh* (Bushey et al., 2007). A forward genetic screen using transposon induced mutation discovered *sleepless* (*sss*), which encodes a brain-enriched glycosylphosphatidylinositol (GPI)-anchored protein (Koh et al., 2008). *sss* loss of function severely decreases total sleep in *Drosophila*. It has been reported that mutation of *sss* reduces SH protein level, alters SH localization and causes abnormal SH current kinetics (Koh et al., 2008; Wu et al., 2010). Unlike *Sh* mutant flies, mutation of *sss* leads to impaired sleep recovery after deprivation, possibly indicating novel function of *sss* besides modulating potassium currents (Koh et al., 2008; Wu et al., 2010).

PDF neurons are known as pacemaker neurons and they maintain circadian clock in constant darkness. *pdf* mutants show elevated total sleep and decreased sleep latency, indicating that PDF serves as a functional homolog of the mammalian wake-promoting neuropeptide hypocretin/orexins (Chung et al., 2009; Parisky et al., 2008; Sakurai, 2007). Short neuropeptide F (sNPF) is secreted by small ventral lateral neurons (sLNvs) and suppresses the activity of large ventral lateral neurons (lLNvs) to promote sleep (Shang et al., 2013). This interaction between lLNvs and sLNvs indicates that PDF neurons are one of the sleep centers in the fly brain.

More neuropeptides and their receptors have been found to regulate sleep (Helfrich-Forster, 2018), including wake-promoting diuretic hormone 31 (DH31) (Kunst et al., 2014), diuretic hormone 44 (DH44) (Cavanaugh et al., 2014), adipokinetic hormone (AKH) (Lee and Park, 2004), leucokinin (Murphy et al., 2016), and neuropeptide F (NPF) (Chung et al., 2017); sleep-promoting epidermal growth factor receptor (EGFR) (Foltenyi et al., 2007), sex peptide (Oh et al., 2014), AYRKPPFNFSIFamide (SIFamide) (Park et al., 2014), amnesiac (Liu et al., 2008), and allatostatin A (AstA) (Chen et al., 2016). However, the detailed mechanisms are not yet well understood.

Sleep disruption and immune response have been shown to interact with each other. Immune factor NF-κB deficient flies exhibit reduced nighttime sleep and sleep-deprived flies are more resistant to bacterial infection (Williams et al., 2007). Core circadian clock genes *per* and *tim* have been shown to play important roles in defense response to bacterial infection (Allen et al., 2016; Shirasu-Hiza et al., 2007; Stone et al., 2012). *nemuri* (*nur*), an antimicrobial peptide, was discovered in a large-scale gain-of-function screen (Toda et al., 2019). Overexpression of *nur* promotes sleep and *nur* expression is activated after bacterial infection. A secreted immunoglobulin (Ig) domain protein Noktochor (Nkt) is identified as sleep-promoting, knocking down of which decreases nighttime sleep (Sengupta et al., 2019).

Protein degradation pathways serve a vital role in regulating sleep. A forward genetic screen identified *insomniac* (*inc*), which is believed to encode an adaptor protein for the Cullin-3 (Cul3) ubiquitin ligase complex (Stavropoulos and Young, 2011). Mutation of either *inc* or *Cul3* results in significant sleep reduction. $GABA_A$ receptor RDL undergoes rhythmic degradation, which is mediated by E3 ligase F box and leucine-rich-repeat gene 4 (Fbxl4), providing a possible interaction between the processes of sleep regulation and protein degradation (Li et al., 2017).

Perhaps most strikingly, reducing the protein levels of cell cycle proteins cyclin A (CycA) or regulator of cyclin A1 (Rca1) causes severe reduction of sleep and disrupts sleep recovery after deprivation (Rogulja and Young, 2012). This process also involves transcriptional co-regulator TARANIS (TARA) and cell cycle protein Cyclin-dependent kinase 1 (Cdk1) (Afonso et al., 2015). It remains a mystery why and how cell cycle pathways could regulate sleep.

Some of the genes and pathways mentioned above have also been shown to regulate sleep or sleep-like behavior in other organisms, including mammals, zebrafish, and worms (Crocker and Sehgal, 2010; Sehgal and Mignot, 2011). Meanwhile, *D. melanogaster* have been used to generate disease models for genetic disorders in humans. The *D. melanogaster fragile X mental retardation* (*Fmr1*) gene, whose human homolog is associated with inherited mental retardation, affects both sleep, synaptic function, and phagocytosis (Bushey et al., 2009, 2011; O'Connor et al., 2017). These discoveries provide a valuable pool of genes and pathways for uncovering the relationship between sleep disruption and longevity reduction.

1.6 Aims of this study

As more and more genetic components are discovered as sleep regulators, it is more likely that there is no single dedicated genetic pathway controlling sleep. The homeostatic mechanisms regulating sleep and its outputs might not be as simple as those regulating circadian rhythm. Given that there are multiple stages during sleep, sleep is probably a complex behavior controlled by both genetic network and neural circuitry. A better way to investigate sleep and the consequences of sleep disruption is to take advantage of the mutants that have been identified to characterize shared features among different mutants with similar phenotypes. This approach is necessary because most sleep related genes are involved in other physiological pathways besides sleep regulation. In order to get a clear picture of any sleep-regulating network, it is crucial to rule out "side effects" that are exhibited by sleep mutants but are not related to sleep.

My thesis research project used RNA-sequencing as an approach to identify shared gene expression changes in sleep-disrupted flies caused by different mutations. Although the documented mutations are involved in different physiological pathways, their longevity curves look quite similar to each other and more interestingly, similar to aged wild type flies. I discovered a significant and robust positive correlation between daily sleep time and median lifespan. Based on this discovery, I hypothesize that sleep disrupting genetic mutations cause early onset of aging in young flies and ultimately lead to reduced longevity. Since the profiled flies all exhibit reduced sleep and similar longevity curves, I took the intersection of significantly changed genes in different experimental groups and identified a series of genetic links between sleep and longevity. I found that the circadian rhythm was changed in both aged flies and sleep mutants, although few genes were found to be shared in both conditions. Furthermore, using differential gene expression analysis, I identified several candidate genes that were consistently changed in all experimental groups. Among these candidate genes, I studied *Neuropeptide-like precursor 3* (*Nplp3*) systematically. I found that *Nplp3* expression levels were altered in sleep mutants and abnormal *Nplp3* expression resulted in longevity reduction. My discoveries could provide insight for effects of sleep disruption on lifespan and for treating health problems associated with sleep disorders.

CHAPTER 2. ESTABLISHING RELATIONSHIP BETWEEN SLEEP AND LONGEVITY

Over the past decades, more than a dozen mutant fly strains have been isolated with abnormal sleep phenotypes. Majority of these mutations were generated using mutagenesis-based genetic screens. As a result, these mutants are from different genetic background, i.e. each mutant strain carry a unique combination of genetic materials. Genetic background affect animal behaviors including sleep due to presence of different modifier genes in different genetic backgrounds (Chow, 2016; Faville et al., 2015; Zimmerman et al., 2012). Before establishing any relationship between sleep and longevity, it is crucial to backcross each mutant strain to the same genetic background so that the phenotypes caused by different mutations can be compared directly.

In this chapter, I first backcrossed a panel of sleep mutants to the same genetic background and measured their sleep and longevity. Then, I looked for relationship between sleep and longevity from pooled experimental results of various sleep mutants. Furthermore, based on my hypothesis, I designed experimental approaches to elucidate the genetic linkage between sleep and longevity.

2.1 Sleep and longevity phenotypes of sleep mutants

Since the establishment of *D. melanogaster* as an animal model for sleep study, sleep researchers have utilized unbiased genetic screen to search for sleep regulating genes. More than a dozen genes have been identified, whose mutation would result in disrupted sleep (**Table 2.1**). Most of these mutations reduce total sleep time, indicating that in laboratory environment sleep duration is maintained at or close to its upper limit.

The identified genes are involved in different biological pathways, including potassium channel function, neurotransmitter signaling, protein degradation, cell cycle control, and immune response. Unlike circadian clock, it is not likely that sleep is regulated by a set of dedicated genes. However, the behavioral readouts of these mutations converge onto sleep disruption, and in most of the cases, longevity reduction. It is interesting that longevity reduction tends to accompany sleep loss, suggesting some connection between sleep and longevity.

In order to quantitatively determine the correlation between sleep and lifespan, it is necessary to compare the phenotypes across various mutants. However, because these mutants were generated in different genetic backgrounds, it is inconclusive to compare their phenotypes without backcrossing these mutants into the same genetic background.

I used isogenized wild type strain *iso1CJ* (Li et al., 2013), referred to as *WT*, as the target genetic background for backcrossing the sleep mutants. *WT* is an isogenized variant of commonly used w^{1118}, which was derived from the Canton-S wild type strain (Yin et al., 1994). It has been reported that compared to closely related w^{1118} strain, *WT* flies show deeper sleep especially at night (Faville et al., 2015). After backcrossing for at least 5 generations with the *WT* strain, all sleep mutants were considered to be in a homogeneous genetic background (see **Methods** for details). Then I measured the sleep duration and lifespan of the backcrossed sleep mutants.

There are two methods for sleep measurement, infrared beam-based measurement and video tracking (**Figure 2.1**). The widely used infrared beam-based method was adopted from circadian rhythm research. An infrared beam crosses the midline of a glass tube housing a single fly with food. Whenever the fly moves around in the tube and breaks the infrared beam, a signal will be recorded by the computer connected to the *Drosophila* Activity Monitor (DAM). Video tracking can be done by loading individual fly into recording chambers and record their position over time using cameras. Recorded videos are analyzed subsequently using a tracking algorithm to quantify the time that the flies are asleep. Both methods have been demonstrated to be reliable and robust for sleep measurement (Gilestro, 2012). In this study I used infrared beam-based method because this method allows high throughput sleep experiments and does not require devotion of a large space.

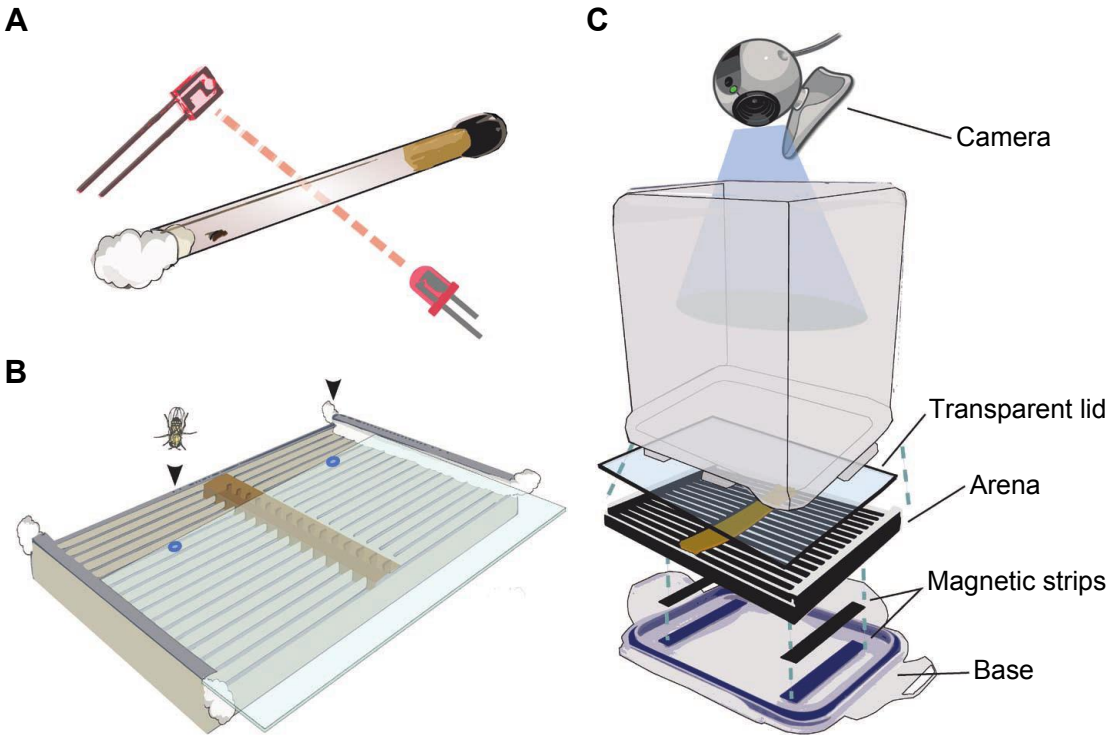
Table 2.1. Summary of identified sleep regulating genes

Gene	Molecular Function	Allele/ Method	Sleep	Longevity	Reference
Sh	Potassium Channel	*mns*	↓	↓	Cirelli et al. (2005a)
Hk	Sh modulator	Hk^{1}, Hk^{2}, Hk^{Y}	↓	↓	Bushey et al. (2007)
DAT	Dopamine transpoter	*fmn*	↓	--*	Kume et al. (2005)
sss	Sh regulator	sss^{P1}	↓	↓	Koh et al. (2008)
inc	Ubiquitin ligase adaptor	inc^{1}, inc^{2}	↓	↓	Stavropoulos and Young (2011)
CycA	Cell cycle factor	(RNAi)	↓	↓	Rogulja and Young (2012)
wake	RDL regulator	$wake^{D1}$, $wake^{D2}$	↓	↓	Liu et al. (2014)
Nmdar1	NMDA receptor	(RNAi)	↓	N.D.**	Tomita et al. (2015)
tara	Cell cycle protein	$tara^{s}$	↓	N.D.**	Afonso et al. (2015)
fbxl4	E3 ligase	$fbxl4^{\Delta 45}$	↓	N.D.**	Li et al. (2017)
Nkt	Ig-domain protein	(RNAi)	↓	N.D.**	Sengupta et al. (2019)
nur	Antimicrobial protein	(Overexpression)	↑	N.D.**	Toda et al. (2019)

* Normal lifespan; ** Not determined

Figure 2.1. Methods for sleep measurement
A. Infrared beam-based measurement. A single fly is housed in a glass tube with food. An infrared beam crosses the midline of the tube and is connected to a computer. Whenever the fly moves and breaks the beam, a digital signal will be sent to the computer.
B. Chambers for video recording. The 3D-printed recording chambers can be customized into different shapes. This example shown here resembles the infrared beam-based method, where individual fly is loaded into small chamber with food and the entire arena is covered with glass.
C. Video recording setup. The chambers shown in **B** are assembled with modules including a base for securing the chamber and a camera for collecting videos.
Modified from Gilestro (2012).

Figure 2.1

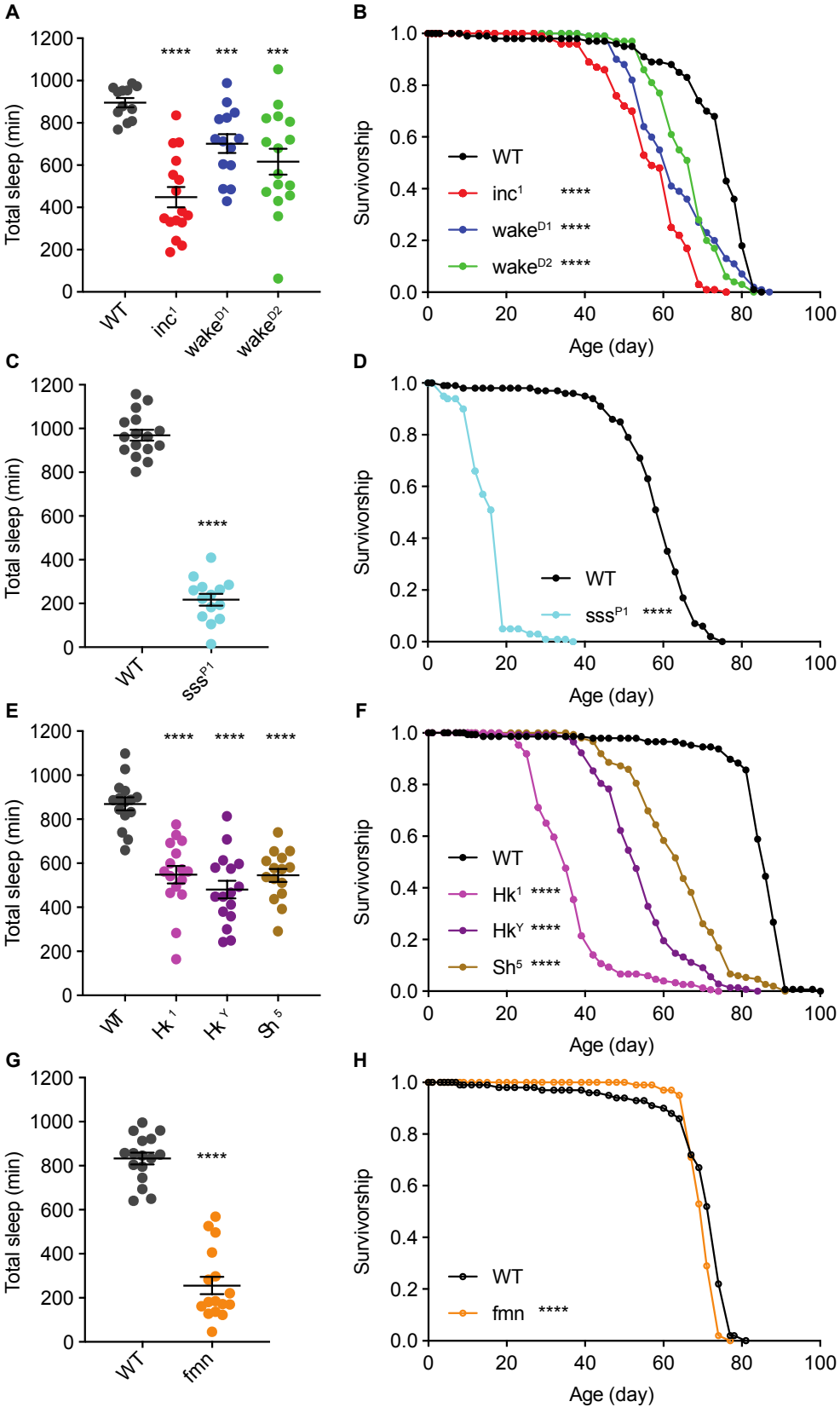


All backcrossed sleep mutants showed a significantly decreased amount of total sleep, demonstrating the robust sleep disruption effect of these mutations (**Figure 2.2**). The severity of sleep reduction depended on the mutation. Different mutations affecting the same gene produced comparable yet different phenotypes, such as $wake^{D1}$ and $wake^{D2}$, Hk^{1} and Hk^{Y} (**Figures 2.2A** and **2.2E**). The most severe sleep reduction was caused by sss^{P1}, followed by *fmn* and inc^{1} (**Figures 2.2A**, **2.2C**, and **2.2G**).

These mutations also led to shortened lifespan, except for *fmn* (**Figure 2.2**). *inc*, *wake*, *sss*, *Hk*, and *Sh* mutations all resulted in robust and significant curtailment in longevity ($p < 0.0001$ in all groups) (**Figures 2.2B**, **2.2D**, and **2.2F**). The strongest longevity reduction phenotype was observed in sss^{P1} flies, same as sleep phenotype. Although the longevity curve of *fmn* was significantly different from that of wild type, it was more convincing that there was a moderate extension of lifespan between Day 40 and Day 70 in *fmn* flies rather than a reduction in lifespan (**Figure 2.2H**). Such phenotype of *fmn* was different from what has been documented in the original study, where *fmn* flies showed normal lifespan, probably due to difference in genetic backgrounds (Kume et al., 2005). It remains a mystery why short-sleeping *fmn* mutants do not show reduced longevity. One possible explanation is that *fmn* mutation only affects sleep temporarily, thus older flies can receive sufficient amounts of sleep for normal survival.

Figure 2.2. Sleep and longevity phenotypes of backcrossed sleep mutants
A. Average total sleep per day of *WT* (black), inc^{1} (red), $wake^{D1}$ (blue), and $wake^{D2}$ (green) male flies. n = 12-16 for all groups.
B. Longevity of *WT* (black), inc^{1} (red), $wake^{D1}$ (blue), and $wake^{D2}$ (green) male flies. n = 145-150 for all groups.
C. Average total sleep per day of *WT* (black) and sss^{P1} (cyan) male flies. n = 16 for *WT* and n = 14 for sss^{P1}.
D. Longevity of *WT* (black) and sss^{P1} (cyan) male flies. n = 152 for sss^{P1}; n = 147 for *WT*.
E. Average total sleep per day for *WT* (black), Hk^{1} (magenta), Hk^{Y} (purple), and Sh^{5} (brown) male flies. n = 15-16 for all groups.
F. Longevity of *WT* (black), Hk^{1} (magenta), Hk^{Y} (purple), and Sh^{5} (brown) male flies. n = 143-150 for all groups.
G. Average total sleep per day of *WT* (black) and *fmn* (orange) male flies. n = 16 for both groups.
H. Longevity of *WT* (black) and *fmn* (orange) male flies. n = 144 for *fmn*; n = 147 for *WT*.
For sleep measurements, mean ± standard error of the mean (SEM) is shown; *** $p < 0.001$; **** $p < 0.0001$, t test with Welch's correction against *WT* control animals.
For longevity measurements, overall survivorship is shown; **** $p < 0.0001$, log-rank test against *WT* control animals.

Figure 2.2


A
Total sleep (min)
WT
inc1
wakeD1
wakeD2
B
Survivorship
Age (day)
WT
inc1 ****
wakeD1 ****
wakeD2 ****
C
Total sleep (min)
WT
sssP1
D
Survivorship
Age (day)
WT
sssP1 ****
E
Total sleep (min)
WT
Hk1
HkY
Sh5
F
Survivorship
Age (day)
WT
Hk1 ****
HkY ****
Sh5 ****
G
Total sleep (min)
WT
fmn
H
Survivorship
Age (day)
WT
fmn ****

2.2 Positive correlation between daily sleep time and median lifespan

Interestingly, the structure of longevity curves was similar among wild type flies and sleep mutants. There was a plateau stage at the beginning of the measurements, followed by a steep descent where most mortality occurred. The difference between sleep mutants and wild type flies was mainly the timing when the descent started, or in other words, the duration of the plateau stage (**Figure 2.2**). The phenomenon observed in the sleep mutants differed from the traditional longevity mutants, where changes in the slope of the descent is the predominant feature (Bjedov et al., 2010; Hwangbo et al., 2004; Lin et al., 1998).

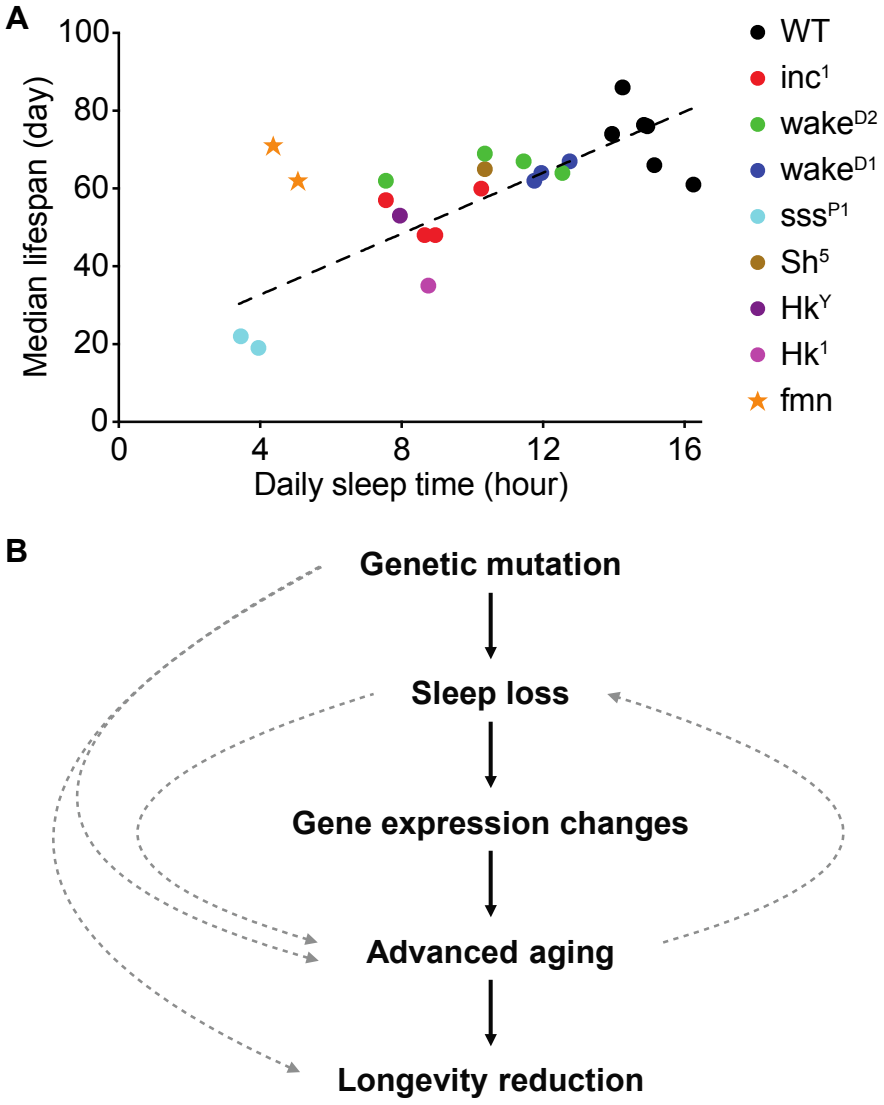
To determine whether there was any correlation between sleep and longevity, I plotted the median lifespan against the daily sleep time (**Figure 2.3A**). Data from all tested sleep mutants were pooled, together with data from wild type animals. Linear regression revealed a significant correlation between daily sleep time and median lifespan (Pearson's $r = 0.66$, $p < 0.001$). When excluding the outlier data collected from *fmn* mutants, the data showed stronger linear correlation (Pearson's $r = 0.83$, $p < 0.0001$). These results demonstrated that daily sleep time positively correlated with median lifespan.

Taken these observations together, my hypothesis was that genetic mutations of sleep genes led to loss of sleep; when sleep was disrupted, certain gene expression changes would occur, which subsequently caused accelerated aging and shortened lifespan (**Figure 2.3B**). However, other mechanisms could not be ruled out. The mutated gene could directly regulate longevity independent of its effects on sleep. Moreover, loss of sleep could be the consequence of advanced aging rather than the cause. Lastly, sleep loss could affect aging and longevity via translation level regulation, bypassing transcription networks. These mechanisms might not be independent and thus elucidating one potential pathway could shed light upon other possibilities. Thanks to recent advances in sequencing technologies, large scale transcriptome profiling experiments became reliable and affordable. I decided to focus on the changes in transcriptome using RNA-sequencing (RNA-seq).

Large scale transcriptomic studies offer great power but also suffer from false positive discoveries. To avoid the problem, the candidate genes identified from the RNA-seq experiments would be investigated individually. Three criteria must be met for a gene to be considered as the connection between sleep disruption and longevity reduction: 1) sleep loss would induce changes in expression levels comparable to the changes observed in RNA-seq datasets; 2) such changes in expression levels would affect longevity; 3) but not affect sleep. The first and second criteria could ensure that the expression levels of the gene regulate longevity in response to sleep disruption. The third criterion was set to rule out the possibility that the identified gene was a sleep regulating gene like *inc* and *wake*.

Figure 2.3. Model for relationship between sleep and longevity
A. Correlation between daily sleep time and median lifespan. Data from wild type animals and sleep mutants are pooled and median lifespan is plotted against daily sleep time. Colors represent genotypes. *fmn* is highlighted with different shape due to absence of longevity reduction. Dashed line represents linear regression fit with all data points; Pearson's $r = 0.66$, $p < 0.001$.
B. Hypothesis about the relationship between sleep and longevity. Genetic mutations cause loss of sleep, which leads to changes in gene expression and eventually results in aging and longevity issues. Dashed lines represent additional possible mechanisms.

Figure 2.3


A
Median lifespan (day)
100
80
60
40
20
0
0
4
8
12
16
Daily sleep time (hour)
WT
inc1
wakeD2
wakeD1
sssP1
Sh5
HkY
Hk1
fmn
B
Genetic mutation
Sleep loss
Gene expression changes
Advanced aging
Longevity reduction

2.3 Experimental design of transcriptome profiling and multiple intersection

Gene expression profiling for sleep and longevity has gained attention in the past years. Microarray analysis was carried out to profile gene expression in spontaneously awake, sleep-deprived and sleeping flies (Cirelli et al., 2005b). Wakefulness- and sleep-related genes were discovered but the sleep-deprived group provided few insights, perhaps because the flies were only acutely deprived from sleep for 8 hours. More importantly, microarray analysis is highly biased towards genes with known identity and is not quantitative enough to detect minor changes in expression levels. A two-dimensional differential in-gel electrophoresis (2D-DIGE) of *sss* brain identified a neuron-glia interaction involving GABA transaminase (GABAT) (Chen et al., 2015). However, 2D-DIGE is limited by low resolution and insufficient genome coverage.

Next-generation sequencing has made it possible to detect transcriptome changes in an unbiased and quantitative manner with high resolution and coverage. RNA-seq using 5- and 55-day-old flies revealed a set of stress-response genes that adopted *de novo* rhythmicity during aging (Kuintzle et al., 2017). Gill et al. (2015) discovered that time-restricted feeding might extend longevity by improving sleep and cardiac function, and gene expression profiling using RNA-seq identified mitochondrial electron transport chain complexes as a potential pathway mediating the benefits of time-restricted feeding. For my thesis project, I decided to use RNA-seq as my approach to profile gene expression in fly heads.

As discussed above, the identified sleep genes are involved in various physiological processes, which raises the concern that there could be a significant portion of transcriptome changes that are related to their molecular functions independent of sleep or longevity. However, if multiple unrelated mutants are profiled and the results are subsequently intersected, it is possible to filter out changes related to their independent functions and enrich for patterns associated with sleep disruption.

I decided to profile two sleep mutants inc^{1} and $wake^{D2}$ for 3 reasons: 1) their sleep and longevity phenotypes were most stable across multiple experiments; 2) both mutants were viable enough to collect a large population of animals for sample preparation; 3) protein degradation (*inc*) and GABA signaling (*wake*) are very distinctive biological pathways. By comparing the profiling results of each mutant and then intersecting the findings, I would be able to identify the transcriptional changes that resulted from sleep disruption. Base on my hypothesis (**Figure 2.3B**), a subset of these changes could lead to advanced aging and shortened lifespan.

In order to further enrich genes that were associated with longevity regulation, the experimental groups included aged wild type flies that were much older than the control wild type animals and two sleep mutants. Aging related effects could be uncovered by comparison between aged wild type animals and control wild type animals. Intersection of all three experimental groups would provide the opportunity to study the changes in transcription that occurred both after chronic sleep deprivation and during aging. The genetic linkage between sleep and longevity could thus be revealed.

Before collecting RNA samples, I conducted longevity assays to determine the age for sample collection (**Figure 2.4A**). In these experiments, instead of only testing male flies, I kept male flies and female flies together over the course of the experiment. As expected, inc^{1} and $wake^{D2}$ flies exhibited significantly shortened lifespan compared to control animals. Based on the longevity curves, I decided to collect control wild type

group and two sleep mutant groups at Day 27, when mortality became evident in both sleep mutants. Aged wild type group was collected at Day 49, when wild type flies reached similar survivorship as that of *inc*^{1} flies at Day 27 (**Figure 2.4A**). Within-group comparison between male flies and female flies did not show significant difference for *WT* ($p > 0.05$) and *wake*^{D2} ($p > 0.05$). *inc*^{1} flies showed significant yet moderate sex-dependent difference in survivorship ($p < 0.05$) and the difference was more evident after Day 50. Same-sex comparison of sleep mutants against control animals all showed significantly reduced longevity in sleep mutants ($p < 0.0001$). Based on these results, I collected samples from pooled population of male and female flies. Four groups of samples were collected, *WT* at Day 27 as control group, *inc*^{1} at Day 27, *wake*^{D2} at Day 27, and *WT_old* at Day 49.

Meanwhile, I collected sleep data from *WT*, *inc*^{1}, and *wake*^{D2} flies throughout their lifetime (**Figure 2.4B**). *WT* flies showed mildly increased levels of sleep during aging. The daily sleep time of *wake*^{D2} animals fluctuated around a fixed level. Most strikingly, total sleep of *inc*^{1} flies increased dramatically (> 60%) as animals aged. This effect could be explained by survivorship bias; flies that managed to get more sleep were more likely to live longer.

Because circadian rhythm serves a pivotal function in sleep regulation, I was also interested in the expression changes of circadian clock genes. Samples were collected every 4 hours over 2 days of 12-hour light/12-hour dark (LD) cycles, yielding 12 timepoints per genotype. In summary, the RNA-seq experiments contained 4 groups with 12 samples in each group, totaling 48 samples.

After cleaning up the RNA-seq datasets and excluding genes with extremely low expression levels, the samples showed high within-group correlations (Pearson's $r > 0.9$) in all 4 groups (**Figure 2.5A**). Furthermore, principle components analysis (PCA) showed nice separation among genotypes and interestingly, samples from *WT* control animals located in the middle region of the plots, indicating that each experimental group was closest to *WT* group (**Figure 2.5B**). These results demonstrated good quality of RNA-seq data.

Figure 2.4. Longevity and sleep phenotypes of profiled flies
A. Longevity of *WT* (black), inc^1 (red), and $wake^{D2}$ (green) male (dashed line) and female (dotted line) flies. Vertical lines indicate dates of sample collection. n = 140-150 for all groups. For same genotype log-rank test, $p > 0.05$ for *WT* and $wake^{D2}$, $p < 0.05$ for inc^1. For cross genotype log-rank test, $p < 0.0001$ for inc^1 and $wake^{D2}$ against *WT* for both males and females.
B. Average total sleep per day of *WT* (black), inc^1 (red), and $wake^{D2}$ (green) male flies over lifetime. Each data point represents flies of corresponding age that were loaded and measured for sleep over 5 consecutive days. Data collected from different loadings are connected by solid line. Mean ± SEM is shown. n = 26-32 for *WT*, n = 4-32 for inc^1, n = 11-32 for $wake^{D2}$.

Figure 2.4

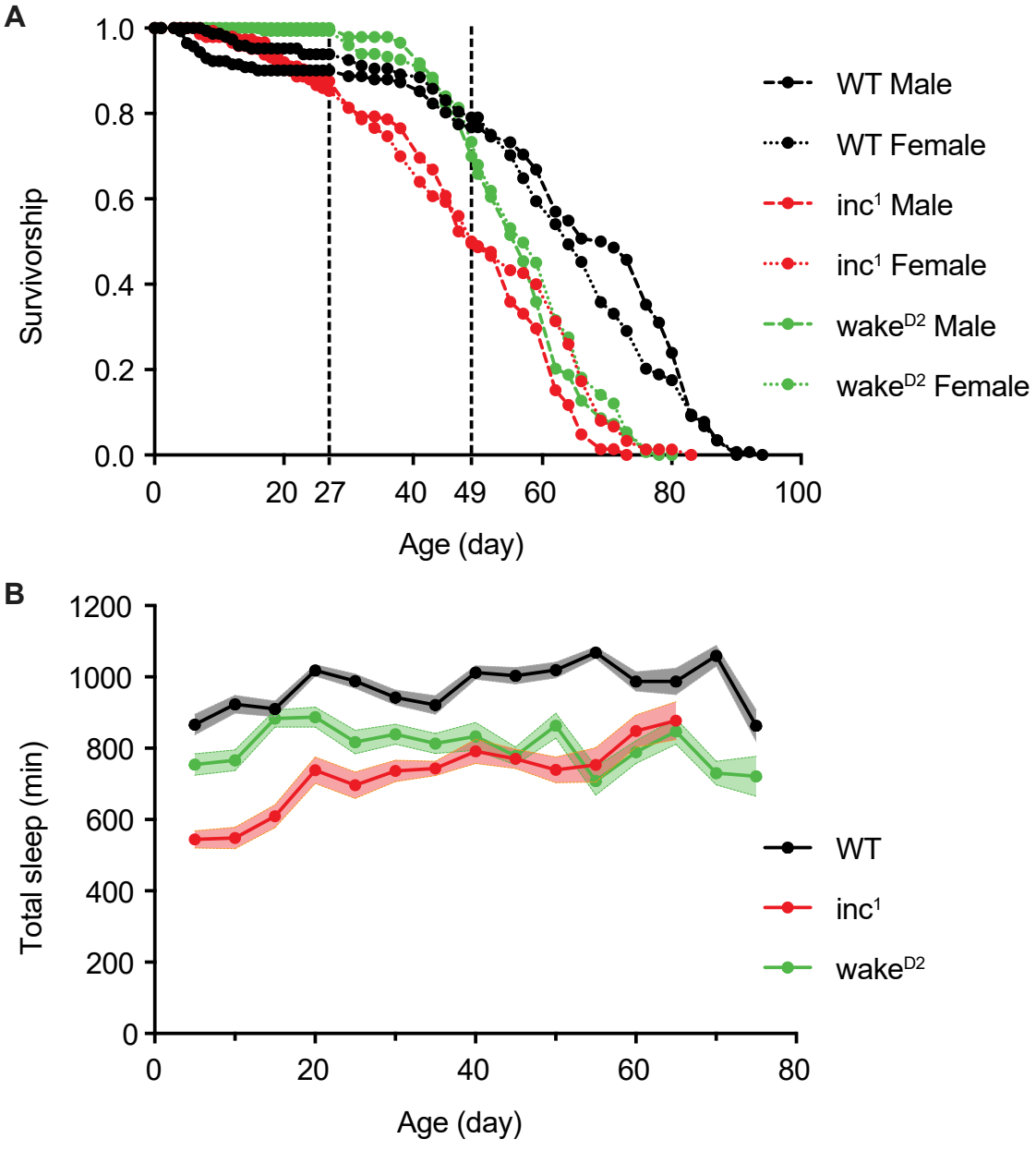
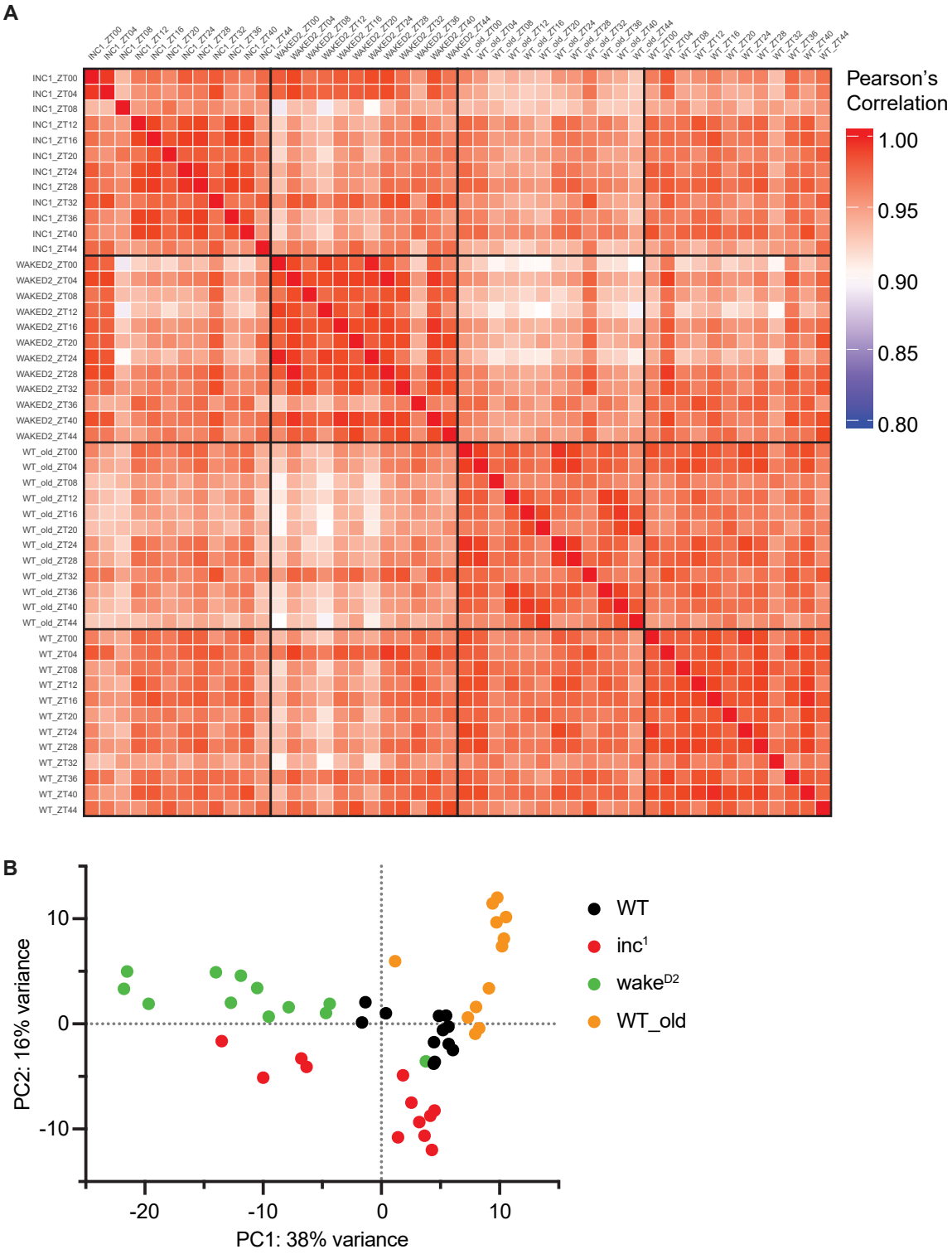

A
1.0
0.8
0.6
0.4
0.2
0.0
Survivorship
0
20
27
40
49
60
80
100
Age (day)
WT Male
WT Female
inc1 Male
inc1 Female
wakeD2 Male
wakeD2 Female
B
1200
1000
800
600
400
200
0
Total sleep (min)
0
20
40
60
80
Age (day)
WT
inc1
wakeD2

Figure 2.5. Quality check for RNA sequencing results
A. Heatmap showing gene expression Pearson's correlation for all RNA-seq datasets.
B. Principle components analysis for all RNA-seq datasets.

Figure 2.5


A
Pearson's Correlation
1.00
0.95
0.90
0.85
0.80
B
WT
inc1
wakeD2
WT_old
PC2: 16% variance
PC1: 38% variance
10
0
-10
-20
-10
0
10

CHAPTER 3. ANALYSIS OF TRANSCRIPTOME SCALE CHANGES OF CIRCADIAN RHYTHM

Animal's locomotion activities show circadian oscillation. Humans, like other diurnal animals, are more active during the day and rest during the night. On the other hand, nocturnal animals such as rats and mice have opposite locomotion patterns. The expression levels of some genes in our genome also exhibit circadian oscillation, behaving like a sine wave (with a phase shift).

Because circadian clock is a major regulator of sleep behavior as illustrated by the two-process model, I was curious about the transcriptome changes affecting the circadian oscillating genes during sleep deprivation as well as aging. In this chapter I first analyzed the circadian clock of each group to identify the genes that showed circadian oscillation. Next, I investigated the changes in rhythmic gene expression resulted from aging or sleep disruption. Finally, the findings were studied in closer detail to identify gene expression changes that were shared in all experimental groups.

3.1 Identification of oscillating genes using circadian analysis

Before digging into the RNA-seq dataset for oscillating genes, I first looked at the core circadian clock genes, *per*, *tim*, *Clk*, and *cry* (**Figure 3.1**). It was clear that the Reads Per Kilobase of transcript, per Million mapped reads (RPKM) from the RNA-seq dataset for all 4 core clock genes oscillated with a 24-hour period in all 4 groups. *per* and *tim* showed peak expression at Zeitgeber time (ZT) 12, when lights went off. Clk displayed the opposite phase, peaking around lights-on time ZT0, and expression levels of cry oscillated with peak expression at ZT4.

Although the results indicated that the central clock was intact in sleep mutants and aged flies, some significant changes were observed (**Figure 3.1**). inc^{1} flies showed significantly lower expression levels of *per* (adjusted $p < 0.0001$), *tim* (adjusted $p < 0.0001$), and *cry* (adjusted $p < 0.0001$). In $wake^{D2}$, *cry* was reduced compared to *WT* (adjusted $p < 0.0001$). Aging had little effect on the core clock gene besides a mild reduction in the expression levels of *Clk* (adjusted $p < 0.05$). These results suggested that sleep disruption and aging could affect circadian gene oscillation, but the effects might be context dependent due to differences in affected core clock genes.

Multiple bioinformatic algorithms are available for discovering oscillating genes in large profiling data, including ARSER (Yang and Su, 2010), JTK_cycle (Hughes et al., 2010), GeneCycle (Ahdesmaki et al., 2007; Ahdesmaki et al., 2005; Wichert et al., 2004), etc. Each algorithm comes with its advantages and disadvantages (Deckard et al., 2013; Wu et al., 2014). For example, ARSER is believed to be less influenced by noise but has low power on data with less timepoints; JTK_cycle offers high computational efficiency yet relatively higher false negative rate.

I tested the RNA-seq datasets with ARSER, JTK_cycle, and GeneCycle at threshold of $p < 0.05$. The number of genes that were found to be oscillating varied greatly in different algorithms. Using RNA-seq data from *WT* samples, ARSER, JTK_cycle, and GeneCycle identified 2180, 977, and 3057 oscillating genes, respectively (**Figure 3.2**). JTK_cycle found the least number of cycling genes, suggesting relatively higher false negative rate. On the other hand, GeneCycle seemed to be the least restrictive method, identifying over 3000 oscillating genes. A large proportion of the oscillating gene lists were shared; 706 genes were found to be rhythmic in all algorithms, indicating robustness and reliability of the algorithms.

Genes that were detected to be significantly oscillating in at least two programs were considered as oscillating (Koike et al., 2012). Using this criterion, I found that 1115 genes exhibited robust oscillation in the control *WT* flies, which was comparable to previous studies (**Figure 3.2**). Using similar experimental approaches, Hughes et al. (2012) identified 870 oscillating transcripts from 3-5-day old flies using JTK_cycle; in the study by Kuintzle et al. (2017), ARSER identified 2036 rhythmic genes in 5-day-old wild type flies. These findings not only emphasized the necessity of using multiple programs, but also indicated that the number of oscillating genes in 5-day-old wild type flies was close to that in 27-day-old flies, the control group used in this study.

Figure 3.1. Expression of core circadian clock genes
Reads Per Kilobase of transcript, per Million mapped reads (RPKM) of *period* (**A**), *timeless* (**B**), *Clock* (**C**), and *cryptochrome* (**D**) for *WT* (black), inc^{1} (red), $wake^{D2}$ (green), and *WT_old* (orange). x axis represents two 12-hour light/12-hour dark (LD) cycles with white bars representing daytime and black bars representing nighttime.
For *period*, **** inc^{1}; n.s. $wake^{D2}$ and *WT_old*. For *timeless*, **** inc^{1}; n.s. $wake^{D2}$ and *WT_old*. For *Clock*, * *WT_old*; n.s. $inc^{1}$and $wake^{D2}$. For cryptochrome, **** $inc^{1}$and $wake^{D2}$; n.s. *WT_old*. All comparisons were against *WT*, using adjusted p values calculated by DESeq2 for time-series analysis (Love et al., 2014). * $p < 0.05$; **** $p < 0.0001$; n.s., not significant.

Figure 3.1

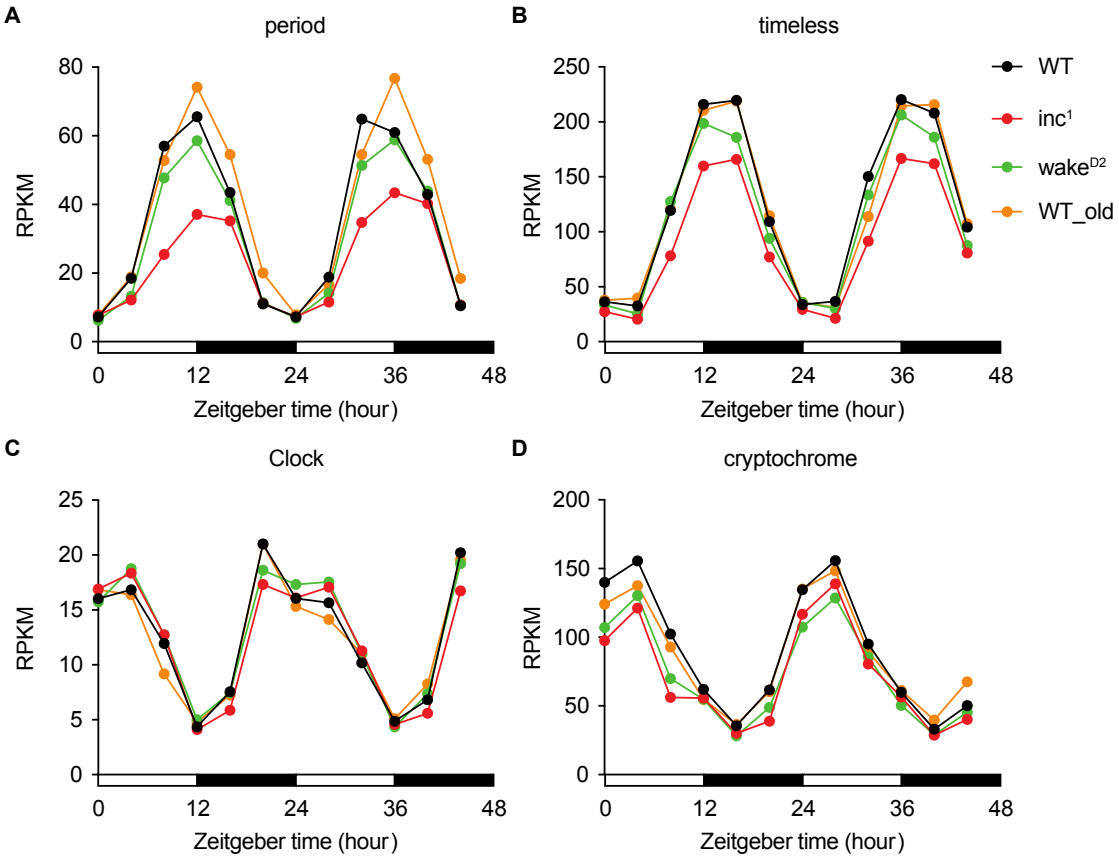
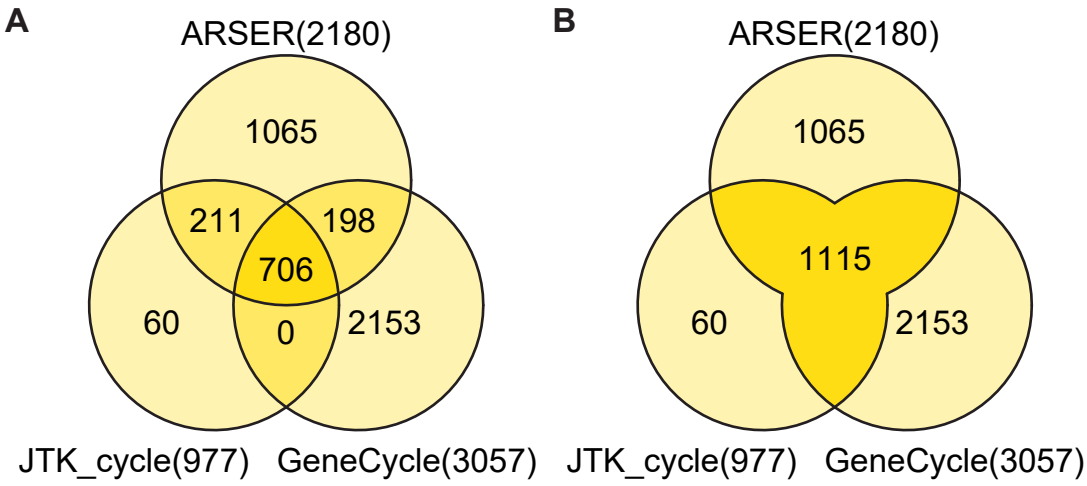

A
period
RPKM
Zeitgeber time (hour)
B
timeless
RPKM
Zeitgeber time (hour)
WT
inc1
wakeD2
WT_old
C
Clock
RPKM
Zeitgeber time (hour)
D
cryptochrome
RPKM
Zeitgeber time (hour)

Figure 3.2. Identification of oscillating genes in *WT* RNA-seq dataset
A. Intersection of identified oscillating gene lists using ARSER, JTK_cycle, and GeneCycle with threshold of $p < 0.05$.
B. 1115 genes were considered oscillating, whose expression levels were found to be significantly oscillating in at least two algorithms.

Figure 3.2


A
ARSER(2180)
1065
211
198
706
60
0
2153
JTK_cycle(977)
GeneCycle(3057)
B
ARSER(2180)
1065
1115
60
2153
JTK_cycle(977)
GeneCycle(3057)

3.2 Overall circadian gene oscillation in control and experimental groups

After identifying the oscillating genes in *WT* group, I plotted the expression levels of all 1115 oscillating genes at every timepoint with heatmap and ordered the genes according to their peak expression time (**Figure 3.3A**). This result demonstrated the waves of gene expression with alternating red and blue stripes representing the phases of rhythmically expressed genes. Interestingly, instead of producing vertical stripes, the gene expression ran diagonally across the panel. This phenomenon indicated that the expression levels of cycling genes were not oscillating with the same phase (day and night), but rather every hour of the day was marked by a unique pattern of gene oscillation.

Next, I produced similar heatmaps using RNA-seq data from the experimental groups for the same set of 1115 rhythmic genes identified in *WT* (**Figures 3.3B-D**). The genes were aligned in the same order as those of *WT*. From these data, the genes were mostly still oscillating with the same phase as the *WT* group. However, differences between peak and trough, or the amplitude of oscillation, became relatively smaller, as indicated by the reduced appearance of dark red and dark blue. Moreover, the alignment was less organized, suggesting phase shift or rhythmicity change for some genes.

To study the changes in rhythmicity of oscillating genes, I performed the same analysis illustrated in previous section to identify oscillating transcripts in three experimental groups, *WT_old*, inc^1, and $wake^{D2}$ (**Figure 3.4**). To my surprise, in *WT_old* 1583 genes were found to be oscillating, 40% more than the number of oscillating genes in *WT*. This result indicated a large scale *de novo* gain of rhythmicity during aging (Kuintzle et al., 2017). Meanwhile, a smaller number of genes were identified as rhythmic in the two sleep mutants, 663 in inc^1 and 809 in $wake^{D2}$, suggesting a strong disruption of circadian gene oscillation compared to *WT*.

The heatmaps also suggested differences in phase distribution of gene oscillation. As demonstrated by the circular histograms representing the distribution of peak expression time of oscillating genes, the time with highest number of gene expression peaks shifted from ZT0 in *WT* to ZT6 in *WT_old* (**Figures 3.5A** and **B**). Such dramatic difference was not observed in inc^1 and $wake^{D2}$, whose phase distribution resembled that of *WT* but with a lower magnitude (**Figures 3.5C** and **D**).

Taken together, these analyses indicated that the effects on circadian gene oscillation of aging and sleep disruption were different. Aging reshaped the circadian rhythm by gain of rhythmicity and phase shift of oscillating genes. On the other hand, mutations of *inc* and *wake* perturbed the circadian rhythm and resulted in loss of rhythmicity for some oscillating genes.

Figure 3.3. Heatmaps of gene expression for genes oscillating in *WT* group
A. Heatmap of RNA-seq gene expression for the 1115 genes that are identifies as rhythmic in *WT* group.
B-**D**. Heatmap for the same set of genes as in **A** using RNA-seq data from *WT_old* (**B**), inc^{1} (**C**), and $wake^{D2}$ (**D**). Data were aligned in the same order as in **A**.
Each row represents one gene and each column represents one timepoint. Expression levels are normalized to each gene itself and data are aligned according to the peak expression time in *WT* group calculated by JTK_cycle. The blocked horizontal bars below the panel correspond to the light-dark cycle.

Figure 3.3

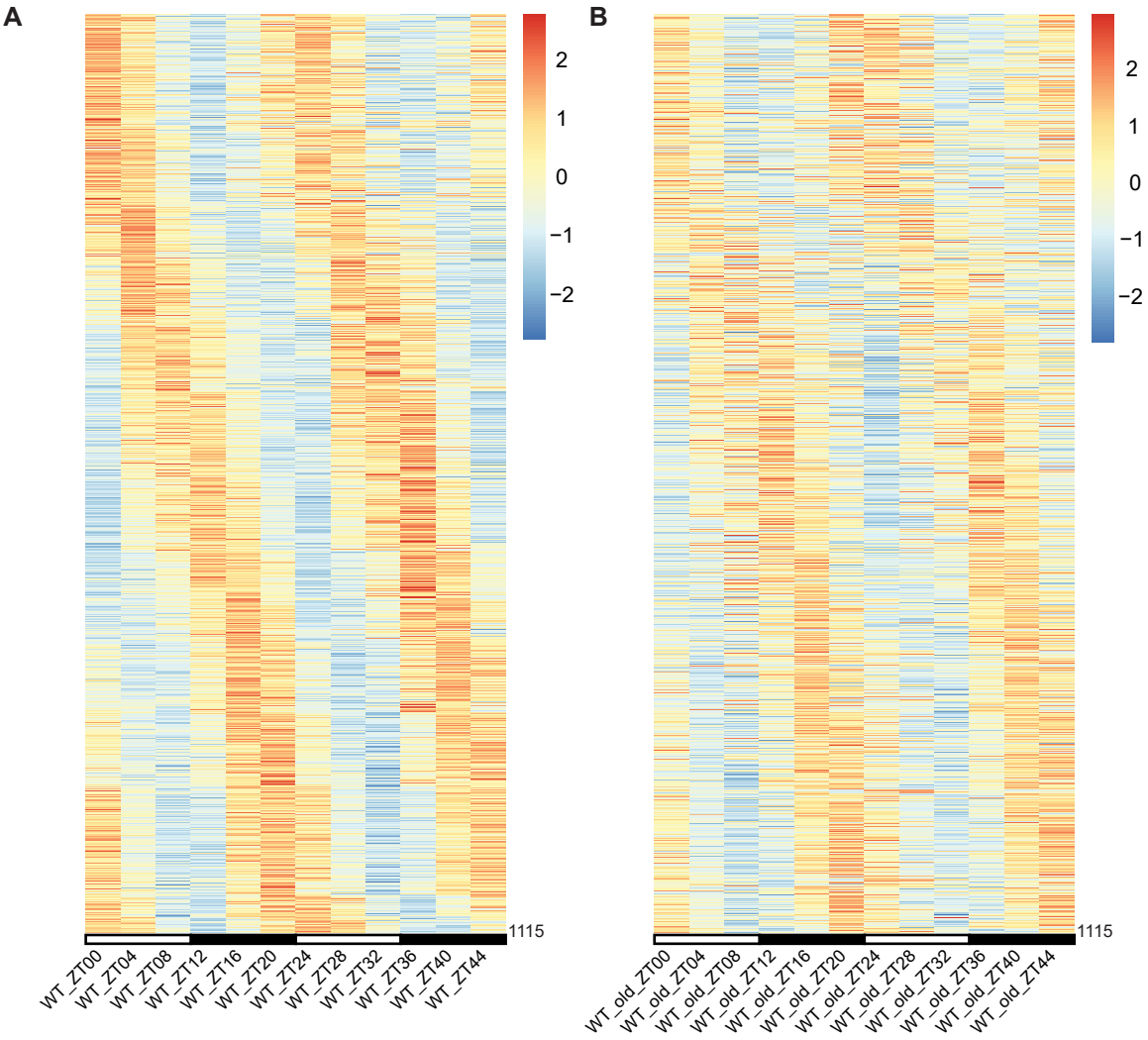

A
2
1
0
−1
−2
1115
WT_ZT00
WT_ZT04
WT_ZT08
WT_ZT12
WT_ZT16
WT_ZT20
WT_ZT24
WT_ZT28
WT_ZT32
WT_ZT36
WT_ZT40
WT_ZT44
B
2
1
0
−1
−2
1115
WT_old_ZT00
WT_old_ZT04
WT_old_ZT08
WT_old_ZT12
WT_old_ZT16
WT_old_ZT20
WT_old_ZT24
WT_old_ZT28
WT_old_ZT32
WT_old_ZT36
WT_old_ZT40
WT_old_ZT44

Figure 3.3 (continued)

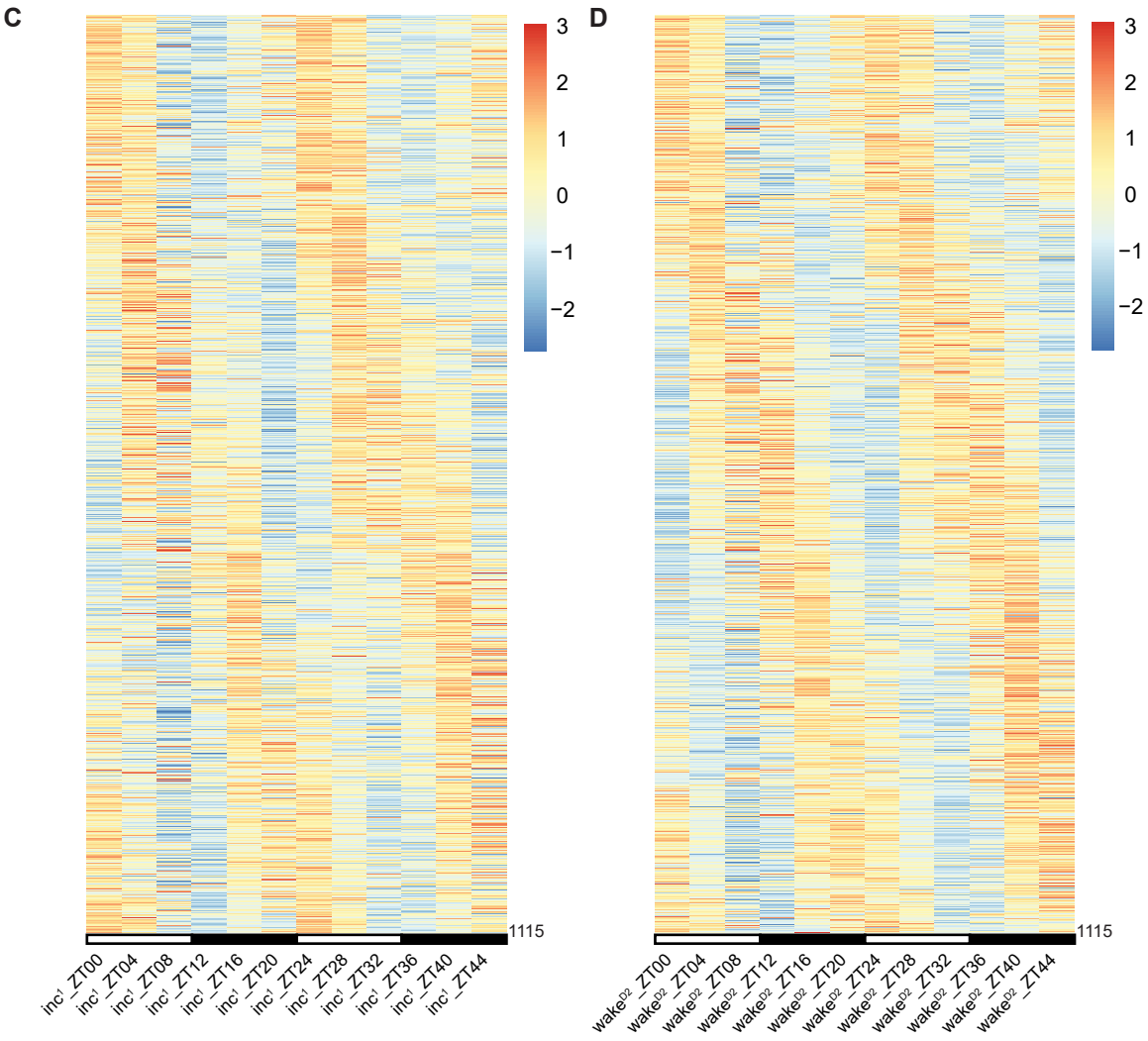


Figure 3.4. Heatmaps of gene expression oscillation in *WT_old*, *inc*^{1}, and *wake*^{D2}
A. Heatmap of RNA-seq gene expression for the 1583 rhythmic genes in *WT_old* group.
B. Heatmap of RNA-seq gene expression for the 663 rhythmic genes in *inc*^{1} group.
C. Heatmap of RNA-seq gene expression for the 809 rhythmic genes in *wake*^{D2} group.
Each row represents one gene and each column represents one timepoint. Expression levels are normalized to each gene itself and data are aligned according to the peak expression time calculated by JTK_cycle. The blocked horizontal bars below the panel correspond to the light-dark cycle.

Figure 3.4

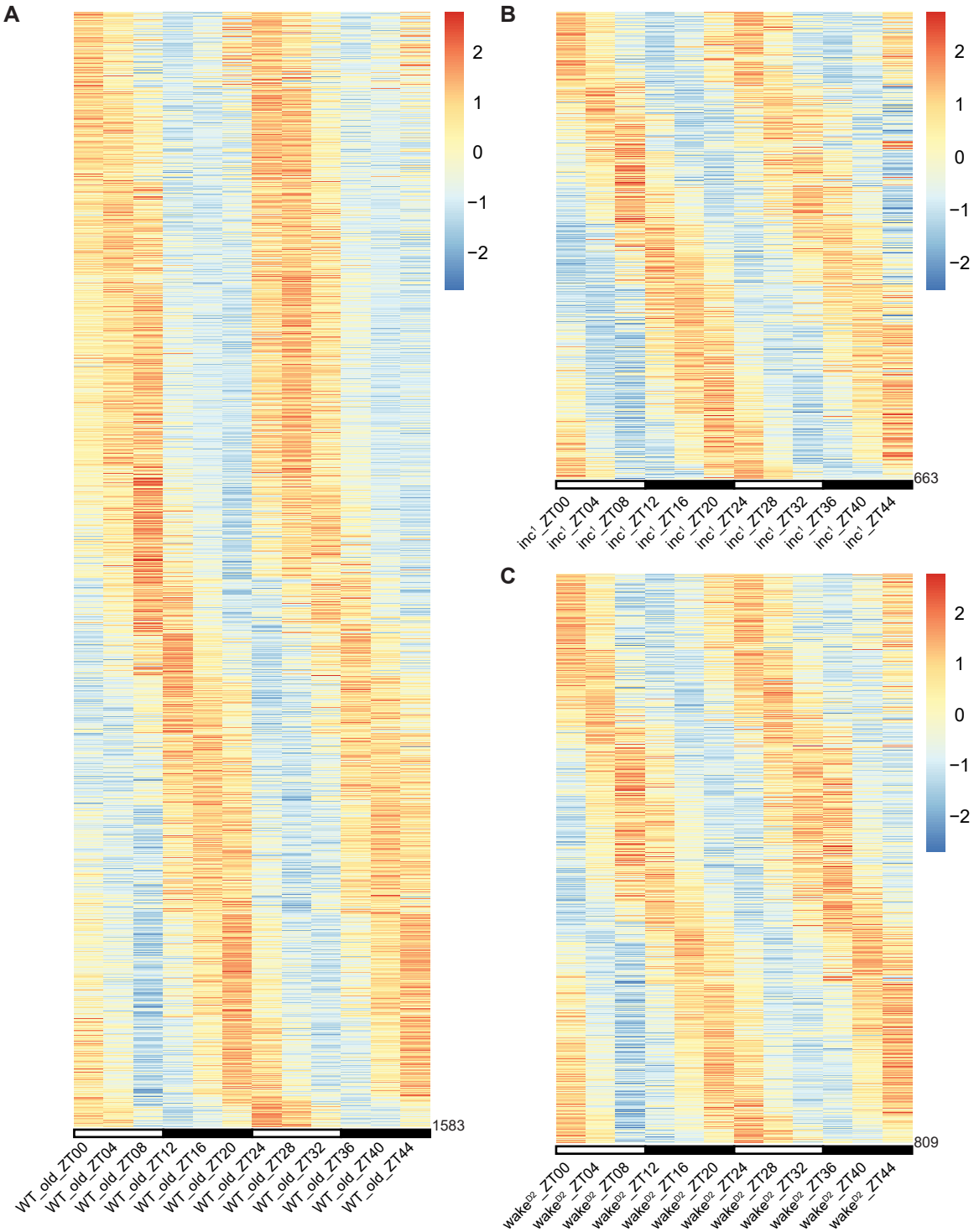
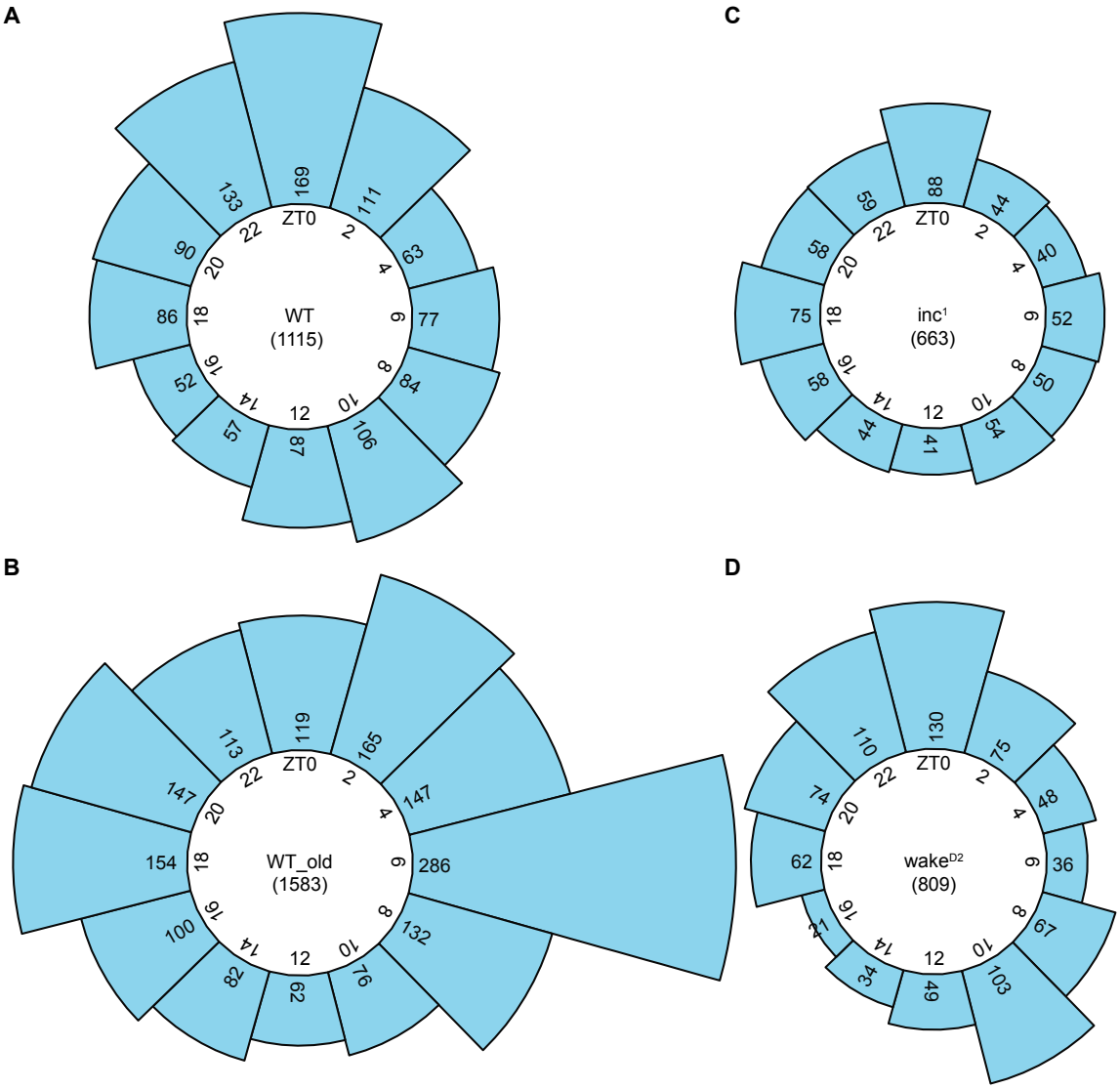

A
2
1
0
−1
−2
1583
WT_old_ZT00
WT_old_ZT04
WT_old_ZT08
WT_old_ZT12
WT_old_ZT16
WT_old_ZT20
WT_old_ZT24
WT_old_ZT28
WT_old_ZT32
WT_old_ZT36
WT_old_ZT40
WT_old_ZT44
B
2
1
0
−1
−2
663
inc¹_ZT00
inc¹_ZT04
inc¹_ZT08
inc¹_ZT12
inc¹_ZT16
inc¹_ZT20
inc¹_ZT24
inc¹_ZT28
inc¹_ZT32
inc¹_ZT36
inc¹_ZT40
inc¹_ZT44
C
2
1
0
−1
−2
809
wake^D2_ZT00
wake^D2_ZT04
wake^D2_ZT08
wake^D2_ZT12
wake^D2_ZT16
wake^D2_ZT20
wake^D2_ZT24
wake^D2_ZT28
wake^D2_ZT32
wake^D2_ZT36
wake^D2_ZT40
wake^D2_ZT44

Figure 3.5. Distribution of peak expression time for oscillating genes
Circular histogram for the distribution of peak expression time for rhythmic genes in *WT* (**A**), *WT_old* (**B**), inc^{1} (**C**), and $wake^{D2}$ (**D**). Peak expression times are calculated using JTK_cycle. Numbers in the inner circle represent Zeitgeber time (ZT) and numbers in the bars represent number of genes in the corresponding bar. Total number of oscillating genes are labeled in parentheses.

Figure 3.5


A
ZT0
2
4
6
8
10
12
14
16
18
20
22
WT
(1115)
169
111
63
77
84
106
87
57
52
86
90
133
C
ZT0
inc1
(663)
88
44
40
52
50
54
41
44
58
75
58
59
B
ZT0
WT_old
(1583)
119
165
147
286
132
76
62
82
100
154
147
113
D
ZT0
wake D2
(809)
130
75
48
36
67
103
49
34
74
62
110

3.3 Comparison of circadian gene oscillation in control group and aged group

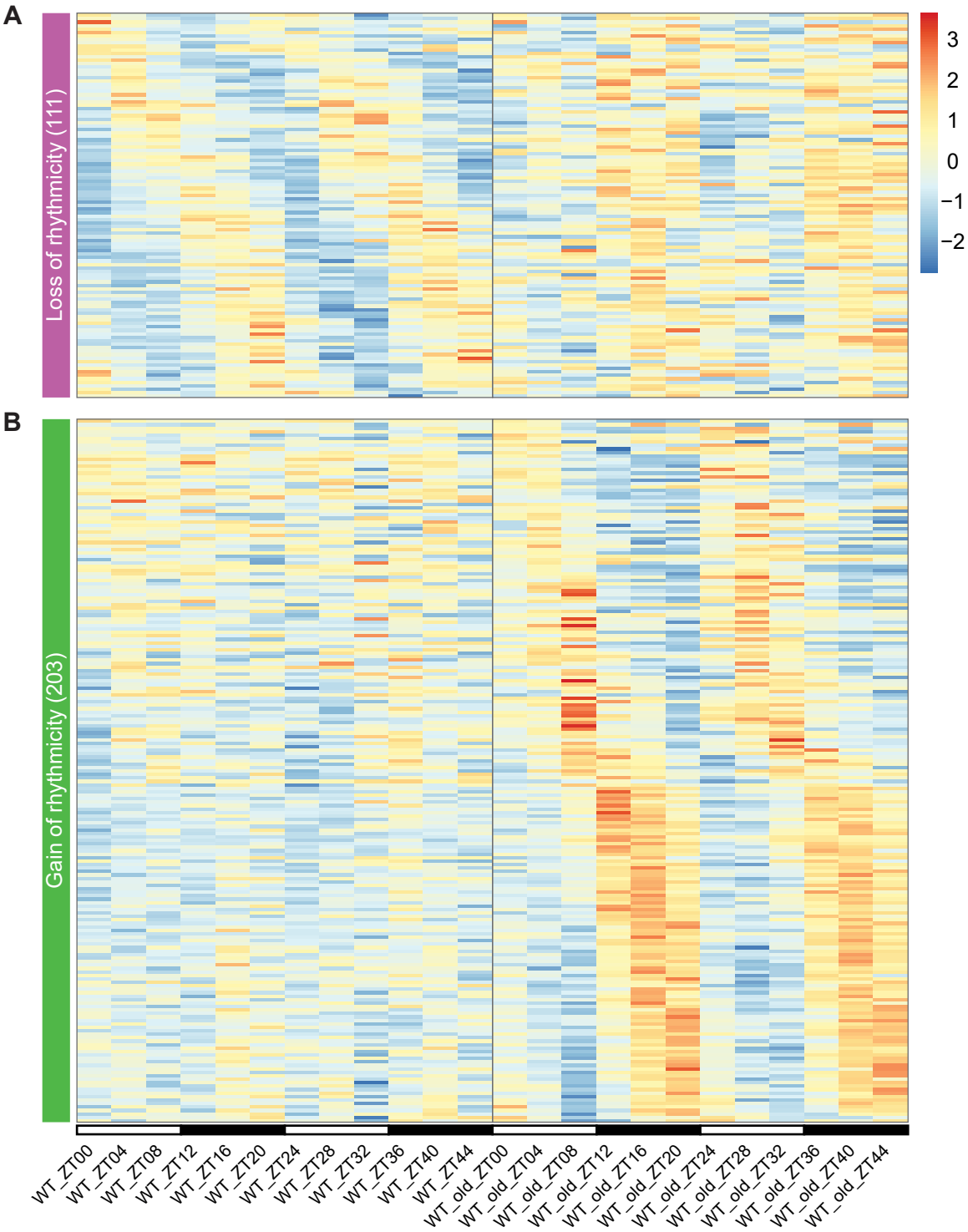
To further investigate the effects of aging on circadian gene oscillation, I compared the lists of rhythmic genes in *WT* and *WT_old* groups. As results in previous section suggested, a set of genes gained *de novo* rhythmicity in aged flies, so I was interested in the genes that were found to be rhythmic in one group but arrhythmic in the other.

Circadian analysis has been known to carry false discoveries (Deckard et al., 2013; Wu et al., 2014). To minimize the effect of false discoveries, results from JTK_cycle were used because this program offers low false positive rate. Out of the 977 oscillating genes identified by JTK_cycle, 917 genes (93.9%) were also identified by either ARSER or GeneCycle (**Figure 3.2A**). Furthermore, highly rhythmic genes were defined as adjusted $p < 0.01$ and highly arrhythmic genes as $p > 0.5$. With these criteria, I identified 557 highly rhythmic genes and 6704 highly arrhythmic genes in *WT* group, and 619 highly rhythmic genes and 5852 highly arrhythmic genes in *WT_old* group.

By intersecting the lists of highly rhythmic genes in one group and highly arrhythmic genes in another group, I discovered genes that lost rhythmicity (111) and genes that gained rhythmicity (203) during aging (**Figure 3.6**). Consistent with previous discussions, there were more genes in the gain of rhythmicity group than the loss of rhythmicity group. Interestingly, the peak expression levels of the genes in the gain of rhythmicity group seemed to be higher in the aged group compared to the control group. Genes that lost rhythmicity showed random fluctuations in the aged group. These results again indicated that although the central clock mostly remained unchanged during aging, the landscape of gene oscillation experienced dramatic modulation.

Figure 3.6. Change of rhythmicity during aging
A. Genes that are highly rhythmic in *WT* and highly arrhythmic in *WT_old*.
B. Genes that are highly rhythmic in *WT_old* and highly arrhythmic in *WT*.
Highly rhythmic genes are defined as adjusted $p < 0.01$ and high arrhythmic genes are defined as adjusted $p > 0.5$. Adjust p values are calculated by JTK_cycle. Each row represents one gene and each column represents one timepoint. Expression levels are normalized to each gene itself and data are aligned according to the peak expression time in the highly rhythmic group calculated by JTK_cycle. The blocked horizontal bars below the panel correspond to the light-dark cycle.

Figure 3.6


A
Loss of rhythmicity (111)
B
Gain of rhythmicity (203)
3
2
1
0
−1
−2
WT_ZT00
WT_ZT04
WT_ZT08
WT_ZT12
WT_ZT16
WT_ZT20
WT_ZT24
WT_ZT28
WT_ZT32
WT_ZT36
WT_ZT40
WT_ZT44
WT_old_ZT00
WT_old_ZT04
WT_old_ZT08
WT_old_ZT12
WT_old_ZT16
WT_old_ZT20
WT_old_ZT24
WT_old_ZT28
WT_old_ZT32
WT_old_ZT36
WT_old_ZT40
WT_old_ZT44

3.4 Comparison of circadian gene oscillation in control group and sleep mutants

I performed the same rhythmicity analysis to compare two sleep mutants individually with *WT* control group (**Figure 3.7**). Interestingly, the results were similar between the two mutants. Compared to *WT*, 161 and 153 genes lost rhythmicity in inc^{1} and $wake^{D2}$, respectively. The gain of rhythmicity group was much smaller, 53 genes for inc^{1} and 56 genes for $wake^{D2}$. These results indicated that in both sleep mutants, circadian gene oscillation was disrupted. Compared to the results of *WT_old*, a slightly larger number of genes lost rhythmicity whereas genes that gained rhythmicity were only about 25% of those observed in *WT_old* dataset.

It was intriguing that the numbers were close in the two sleep mutants, so I intersected the results to test if there were genes that changed rhythmicity in both sleep mutants. More than 50% of the genes that lost rhythmicity were shared between inc^{1} and $wake^{D2}$ (**Figure 3.8A**). Strikingly, however, only less than 10% of the genes that gained rhythmicity were shared (**Figure 3.8B**). These data suggested that majority of the rhythmicity loss possibly resulted from sleep disruption, while the gain of rhythmicity effect was more likely to relate to the molecular function of the mutated genes.

Because the purpose of this project was to identify gene expression changes that occurred both during aging and after sleep disruption, I further looked for consistent changes of rhythmicity in all experimental groups. Although there were fewer genes in the loss of rhythmicity group than genes in the gain of rhythmicity group in *WT_old*, the intersection of genes with rhythmicity loss yielded a much larger subset of genes, consisting of 34 genes (**Figure 3.8C**). On the other hand, none of the gain of rhythmicity genes overlapped across the three experimental groups (**Figure 3.8D**), indicating that the shortened longevity observed in sleep mutants was not mediated by *de novo* gain of rhythmicity activities.

The 34 genes with rhythmicity change in all experimental groups constituted the list of candidate genes from the circadian analysis (**Table 3.1**). Over 50%, or 19 genes were uncharacterized. Among the genes with known function, several biological pathways related to longevity regulation were present. *presenilin enhancer* (*pen-2*) is believed to be required for notch pathway signaling and activity of γ-secretase, a critical player in the development of Alzheimer's disease (Francis et al., 2002). *Pyrokinin 1 receptor* (*PK1-R*) encodes a G-protein coupled receptor (GPCR) and is thought to regulate the production and release of insulin (Alfa et al., 2015). Furthermore, TBC1 domain family member 7 (TBC1d7) has been shown to regulate longevity via regulating the biosynthesis and release of insulin-like peptide 2 (Ilp2) (Ren et al., 2018). Several development related genes and cell cycle related genes were also on the list. These genes are worthy of further investigation for their potential function in the relationship between sleep and longevity.

Figure 3.7. Change of rhythmicity in sleep mutants *inc*^{1}, and *wake*^{D2}
A. Genes that are highly rhythmic in *WT* and highly arrhythmic in *inc*^{1}.
B. Genes that are highly rhythmic in *inc*^{1} and highly arrhythmic in *WT*.
C. Genes that are highly rhythmic in *WT* and highly arrhythmic in *wake*^{D2}.
D. Genes that are highly rhythmic in *wake*^{D2} and highly arrhythmic in *WT*.
Highly rhythmic genes are defined as adjusted $p < 0.01$ and high arrhythmic genes are defined as adjusted $p > 0.5$. Adjust p values are calculated by JTK_cycle. Each row represents one gene and each column represents one timepoint. Expression levels are normalized to each gene itself and data are aligned according to the peak expression time in the highly rhythmic group calculated by JTK_cycle. The blocked horizontal bars below the panel correspond to the light-dark cycle.

Figure 3.7

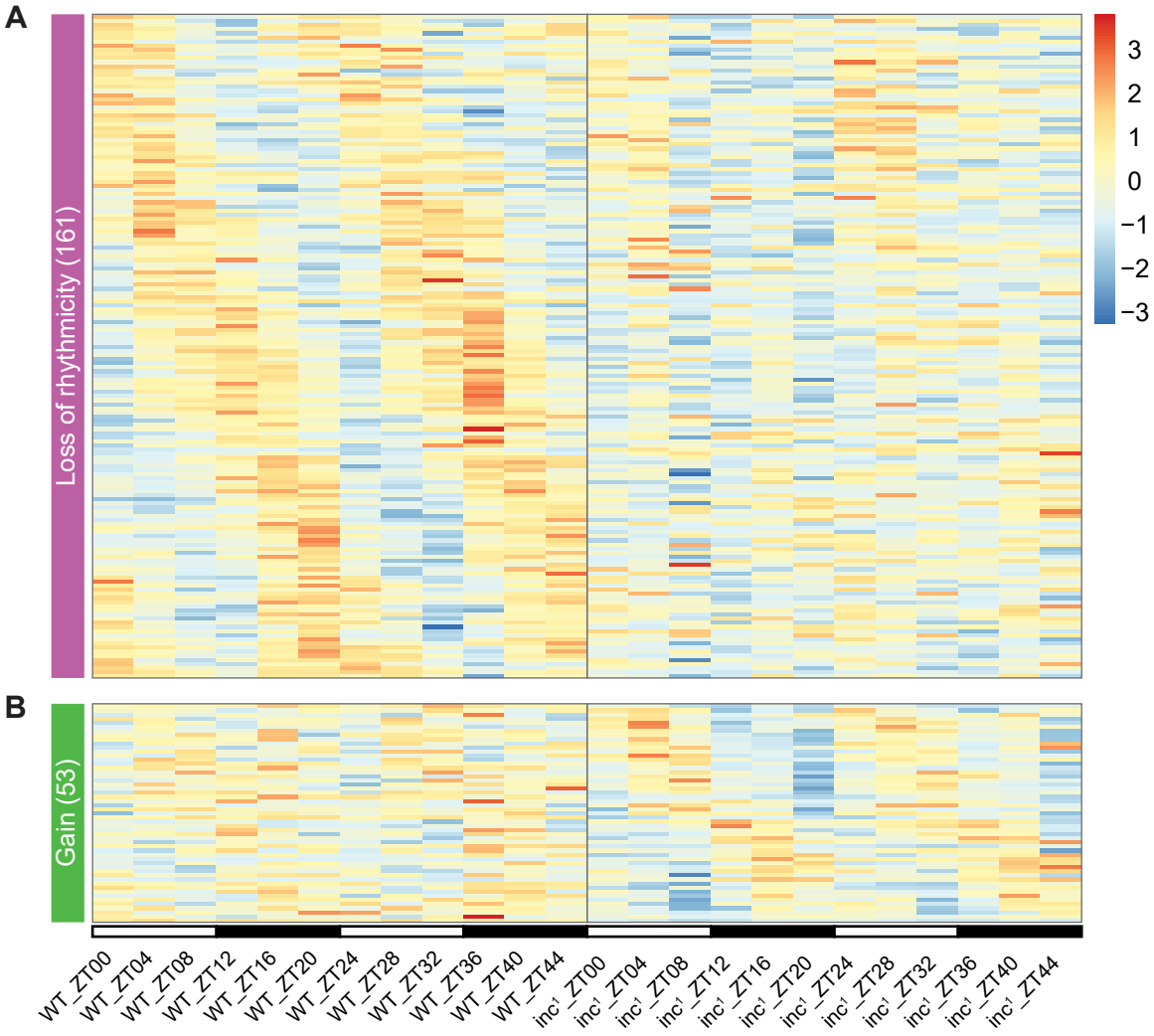

A
Loss of rhythmicity (161)
B
Gain (53)
3
2
1
0
−1
−2
−3
WT_ZT00
WT_ZT04
WT_ZT08
WT_ZT12
WT_ZT16
WT_ZT20
WT_ZT24
WT_ZT28
WT_ZT32
WT_ZT36
WT_ZT40
WT_ZT44
inc⁻¹_ZT00
inc⁻¹_ZT04
inc⁻¹_ZT08
inc⁻¹_ZT12
inc⁻¹_ZT16
inc⁻¹_ZT20
inc⁻¹_ZT24
inc⁻¹_ZT28
inc⁻¹_ZT32
inc⁻¹_ZT36
inc⁻¹_ZT40
inc⁻¹_ZT44

Figure 3.7 (continued)

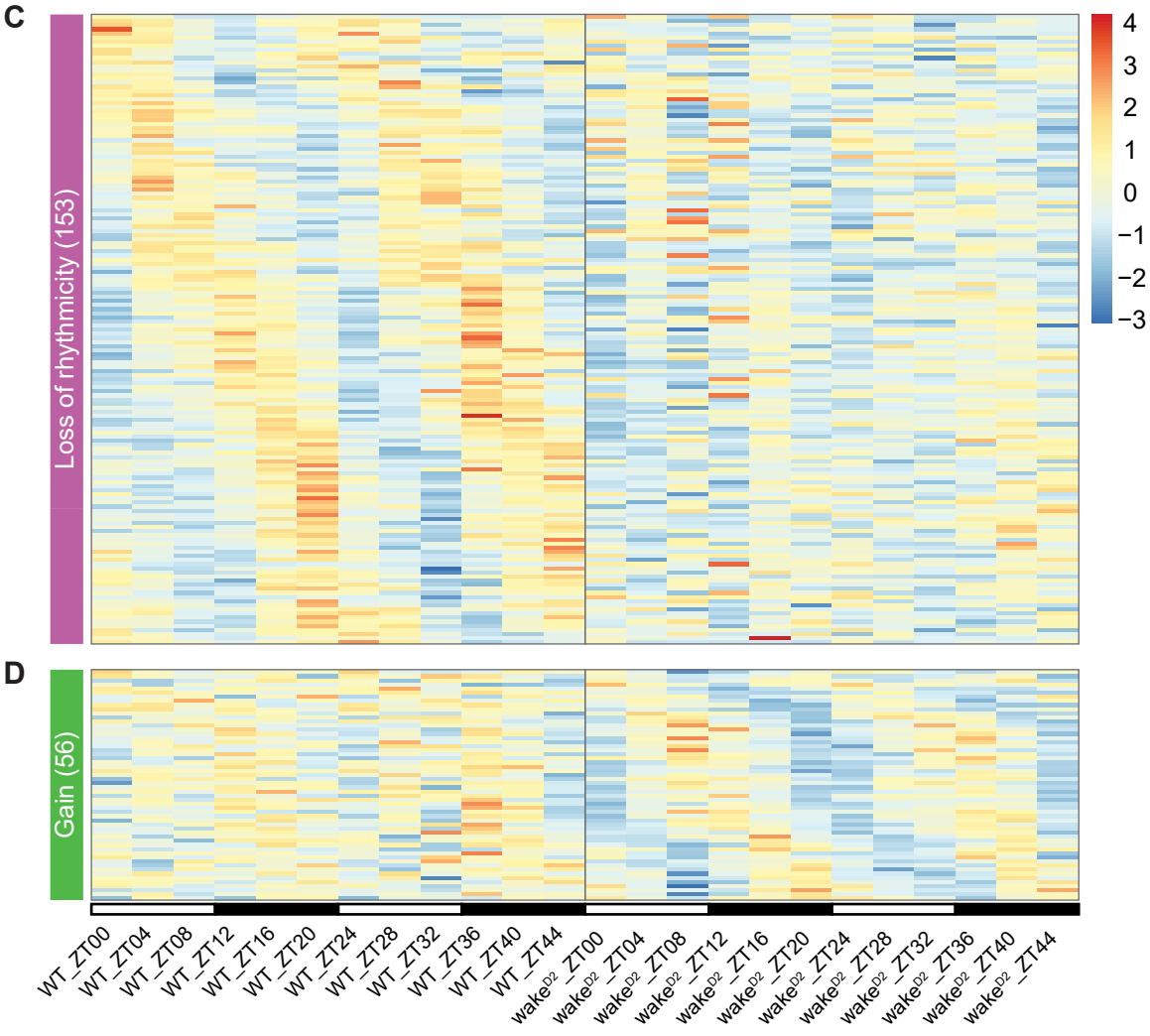


Figure 3.8. Intersection for genes with altered rhythmicity
A. Intersection of loss of rhythmicity genes in *inc*^{1} (red) and *wake*^{D2} (green).
B. Intersection of gain of rhythmicity genes in *inc*^{1} (red) and *wake*^{D2} (green).
C. Intersection of loss of rhythmicity genes in *inc*^{1} (red), *wake*^{D2} (green), and *WT_old* (orange).
D. Intersection of gain of rhythmicity genes in *inc*^{1} (red), *wake*^{D2} (green), and *WT_old* (orange).

Figure 3.8

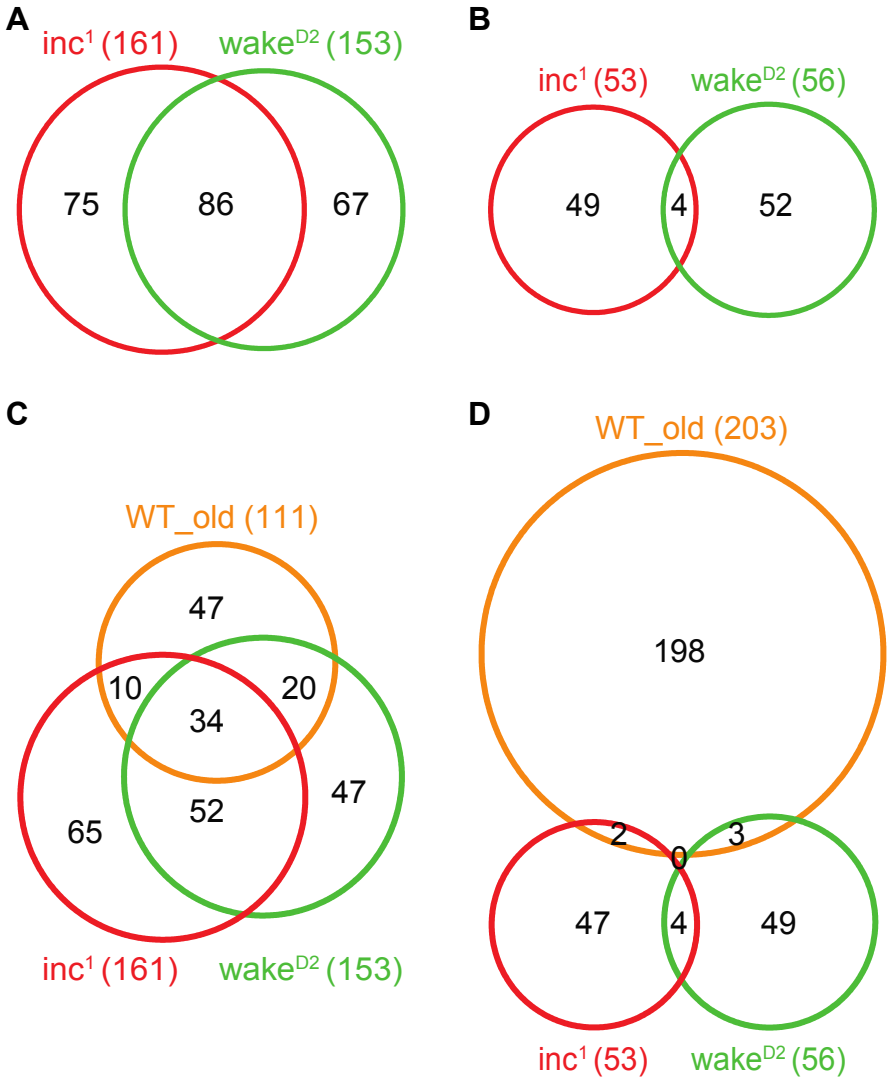

A
inc1 (161)
wakeD2 (153)
75
86
67
B
inc1 (53)
wakeD2 (56)
49
4
52
C
WT_old (111)
47
10
20
34
52
47
65
inc1 (161)
wakeD2 (153)
D
WT_old (203)
198
2
0
3
47
4
49
inc1 (53)
wakeD2 (56)

Table 3.1. Genes with rhythmicity change in all experimental groups

Gene Name	Gene Symbol	Gene Function
presenilin enhancer	*pen-2*	Cleavage of β amyloid precursor protein
Dynactin 1, p150 subunit	*DCTN1-p150*	Dynein receptor subunit
scarface	*scaf*	Embryo development
easter	*ea*	Secreted serine protease for embryo development
dachshund	*dac*	Transcription cofactor for eye development
brain-specific homeobox	*bsh*	Transcription factor for brain development
sans fille	*snf*	Regulation of alternative splicing
Coiled-coil domain containing 53	*CCDC53*	Regulation of cell adhesion
Pyrokinin 1 receptor	*PK1-R*	Regulation of insulin signaling
TBC1 domain family member 7	*TBC1d7*	Regulation of insulin signaling
alien	*alien*	Regulation of protein degradation
MAGE	*MAGE*	Response to DNA damage
Reduction in Cnn dots 4	*Rcd4*	Regulation of cell cycle
suppressor-of-G2-allele-of-skp1	*Sgt1*	Regulation of cell cycle
nazgul	*naz*	Tyramine metabolism
antisense RNA:CR44165	asRNA:CR44165	Unknown
antisense RNA:CR46029	asRNA:CR46029	Unknown
Chorion protein a at 7F	*Cp7Fa*	Unknown
uncharacterized protein	*CG9492*	Unknown
uncharacterized protein	*CG12502*	Unknown

Table 3.1. Genes with rhythmicity change in all experimental groups (continued)

Gene Name	Gene Symbol	Gene Function
uncharacterized protein	*CG13325*	Unknown
uncharacterized protein	*CG31809*	Unknown
uncharacterized protein	*CG8303*	Unknown
uncharacterized protein	*CG1622*	Unknown
uncharacterized protein	*CG2556*	Unknown
uncharacterized protein	*CG7011*	Unknown
uncharacterized protein	*CG7607*	Unknown
uncharacterized protein	*CG7133*	Unknown
uncharacterized protein	*CG17454*	Unknown
uncharacterized protein	*CG3420*	Unknown
uncharacterized protein	*CG14135*	Unknown
uncharacterized protein	*CG15535*	Unknown
uncharacterized protein	*CG31759*	Unknown
uncharacterized protein	*CG8067*	Unknown

3.5 Conclusion

In this chapter, I sought to discover the changes of circadian rhythm in response to aging and sleep disruption. I first analyzed the expression of core circadian clock genes and then identified oscillating genes in the RNA-seq datasets using multiple algorithms. Furthermore, the genes with rhythmic expression were compared between each experimental group and control *WT* group to detect changes in rhythmicity.

I found that the central clock seemed to be intact in sleep mutants and aged flies, but some significant changes were observed. Sleep disruption and aging affected different core clock genes, suggesting context-dependent effects on circadian rhythm.

Circadian analysis combining the power of three algorithms identified over 1000 oscillating transcripts in *WT* control group. Over 1500 genes were found to be rhythmic in *WT_old*, while only 663 and 809 genes were cycling in inc^{1} and in $wake^{D2}$. Comparisons of gene expression heatmaps and oscillation phase distributions indicated that aging reshaped the circadian rhythm by gain of rhythmicity and phase shift, and that sleep mutants exhibited perturbed circadian rhythm with loss of rhythmicity for some oscillating genes.

Change of rhythmicity analysis supported these discoveries. In *WT_old*, I observed large scale gain of rhythmicity activities during aging, which were absent in sleep mutants. Genes that lost rhythmicity were more abundant in inc^{1} and in $wake^{D2}$, and most of these genes were shared. Further intersection of genes with altered rhythmicity in all three experimental groups led to a list of 34 genes, all of which lost rhythmicity in the experimental groups. These 34 genes served as an initial list of candidate genes that could be investigated individually.

CHAPTER 4. ANALYSIS OF DIFFERENTIAL GENE EXPRESSION

In the previous chapter, I analyzed the RNA-seq datasets using circadian analysis to detect large-scale changes in rhythmicity. In this chapter, I would take a different approach with differential gene expression (DGE) analysis, focusing on individual genes. I developed a multi-step pipeline to compare the gene expression levels of each gene in experimental groups with control group. Such high-resolution analysis allowed statistical analysis on single gene level and the results were informative in revealing gene candidates for the relationship between sleep disruption and longevity reduction.

In this chapter, I first explained the principles and steps of the pipeline for DGE analysis. Next, using the least biased results as input for gene ontology analysis, I studied the biological processes that were enriched in the results. The gene ontology analysis suggested further measures for the pipeline. Finally, I inspected the expression data in closer detail and revealed candidate genes worthwhile for further investigation.

4.1 Pipeline of differential gene expression analysis

Figure 4.1A illustrated the workflow of the DGE analysis. The RNA-seq datasets were aligned to *D. melanogaster* reference genome assembly (Release 6.13) consisting of 17,490 annotated genes (dos Santos et al., 2015). Because the total RNA samples were collected from fly heads, not all genes were abundantly represented in the datasets. To remove genes with low expression levels, the raw counts were first normalized to count per million (cpm), accounting for variabilities in library sizes. Subsequently, a gene was kept in the dataset if cpm values were greater than 1 in at least 20 samples in the entire dataset. The thresholds were determined by simulating multiple combinations of possible values. After removal of lowly expressed genes, 9,578 genes remained, which served as the input data for DGE analysis.

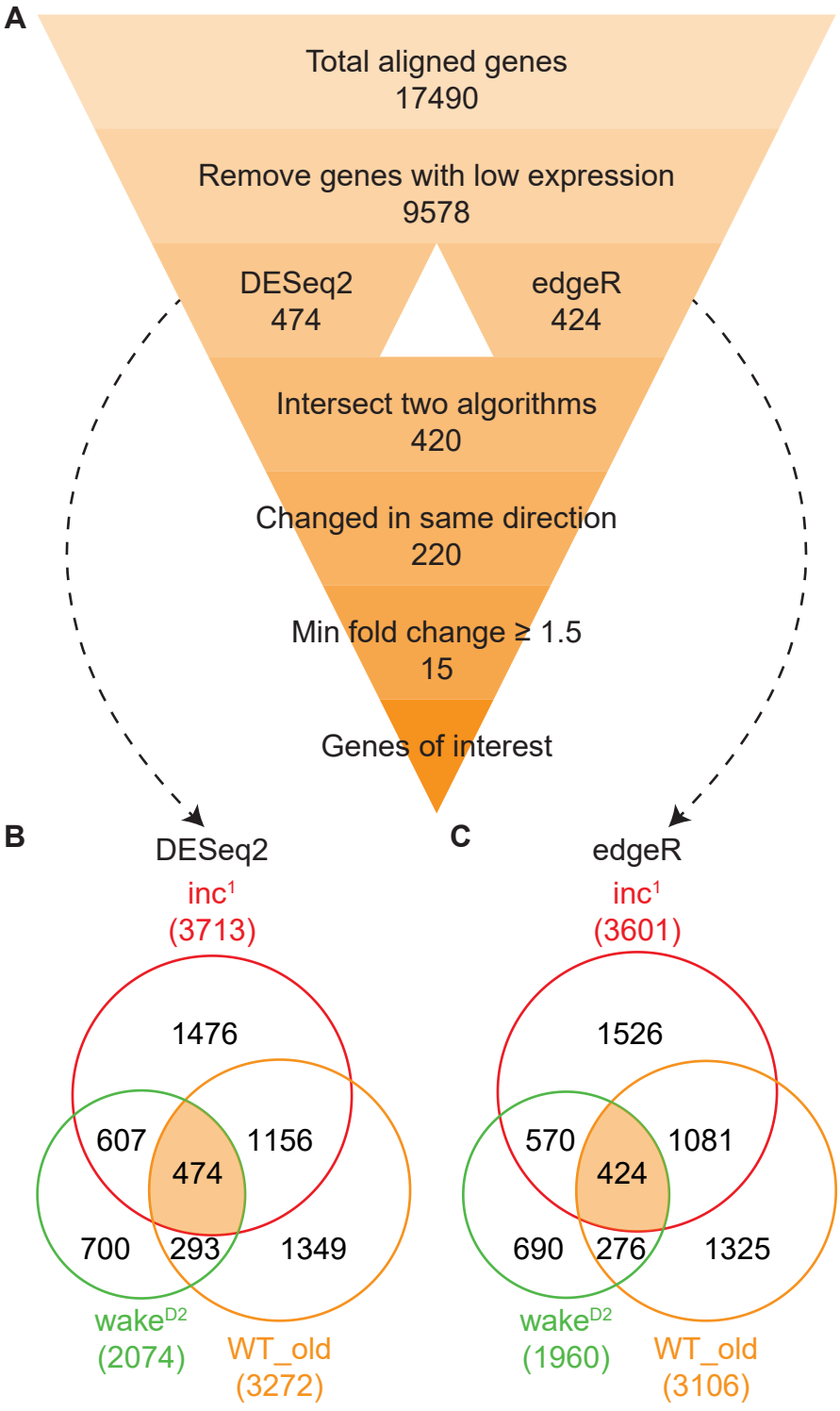
I used two algorithms for DGE analysis, DESeq2 (Love et al., 2014) and edgeR (McCarthy et al., 2012; Robinson et al., 2010). Both algorithms have been used extensively for DGE analysis and support experimental design with multiple factors. Because the parameters in the RNA-seq experiments included time and genotype, it was necessary to construct a complex structure that would take both parameters into consideration. Specifically, the bioinformatic programs were used to identify genes whose expression levels in the experimental group were significantly different from those of control group at one or more timepoints.

When compared individually against *WT* samples, the experimental groups yielded quite different results (**Figures 4.1B** and **4.1C**). Over 3,000 genes were found to be differentially expressed in inc^{1} and *WT_old*, while the algorithms identified about 2000 differentially expressed genes in $wake^{D2}$. Because I was interested in gene expression changes that occurred in all experimental groups, the results from each group were subsequently intersected to identify consistent changes regardless of genotype. Using these criteria, DESeq2 detected 474 genes and edgeR 424 genes. Further intersection of the two algorithms resulted in a list consisting of 420 genes.

These numbers indicated that my RNA-seq experiments were well designed, whereas the choice of bioinformatic programs for DGE did not pose great influence on the results. If the experimental design had included just one sleep mutant, for example inc^{1}, there would be over 1,500 differentially expressed genes, an overwhelming list of gene candidates. By taking an intersectional approach of two sleep mutants, I obtained an initial candidate list with 420 genes.

Figure 4.1. Differential gene expression analysis
A. Flowchart showing the pipeline for differential gene expression (DGE) analysis. Number in each box represents number of genes remained in the datasets.
B. Intersection of differentially expressed genes identified by DESeq2 (adjusted $p < 0.05$) in *inc*^{1} (red), *wake*^{D2} (green), and *WT_old* (orange).
C. Intersection of differentially expressed genes identified by edgeR ($p < 0.05$) in *inc*^{1} (red), *wake*^{D2} (green), and *WT_old* (orange).

Figure 4.1


A
Total aligned genes
17490
Remove genes with low expression
9578
DESeq2
474
edgeR
424
Intersect two algorithms
420
Changed in same direction
220
Min fold change ≥ 1.5
15
Genes of interest
B
DESeq2
inc1
(3713)
1476
607
1156
474
700
293
1349
wakeD2
(2074)
WT_old
(3272)
C
edgeR
inc1
(3601)
1526
570
1081
424
690
276
1325
wakeD2
(1960)
WT_old
(3106)

4.2 Gene ontology analysis

With the initial differentially expressed gene list of 420, I performed gene ontology analysis using the Database for Annotation, Visualization, and Integrated Discovery (DAVID, v6.8), to examine the biological processes, cellular components, and molecular functions that were enriched in the results (Huang da et al., 2009a, b).

The enriched biological processes included proteolysis, GPCR signaling pathway, sleep, several defense response pathways, and most importantly, determination of adult lifespan (**Figure 4.2A**). These biological processes further demonstrated that sleep disruption by genetic mutations affected a series of biological processes that were crucial for survival. The majority of the genes was found to encode membrane-related components (**Figure 4.2B**). For molecular functions, the most represented categories were calcium ion binding and oxidoreductase activity (**Figure 4.2C**). The over representation of membrane proteins and calcium-related functions suggested that the gene expression changes connecting sleep and longevity were likely to involve signaling pathways such as neurotransmitters and neuropeptides.

The genes that belonged to the biological process of determination of adult lifespan were inspected in further detail (**Figure 4.3**). *Target of rapamycin* (*Tor*) is a major regulator of lifespan by sensing nutrient and inhibition of *TOR* signaling pathway is shown to mediate the life extension effect of dietary restriction, in parallel with insulin signaling pathway (Kapahi et al., 2004). *ATP synthase, subunit D* (*ATPsynD*) and *raptor* have been shown to interact with TOR signaling pathway to regulate lifespan in *Drosophila* (Hatfield et al., 2015; Sun et al., 2014). Studies indicated that *CG10383*, a hydrolase in glycosylphosphatidylinositol metabolism, is involved in both sleep regulation and lifespan modulation (Paik et al., 2012; Thimgan et al., 2015). *Na pump α subunit* (*Atpα*) encodes an integral membrane cation antiporter protein that maintains membrane potential, mutation of which leads to neurodegeneration and reduced lifespan in *Drosophila* (Palladino et al., 2003). Overexpression of *four wheel drive* (*fwd*), a Golgi-localized lipid kinase, results in mild increase of longevity (Landis et al., 2003). Nicotinamide amidase (NAAM) is a nicotinamidase that regulates lifespan and oxidative stress response with dependence on sirtuin (Balan et al., 2008). p38b MAP kinase (p38b) constitutes the mitogen-activated protein kinase (MAPK) signaling cascades, which play critical roles in stress responses and immune responses (Chen et al., 2010; Vrailas-Mortimer et al., 2011). *methuselah-like 8* (*mthl8*) and *methuselah-like 10* (*mthl10*) encodes GPCRs that have structure similarity with longevity regulator *methuselah* (*mth*) (Lin et al., 1998); mutation of *mth* has lifespan extension effect and Mth has recently been shown to trigger insulin release in response to its ligand Stunted (Sun) (Cvejic et al., 2004; Delanoue et al., 2016).

Figure 4.2. Gene ontology analysis for differentially expressed genes
A. Biological processes
B. Cellular components
C. Molecular functions

Figure 4.2

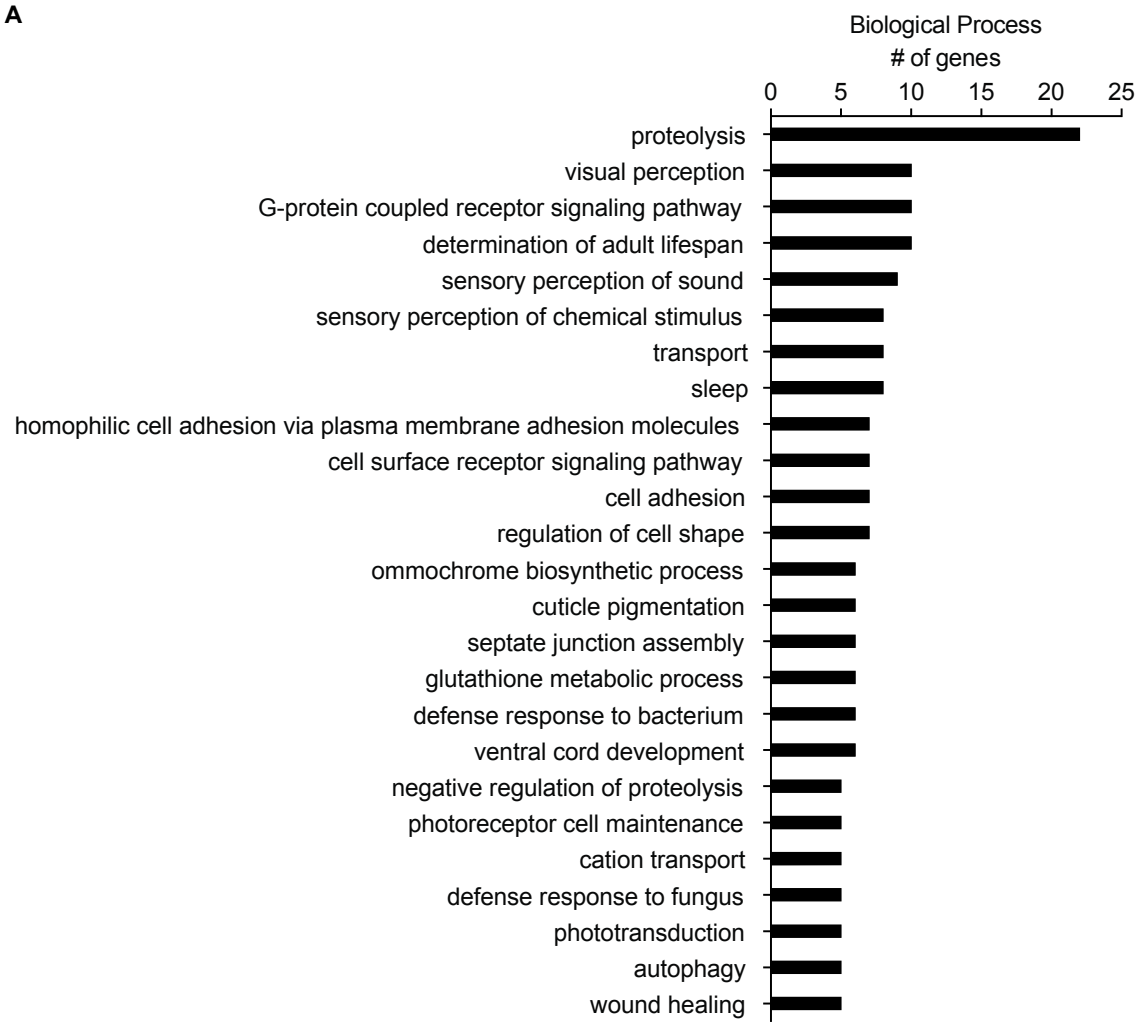

A
Biological Process
# of genes
0
5
10
15
20
25
proteolysis
visual perception
G-protein coupled receptor signaling pathway
determination of adult lifespan
sensory perception of sound
sensory perception of chemical stimulus
transport
sleep
homophilic cell adhesion via plasma membrane adhesion molecules
cell surface receptor signaling pathway
cell adhesion
regulation of cell shape
ommochrome biosynthetic process
cuticle pigmentation
septate junction assembly
glutathione metabolic process
defense response to bacterium
ventral cord development
negative regulation of proteolysis
photoreceptor cell maintenance
cation transport
defense response to fungus
phototransduction
autophagy
wound healing

Figure 4.2 (continued)

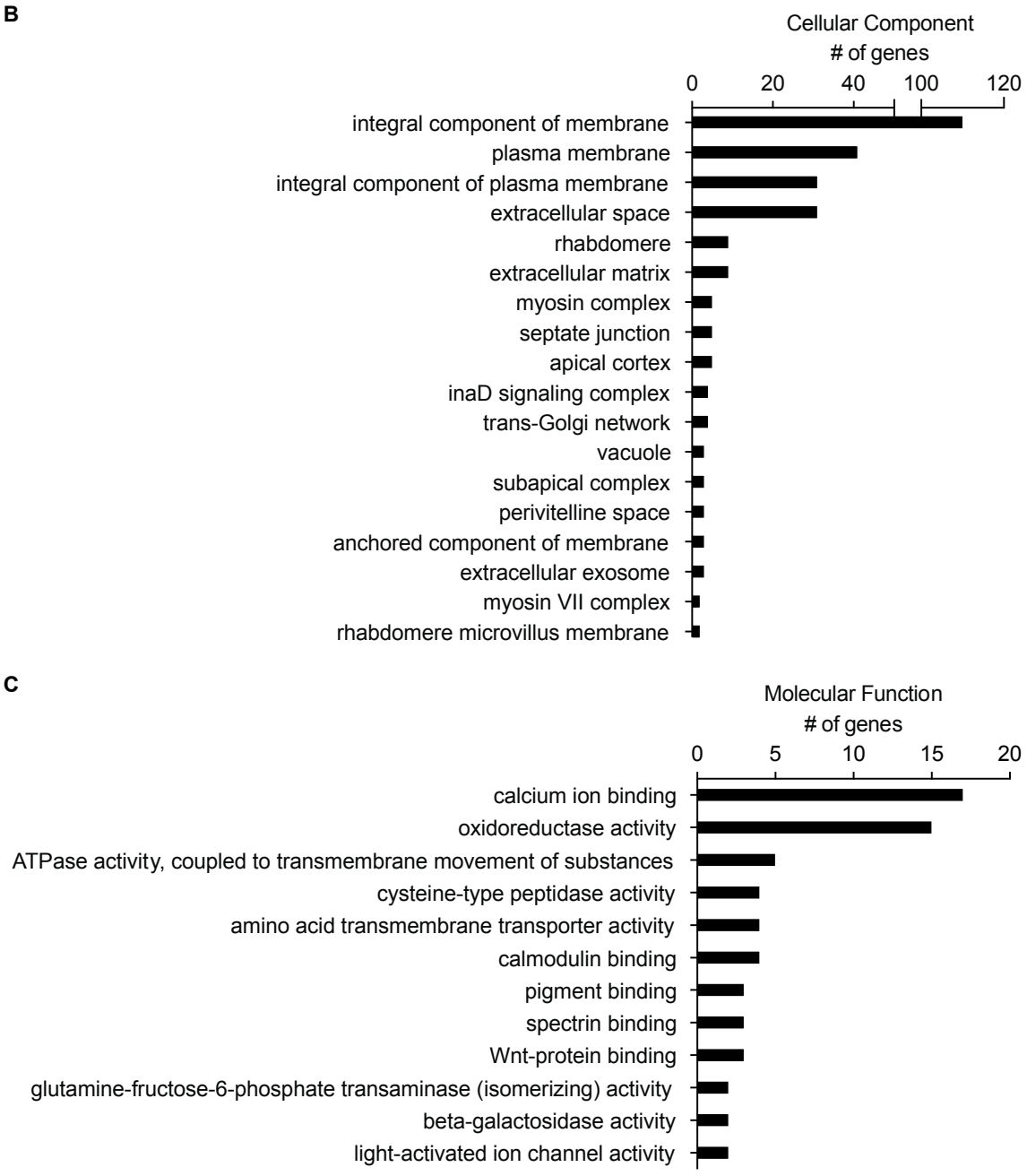


Figure 4.3. Expression of genes involved in determination of adult lifespan
WT (black), inc^{1} (red), $wake^{D2}$ (green), and *WT_old* (orange). Gene names are labeled on top of the plots. y axis represents Reads Per Kilobase of transcript, per Million mapped reads (RPKM). x axis represents two LD cycles with white bars representing daytime and black bars representing nighttime.

Figure 4.3

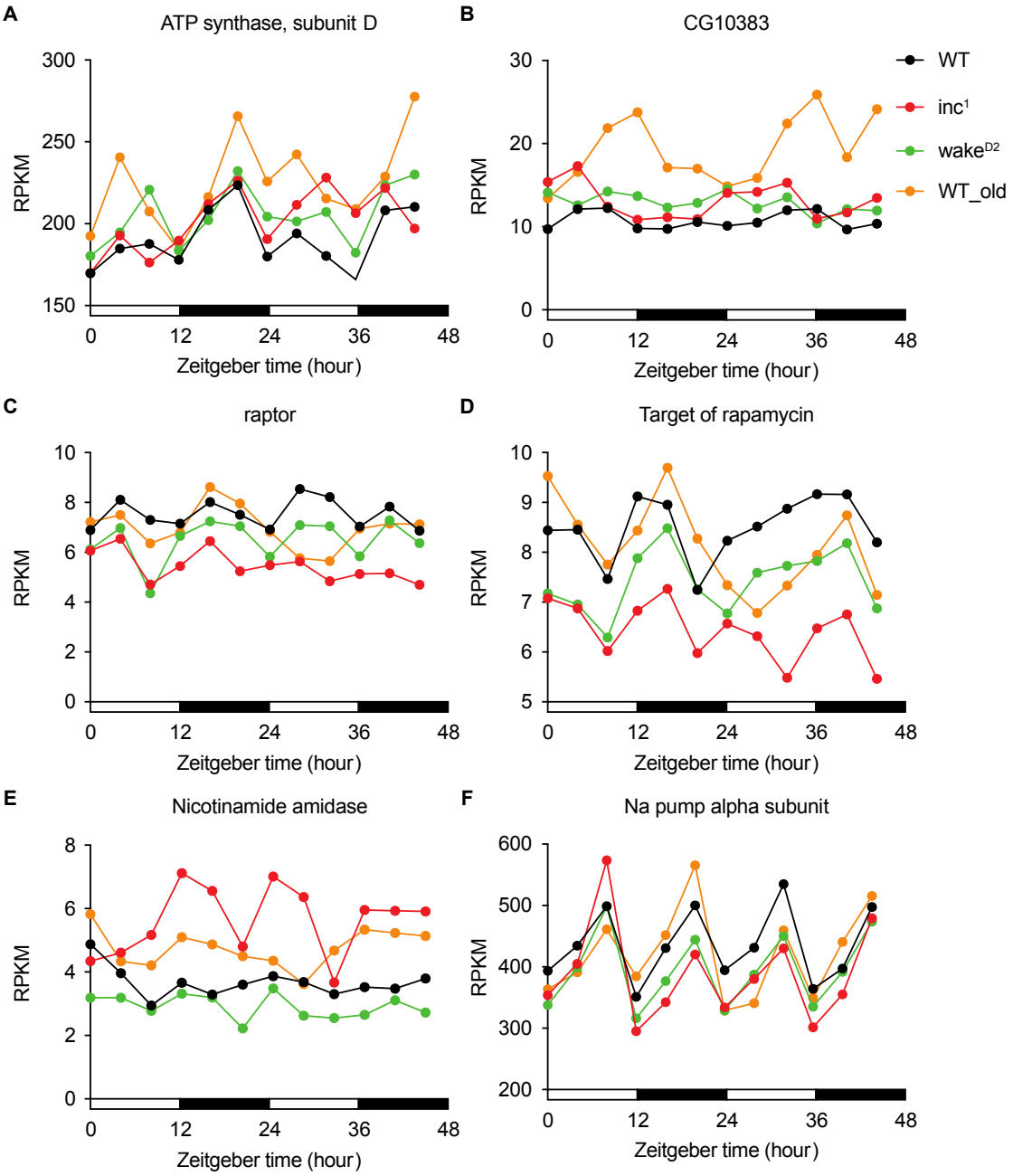
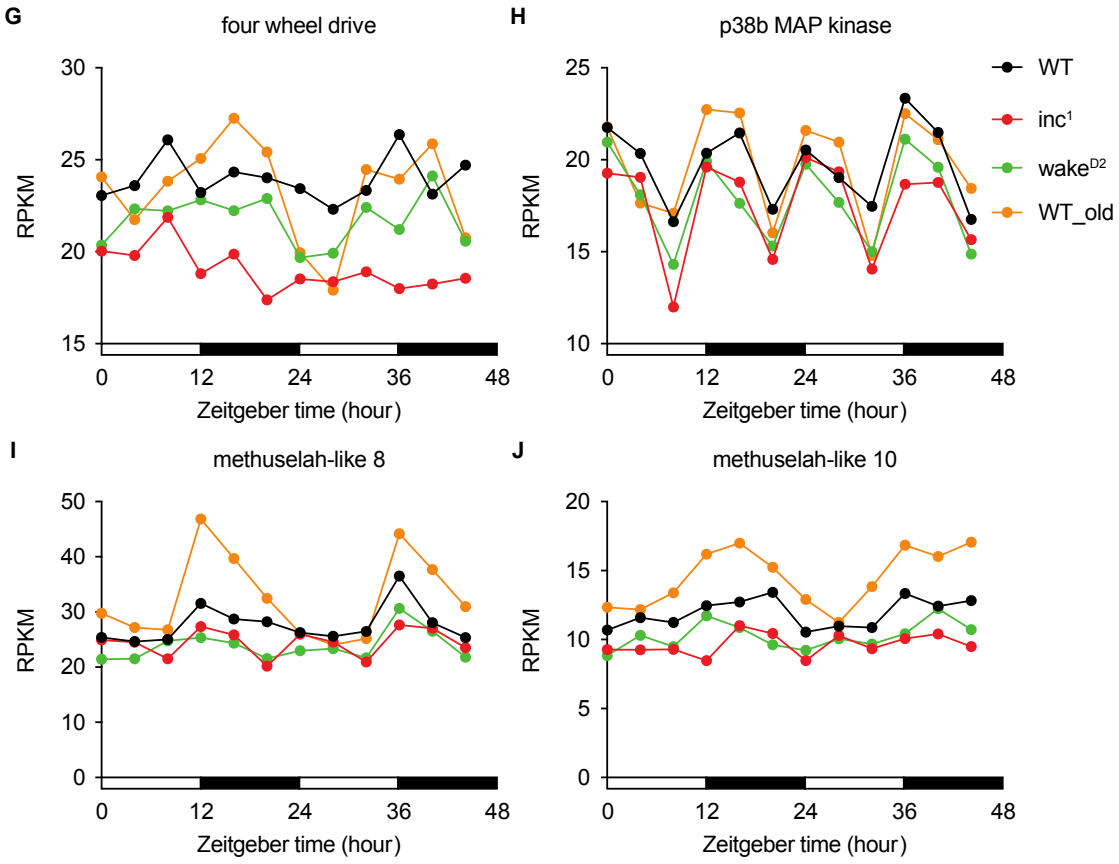

A
ATP synthase, subunit D
RPKM
300
250
200
150
0
12
24
36
48
Zeitgeber time (hour)
B
CG10383
RPKM
30
20
10
0
Zeitgeber time (hour)
WT
inc1
wakeD2
WT_old
C
raptor
RPKM
10
8
6
4
2
0
Zeitgeber time (hour)
D
Target of rapamycin
RPKM
10
9
8
7
6
5
Zeitgeber time (hour)
E
Nicotinamide amidase
RPKM
8
6
4
2
0
Zeitgeber time (hour)
F
Na pump alpha subunit
RPKM
600
500
400
300
200
Zeitgeber time (hour)

Figure 4.3 (continued)



4.3 Identification of candidate genes

Although the 10 genes in determination of adult lifespan seemed promising as the genetic connection between sleep deprivation and longevity reduction, the expression data from RNA-seq datasets excluded them from further individual investigation. There were two reasons. First, the direction of change was not the same across all three experimental groups in most cases, which suggested that the effects were not consistent. Second, the differences between control group and experimental groups were not robust, as indicated by the frequent crossovers in the gene expression plots (**Figure 4.3**).

Because of these two problems, I applied two more subjective criteria to narrow down the list (**Figure 4.1A**). First, genes that were changed in the same direction in all three experimental groups were kept. This step was necessary to focus on gene expression changes that were comparable in sleep mutants and aged flies. Second, a minimal 1.5-fold change on mean expression levels was set for candidate genes, which would help to increase robustness of results and thus reduce false positive rate. After applying these two filters, 15 genes remained, among which the final genes of interest were selected (**Figure 4.4**).

Neuropeptide-like precursor 3 (*Nplp3*) encodes a small peptide whose expression levels oscillated in all groups of the RNA-seq datasets. *CG9377* levels oscillated in all groups excepted for inc^{1}. For both *Nplp3* and *CG9377*, the expression levels were significantly reduced in experimental groups compared to control *WT* group. Sequence prediction indicated that *CG13423* and *CG6337* are involved in proteolysis process (Gaudet et al., 2011). Similarly, *CG5653* and *sepia* (*se*) are predicted to play roles in oxidation-reduction process (Gaudet et al., 2011). *Tetraspanin 2A* (*Tsp2A*) has been shown to regulate septate junction formation in *Drosophila* midgut (Izumi et al., 2016). Four genes, *CG5653*, *CG13071*, *CG13840*, and *CG8100*, encode small peptides with unknown function or structures. The last four candidates, Cuticular protein 49Ae (Cpr49Ae), Cuticular protein 76Bc (Cpr76Bc), Vajk2, and Vajk4, are predicted to be involved in cuticle development (Cinege et al., 2017; Karouzou et al., 2007), which suggested abnormal exoskeleton structures in sleep mutants and aged flies. For the scope of this project, I decided to focus on *Nplp3*.

Figure 4.4. Expression of 15 candidate genes
WT (black), inc^{1} (red), $wake^{D2}$ (green), and *WT_old* (orange). Gene names are labeled on top of the plots. y axis represents Reads Per Kilobase of transcript, per Million mapped reads (RPKM). x axis represents two LD cycles with white bars representing daytime and black bars representing nighttime.

Figure 4.4

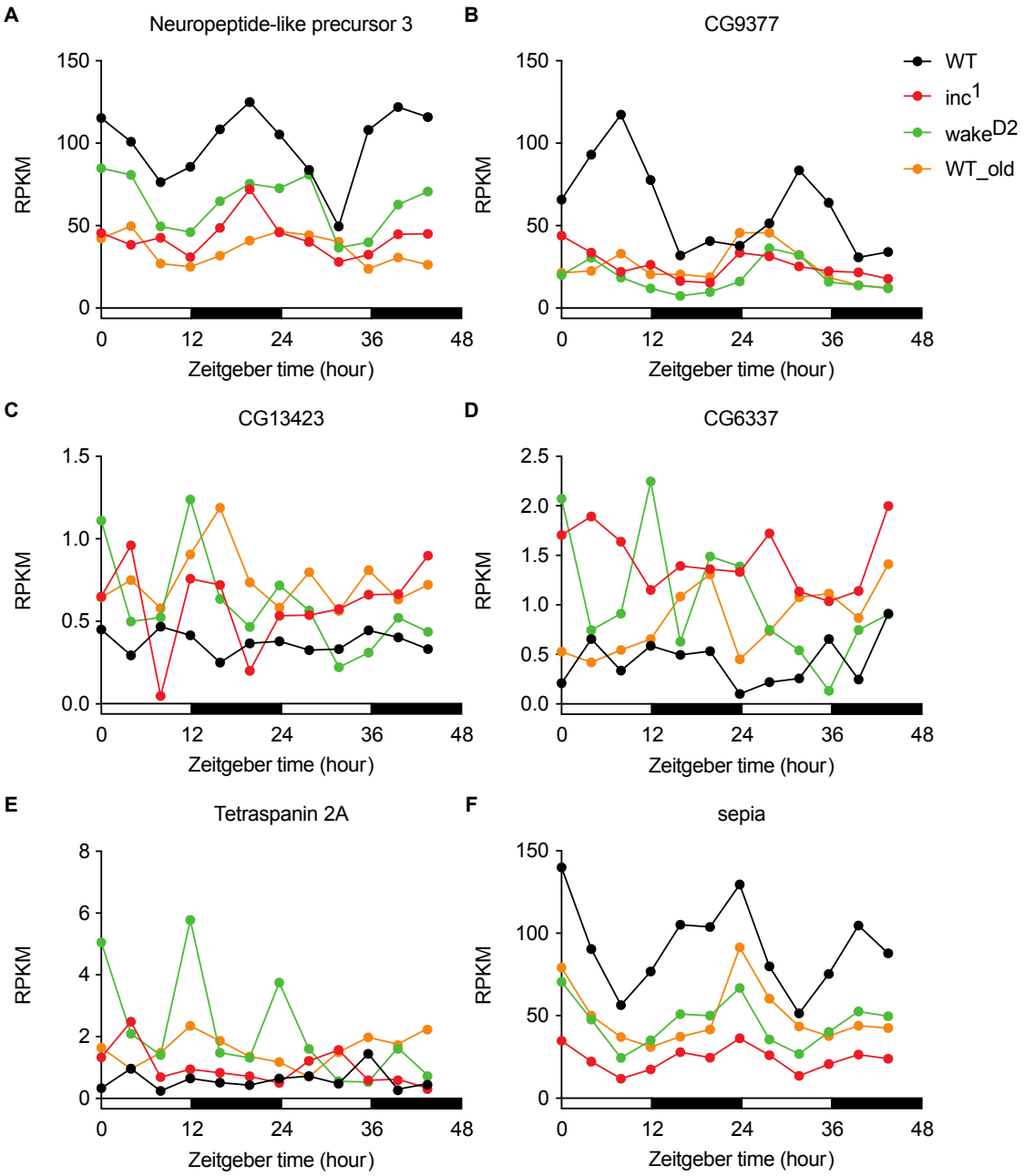

A
Neuropeptide-like precursor 3
B
CG9377
C
CG13423
D
CG6337
E
Tetraspanin 2A
F
sepia
RPKM
Zeitgeber time (hour)
WT
inc1
wakeD2
WT_old

Figure 4.4 (continued)

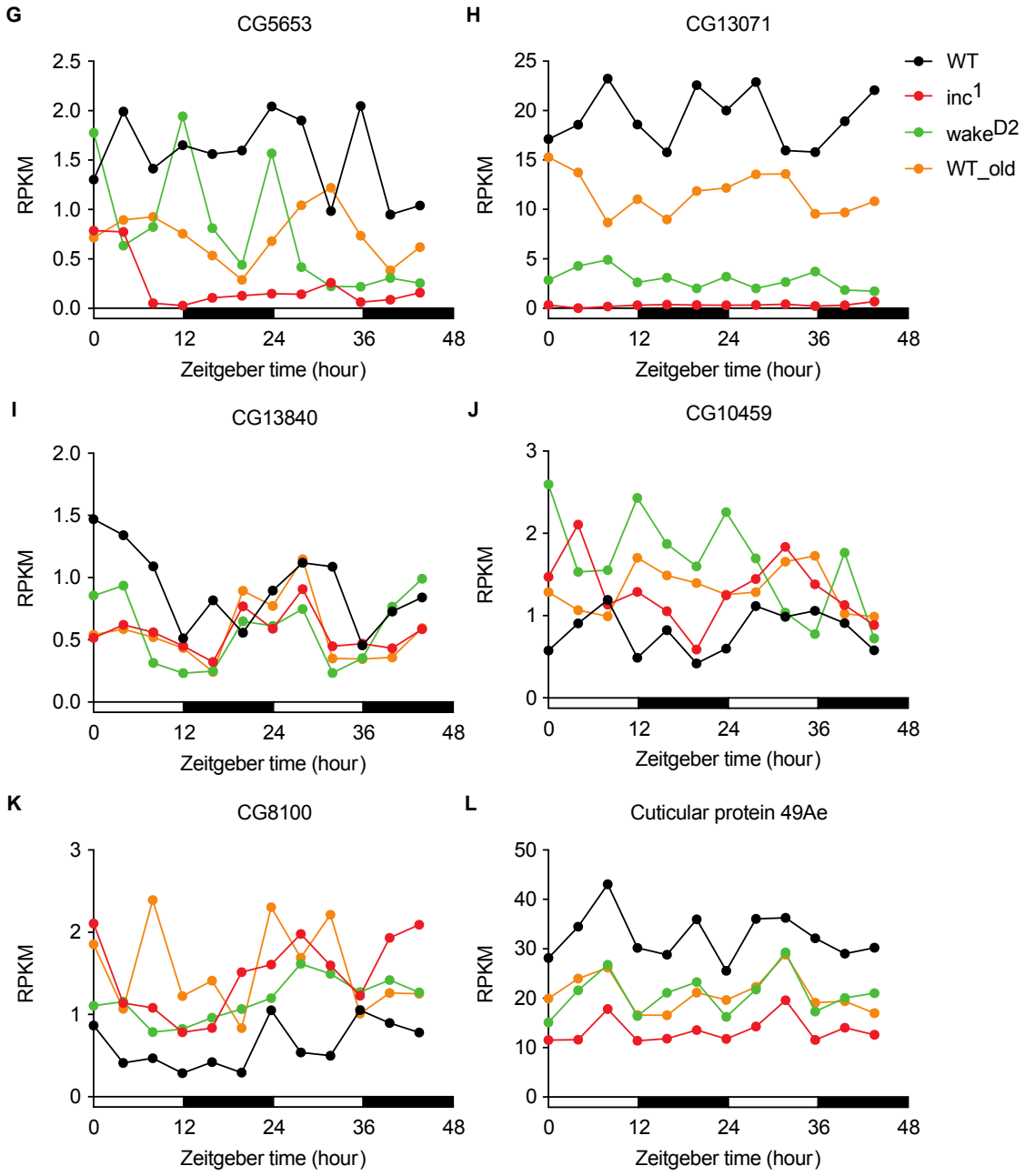
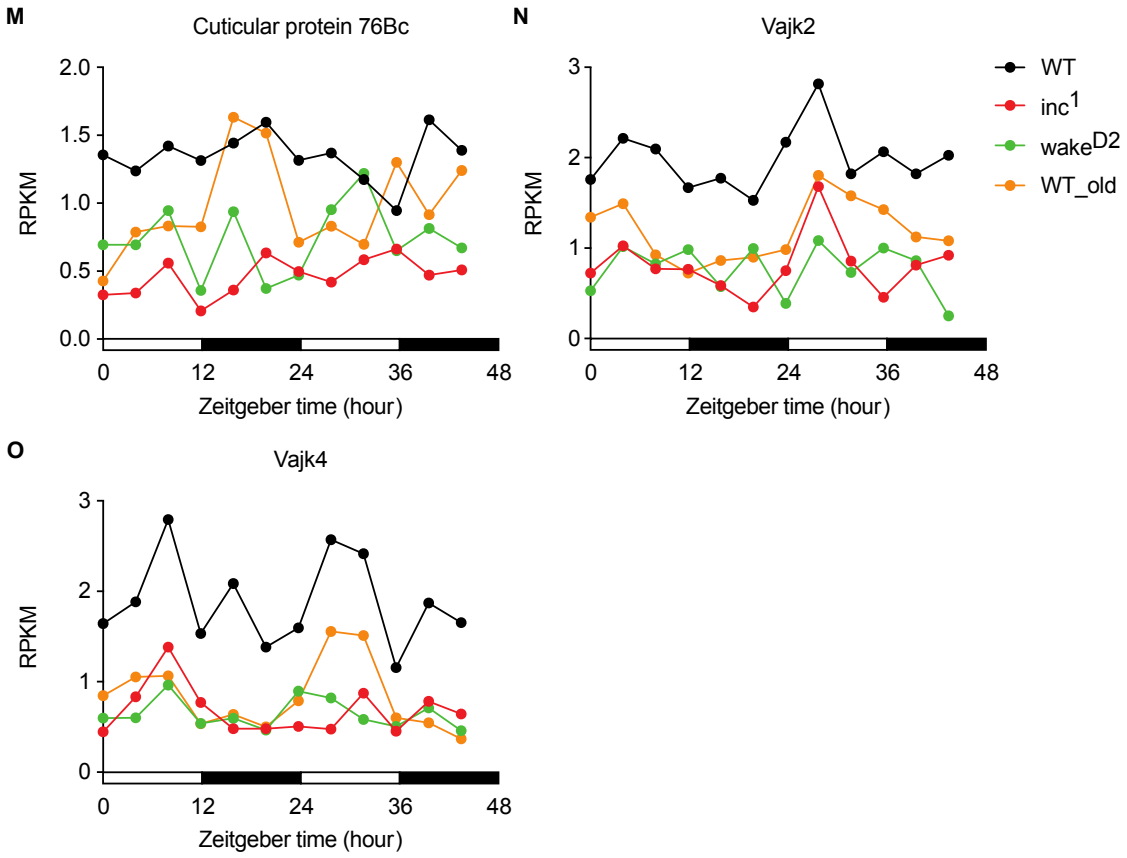


Figure 4.4 (continued)



CHAPTER 5. *NEUROPEPTIDE-LIKE PRECURSOR 3* (*NPLP3*)

As the name suggests, *Nplp3* is believed to encode a precursor peptide that might be processed into a neuropeptide. The expression data from the RNA-seq experiments showed that *Nplp3* RNA levels oscillated with 24-hour period and peak expression at ZT 20 (**Figure 4.4A**). Furthermore, mutations of *inc* or *wake* did not abolish the rhythmicity but decreased the expression levels of *Nplp3*. I observed similar results in the aged group, oscillating with lower amplitude as well as reduced expression level.

In this chapter I discussed the discoveries I made regarding *Nplp3*. I first discussed the structure of the gene and what was already known about *Nplp3*. Next, I examined the expression levels of *Nplp3* in multiple sleep mutants, not only to validate the RNA-seq results, but also to test if other sleep mutants had altered *Nplp3* expression. Furthermore, I investigated the effects on sleep and longevity when *Nplp3* expression levels were manipulated.

5.1 *Nplp3* background

Nplp3 is a small gene with 667 base pairs (bp) and it encodes a short peptide with 90 amino acids (aa) (**Figure 5.1**). The function of *Nplp3* is not clear. A liquid chromatography–mass spectrometry (LC-MS) based study in the central nervous system of larval *D. melanogaster* identified two short peptide that were believed to originate from NPLP3 (Baggerman et al., 2002). Peptide prediction algorithms predicted a 16-aa long signal peptide at the N-terminal of the peptide and the rest to be non-cytoplasmic (Almagro Armenteros et al., 2019; Kall et al., 2004). Although no *Nplp3* orthologs were found in mammalian species, the peptide sequence of NPLP3 is highly conserved in *Drosophila* species (Sievers et al., 2011) (**Figure 5.1C**).

A transcriptome profiling study of the *Drosophila* surface glia cells, components of the fly blood-brain barrier (BBB), showed that *Nplp3* is highly enriched in surface glia cells, 453 fold compared with neurons (DeSalvo et al., 2014). These results suggested that *Nplp3* might be involved in neuropeptide signaling pathway in glia cells.

Figure 5.1. *Nplp3* gene structure
A. Schematic of *Nplp3* genome locus. Blue boxes indicate untranslated region (UTR) and magenta boxes indicate coding sequence (CDS). Introns are shown as lines. Triangle indicates the *PBac{RB}Nplp3^{e01799}* insertion site in the *Nplp3^{e}* mutant.
B. Sequence of *Nplp3* gene. Upper case indicates transcribed sequence and lower case indicates intron. Blue letters indicate UTR and magenta letters indicate CDS. Arrowhead indicates the *PBac{RB}Nplp3^{e01799}* insertion site in the *Nplp3^{e}* mutant.
C. Alignment of NPLP3 protein sequences for various *Drosophila* species. * indicates fully conserved residue. The signal peptide is boxed in green.

Figure 5.1

B

>3L:16318024..16318690

ATGTCAAATCGTTGCAAACCATCAACAAAACAAAATGTTCAAGCTGgtgagttctttaag
acaatcaagagtattccttgtgcaaataaaggattctatgaaaaaaaaagtgaacatatg
ctgattcaagtgacatccatctaatatatgtatatatatttgcattgttttccagTGCGT
CTTCGTTGCTCTCCTTAGCCTGGCTGCTGCTGCCCCAGCTCCCGCTCCTGCTCCTGCTCC
TGCTCCTGGTCTCATTGGCCCTGGCATTGTGGCACCTGGAATCTGGGGACCCACCGTTGT
CGGATCTCCTCTGCTTGCTCCTCAGGTGGTGAGCGTGGTGCCCGGTGCCATTTCTCATGC
TGCCATCACCCAGGTGCATCCTTCTCCTCTGCTGATCAAGAGCGTCCATGGCCTGGGACC
AGTTGTGATCGGTTAAACCAGTTACCCATTGGAGATCCAACCATCCATATAGCTCACCCA
CACGCCAGCCAATCAGCGCTCCTGTTCAACCATAACATTTCCTGCGGATTACTTCAACCA
CAAGTTCTGTGCCCTGTTTATAATGCAAGCAAATTCAGCAATGAGAAGGAAAAAATCACA
AAAAAATGTTTAGACACTAAAATACATTCCAAGCAATAAATACCTACCATACATGTTATA
GCGAGCG

C

```
D.melanogaster    MFKLCVFVALLSLAAAAPAPA----PAPAPAP-GLIGPGIVAPGIWGPTVVGSPLLAPQV
D.simulans        MFKLCVFVALLSLAAAAPAPA----PAPAPAP-GLIGPGIVAPGIWGPTVVGSPLIAPHV
D.erecta          MFKLCVFVALLSLAAAAPAPAPAPAPAPAPAP-GLIAPGLVAPGIWGPTVVGSPLVAPQV
D.yakuba          MFKLCVFVALLSLAAAAPA--------PAPAP-GLIAPGLVAPGIWGPTVVGSPLVAPQV
D.suzukii         MFKLCVFVALLSLAAAAPAPAP--VPAPAPAP-GLIHPGLVAPGIWGPTVVGSPLLAPQV
D.pseudoobscura   MFKLCVFVALLSLAAAAPA--PAPAPVPEPAPSGLWPGGLVAPGIWGPTIIGGP----HV
D.persimilis      MFKLCVFVALLSLAAAAPAPAPAPAPVPEPAPSGLWPGGLVAPGIWGPTIIGGP----HV
                  ********************        * *** **   * *********  * *     *

D.melanogaster    VSVVPGAISHAAITQVHPSPLLIKSVHGL-GPVVIG
D.simulans        VSVVPGAISHAAITQVHPSPLLIKSVHGL-GPVVIG
D.erecta          VSVLPGAISHAAITQVHPSPLLIKSVHGL-GPVVIG
D.yakuba          VSVVPGAISHAAITQVHPSPLLIKSVHGL-GPVVIG
D.suzukii         VSVVPGAISHAAITQVHPSPLLVKSIHGL-GPVVIG
D.pseudoobscura   VSVVPHAISHAAITQVHPSPLVLKSVHGIHGPVLIG
D.persimilis      VSVVPHAISHAAITQVHPSPLVLKSVHGIHGPVLIG
                  *** * ***************  ** **  *** **
```

5.2 *Nplp3* expression in sleep mutants

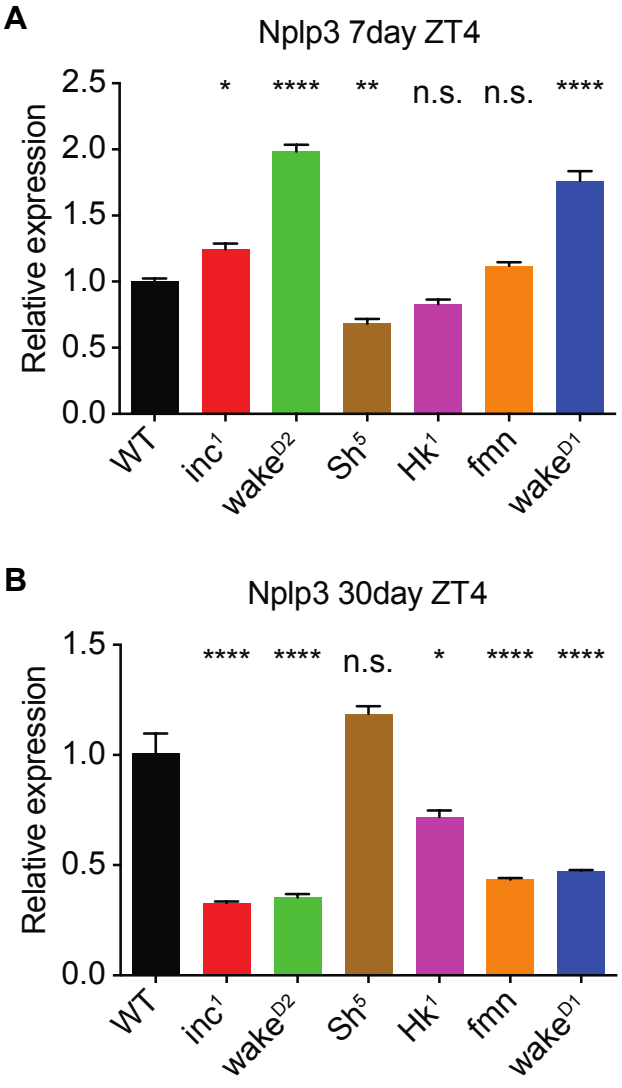
As discussed in **Section 2.2**, the determination of the genetic connection between sleep disruption and longevity reduction relies on three criteria: 1) sleep loss would induce changes in expression levels comparable to the changes observed in RNA-seq datasets; 2) such changes in expression levels would affect longevity; 3) but not affect sleep.

To validate the gene expression changes I observed in RNA-seq and test if sleep loss would induce such changes, I performed quantitative reverse transcription polymerase chain reaction (RT-qPCR) using RNA samples collected from multiple sleep mutants. When tested with samples collected from 7-day-old flies, I did not observe the reduction of *Nplp3* expression in inc^{1} and $wake^{D2}$ compared to *WT* (**Figure 5.2A**). Results using samples collected at ZT4 showed that the expression levels of *Nplp3* increased in *inc* and *wake* mutants compared to *WT* (**Figure 5.2A**). Mutations of *Sh*, *Hk*, and *fmn* resulted in little to no change in *Nplp3* expression (**Figure 5.2A**).

Because the RNA-seq samples were collected from 29-day-old animals, it was possible that the effects I observed in RNA-seq were age-dependent. Using RNA samples from 30-day-old flies, I tested whether the expression levels of *Nplp3* would change in sleep mutants (**Figure 5.2B**). The results demonstrated that *Nplp3* expression levels decreased significantly in inc^{1} and $wake^{D2}$. More importantly, *Nplp3* expression levels showed signification reduction in Hk^{1} and *fmn* mutants, indicating that sleep disruption indeed could reduce *Nplp3* expression (**Figure 5.2B**).

Figure 5.2. *Nplp3* expression in sleep mutants
A. Relative expression of *Nplp3* RNA in 7-day-old animals at ZT4.
B. Relative expression of *Nplp3* RNA in 30-day-old animals at ZT4.
Mean ± SEM is shown; * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$, one-way ANOVA with multiple comparisons against *WT* with Bonferroni correction.

Figure 5.2


A
Nplp3 7day ZT4
Relative expression
2.5
2.0
1.5
1.0
0.5
0.0
*
****
**
n.s.
n.s.
****
WT
inc1
wakeD2
Sh5
Hk1
fmn
wakeD1
B
Nplp3 30day ZT4
Relative expression
1.5
1.0
0.5
0.0
****
****
n.s.
*
****
****
WT
inc1
wakeD2
Sh5
Hk1
fmn
wakeD1

5.3 Effects of reduced *Nplp3* expression on sleep and longevity

To study the effects of reduced *Nplp3* expression, I obtained a hypomorph mutant allele of *Nplp3*, named *Nplp3^{e}*. The mutation was generated by inserting transposable element *PBac{RB}Nplp3^{e01799}* into the intron region of *Nplp3* gene (**Figure 5.1**) (Bellen et al., 2011; Thibault et al., 2004). RT-qPCR results demonstrated that the mutation led to significant reduction (~80%) in *Nplp3* RNA levels (**Figure 5.3A**).

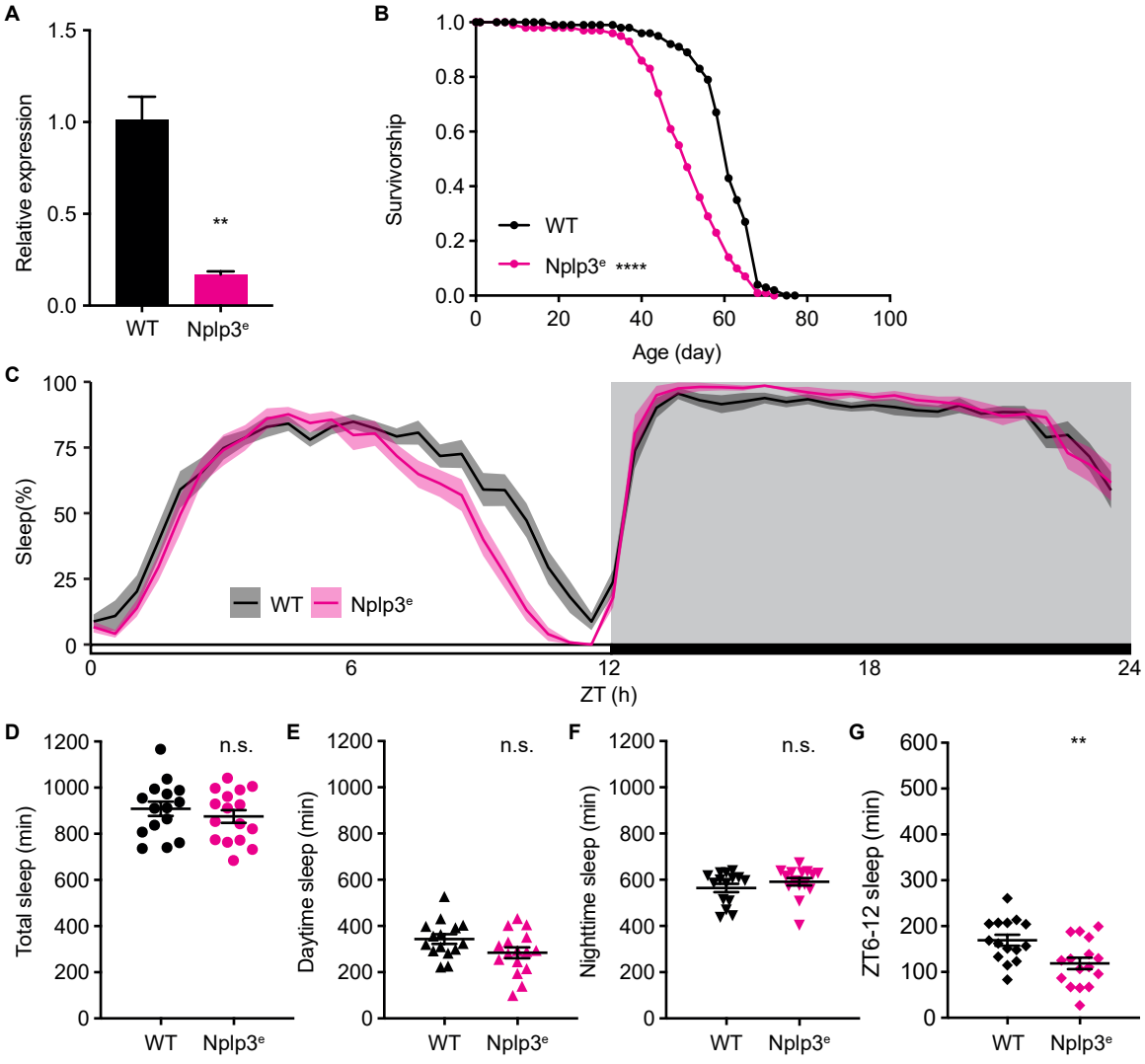
The *Nplp3^{e}* mutant flies offered the opportunity to investigate the effects of decreased *Nplp3* expression. Compared to *WT*, the *Nplp3^{e}* mutants showed significant reduction in lifespan (**Figure 5.3B**), indicating that *Nplp3* misexpression could affect longevity. Interestingly, the longevity curve of *Nplp3^{e}* was different from those of the sleep mutants, where I observed structural similarities of longevity curves between wild type animals and sleep mutants. When comparing the longevity curves of *Nplp3^{e}* animals and *WT* animals, I found that the curve from *WT* animals descended more rapidly. Furthermore, the maximum longevity was comparable between *Nplp3^{e}* and *WT*. These results indicated that *Nplp3^{e}* affected longevity in a different manner from the sleep mutants.

The *Nplp3^{e}* mutants were also tested for sleep behavior. There was no significant change in total sleep amount compared to *WT* (**Figure 5.3C** and **D**). When daytime sleep and nighttime sleep were quantified separately, *Nplp3^{e}* mutant flies were not significantly different from wild type animals (**Figures 5.3E** and **F**). However, the sleep profiles suggested minor difference of sleep in the second half of light phase, which was confirmed by calculating sleep between ZT6 and ZT12 (**Figure 5.3G**). These data suggested that decreased *Nplp3* expression had limited effect on sleep.

Taken together, the hypomorph mutation *Nplp3^{e}* decreased *Nplp3* expression levels, shortened lifespan of animals, but did not dramatically affect sleep.

Figure 5.3. Effects of reduced *Nplp3* expression on sleep and longevity
A. Relative expression of *Nplp3* RNA levels in *WT* (black) and *Nplp3*^{e} (pink). Mean ± SEM is shown; ** $p < 0.01$, unpaired t test.
B. Longevity of *WT* (black) and *Nplp3*^{e} (pink) male flies. n = 293 for *WT*; n = 311 for *Nplp3*^{e}. Overall survivorship is shown; **** $p < 0.0001$, log-rank test.
C-G. Sleep profiles (**C**), Average total sleep per day (**D**), average daytime sleep per day (**E**), average nighttime sleep per day (**F**), and average sleep between ZT6 and ZT12 (**G**) of *WT* (black) and *Nplp3*^{e} (pink) male flies. Note that ZT6-12 sleep (**G**) is plotted on different scale. n = 15 for *WT*; n = 16 for *Nplp3*^{e}. Sleep profile is displayed as the average proportion of time spent sleeping in consecutive 30-min segments during LD cycle. Mean ± SEM is shown; ** $p < 0.01$; n.s., not significant, t test with Welch's correction.

Figure 5.3


A
Relative expression
1.5
1.0
0.5
0.0
**
WT
Nplp3e
B
Survivorship
1.0
0.8
0.6
0.4
0.2
0.0
WT
Nplp3e ****
0
20
40
60
80
100
Age (day)
C
Sleep(%)
100
75
50
25
0
WT
Nplp3e
0
6
12
18
24
ZT (h)
D
Total sleep (min)
1200
1000
800
600
400
200
0
n.s.
WT
Nplp3e
E
Daytime sleep (min)
1200
1000
800
600
400
200
0
n.s.
WT
Nplp3e
F
Nighttime sleep (min)
1200
1000
800
600
400
200
0
n.s.
WT
Nplp3e
G
ZT6-12 sleep (min)
600
500
400
300
200
100
0
**
WT
Nplp3e

5.4 Effects of *Nplp3* overexpression on sleep and longevity

After learning that reduced *Nplp3* expression levels led to shortened lifespan, I was curious about the effects of *Nplp3* overexpression. To drive the overexpression of *Nplp3* with its own promoter, I exploited the Gal4/UAS system, which had been widely used in *Drosophila* research to achieve targeted gene expression with selected promoter (Brand and Perrimon, 1993). I generated transgenic animals carrying *Gal4* transgene, directed by *Nplp3* promoter extending from -1.7 kilobase (kb) to the start codon (**Figure 5.4A**) (Pfeiffer et al., 2008). The transgenic fly would theoretically express *Gal4* proteins in the same cells that *Nplp3* was expressed. Furthermore, the CDS of *Nplp3* was cloned and placed at the downstream of *UAS* sequence (Pfeiffer et al., 2010). The resulted construct was used to generate the *UAS-Nplp3* transgenic fly (**Figure 5.4B**). I also generated *UAS-Nplp3*$^{\Delta}$ fly with similar approach but removed the signal peptide of *Nplp3* (**Figure 5.4C**). The combination of *Nplp3-Gal4* with *UAS-Nplp3* and *UAS-Nplp3*$^{\Delta}$ would specifically overexpress the wild type form and truncated form of *Nplp3* in *Nplp3* expressing cells, respectively.

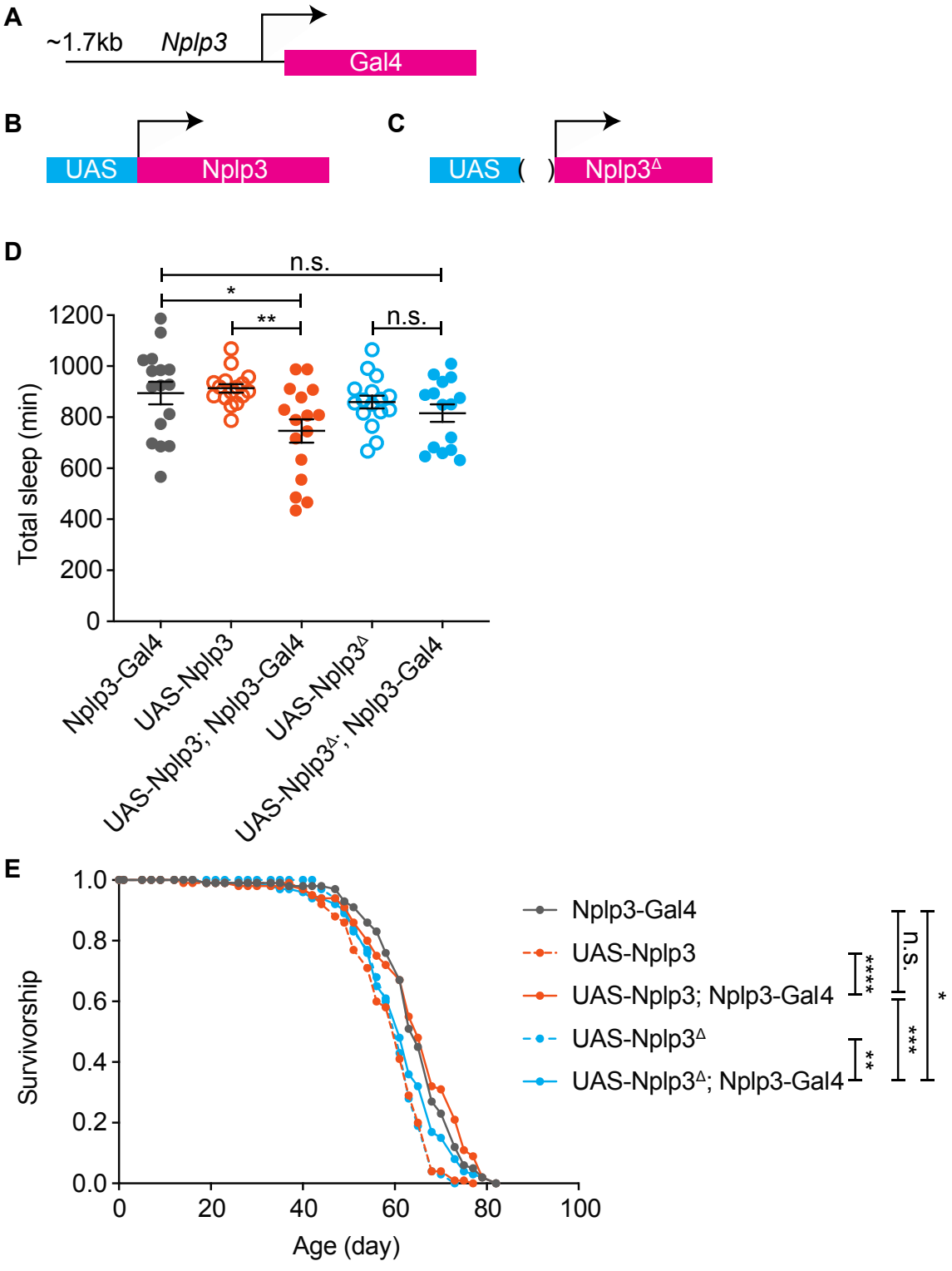
Sleep measurement showed that overexpression of wild type version of *Nplp3* reduced total sleep amount significantly (**Figure 5.4D**), suggesting that *Nplp3* functioned at least partially within the sleep regulation network. However, overexpression of *Nplp3* without the signal peptide did not significantly affect total sleep (**Figure 5.4D**). These data suggested that artificially increasing *Nplp3* expression level affected sleep and the effect depended on the presence of signal peptide.

When tested for survivorship, different genotypes did not show dramatic variations, but subtle changes were observed (**Figure 5.4E**). *Nplp3* overexpression flies lived significantly longer than flies carrying only the *UAS-Nplp3* transgene ($p < 0.0001$). Although comparison between *Nplp3-Gal4* and *Nplp3* overexpression was not significant, overexpression of *Nplp3*$^{\Delta}$ resulted in significantly shorter lifespan compared to *Nplp3-Gal4* ($p < 0.05$). Most importantly, overexpression of wild type version of *Nplp3* showed significantly better survivorship compared to overexpression of *Nplp3*$^{\Delta}$ ($p < 0.001$).

In summary, these results indicated that overexpression of *Nplp3* could mildly disrupt sleep, yet the exact effect on longevity needed more evidence. The differences between overexpression of normal *Nplp3* and overexpression of *Nplp3*$^{\Delta}$ hinted the importance of signal peptide and supported the possibility that NPLP3 functioned as a secreted molecule.

Figure 5.4. Effects of *Nplp3* overexpression on sleep and longevity
A. Schematic of *Nplp3-Gal4* construct. Solid line indicates ~1.7 kb DNA upstream of *Nplp3* start codon. Arrow indicates transcription starting site.
B. Schematic of *UAS-Nplp3* construct. Arrow indicates transcription starting site.
C. Schematic of *UAS- Nplp3$^{\Delta}$* construct. Arrow indicates transcription starting site. Parenthesis indicates removed signal peptide CDS.
D. Average total sleep per day for male flies with overexpression of *Nplp3* or *Nplp3$^{\Delta}$*. n = 15-16 for all groups. Mean ± SEM is shown; * $p < 0.05$; ** $p < 0.01$; n.s., not significant, t test with Welch's correction.
E. Longevity of male flies with overexpression of *Nplp3* or *Nplp3$^{\Delta}$*. n = 145-150 for all groups. Overall survivorship is shown; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; n.s., not significant, log-rank test.

Figure 5.4


A
~1.7kb
Nplp3
Gal4
B
UAS
Nplp3
C
UAS
Nplp3Δ
D
Total sleep (min)
n.s.
*
**
n.s.
Nplp3-Gal4
UAS-Nplp3
UAS-Nplp3; Nplp3-Gal4
UAS-Nplp3Δ
UAS-Nplp3Δ; Nplp3-Gal4
E
Survivorship
Age (day)
Nplp3-Gal4
UAS-Nplp3
UAS-Nplp3; Nplp3-Gal4
UAS-Nplp3Δ
UAS-Nplp3Δ; Nplp3-Gal4
****
**
n.s.
***
*

5.5 Conclusion

In this chapter, I shifted from genome scale analysis to single gene investigation. The differential gene expression analysis highlighted *Nplp3* as promising candidate for the genetic connection between sleep disruption and longevity reduction. RNA quantification demonstrated that the reduction of *Nplp3* expression levels observed in RNA-seq data was preserved in multiple sleep mutants. Given that the affected genes were involved in quite different biological processes, the change of *Nplp3* expression was possibly the consequence of sleep disruption, the shared phenotype across various sleep mutants.

To study the effects of decreased *Nplp3* expression, I obtained a hypomorph mutation $Nplp3^{e}$. Animals carrying $Nplp3^{e}$ mutation exhibited robust reduction in *Nplp3* RNA levels. The mutant flies suffered from shortened lifespan but interestingly, showed normal amount of total sleep. These results satisfied the three criteria for determination of genes associated with reduced longevity in response to sleep disruption.

Overexpression of *Nplp3* was achieved by generating *Nplp3-Gal4* and *UAS-Nplp3* transgenic flies. Results showed that *Nplp3* overexpression led to decreased amount of total sleep. The effects on longevity was not robust despite mild trend of lifespan extension. The implications of sleep reduction by *Nplp3* overexpression is not yet clear, but it is interesting that such sleep reduction was not accompanied by shortened lifespan.

The importance of the *Nplp3* signal peptide was characterized by generating *UAS-*$Nplp3^{\Delta}$, a truncated *Nplp3* with signal peptide removed. Overexpression of $Nplp3^{\Delta}$ did not affect sleep. Significant lifespan shortening was observed in $Nplp3^{\Delta}$ overexpressed animals when compared with overexpression of *Nplp3*. Although these observations supported that *Nplp3* might serve as secreted neuropeptide, direct *in vitro* and *in vivo* assays would be necessary to test this hypothesis.

CHAPTER 6. DISCUSSION AND FUTURE DIRECTIONS

My thesis project was aimed at elucidating the gene expression changes that occurred in sleep mutants and could result in reduced longevity. By pooling experimental results from a panel of sleep mutants with results from wild type animals, I observed a positive correlation between daily sleep time and median longevity. I hypothesized that transcriptome scale gene expression changes might serve as the connection between sleep and longevity. RNA-sequencing experiments were carried out to profile two sleep mutants, wild type animals and aged wild type animals at multiple timepoints. Circadian analysis and differential gene expression analysis were performed to identify rhythmicity change and candidate genes, respectively. Lastly, I investigated *Nplp3* in closer detail. Expression levels of *Nplp3* decreased in several mutants suffering from sleep loss. Reduced *Nplp3* expression resulted in shortened lifespan but did not significantly affect sleep. In this chapter, I revisit these results and propose future experiments.

6.1 Correlation between sleep and longevity

Although sleep deprivation has become a common problem in modern society, the actual physiological consequences of sleep deprivation remains unclear. Using *Drosophila* as model organism, my thesis project studied the relationship between sleep disruption and longevity reduction.

Over the past decades, dozens of gene mutations have been associated with abnormal sleep behavior. Interestingly, in most of the cases, reduction in sleep amount coincides with shortening in lifespan. After backcrossing a panel of sleep mutants into the same isogenic genetic background, I observed a positive correlation between daily sleep time and median longevity using pooled data from sleep mutants and wild type flies. Given that the mutated genes were involved in diverse biological pathways, this discovery led to the hypothesis that longevity reduction resulted from sleep loss, rather than the disruption of gene function. On the other hand, these data supported the hypothesis that sleep loss would cause longevity problems. Since most of the sleep and longevity regulation pathways are conserved from flies to mammals, it is possible that such a positive correlation between sleep and longevity would be preserved in humans.

It is also worth noting that when testing the correlation between sleep and longevity, sleep data was collected from 2- to 7-day-old animals. Although sexual maturity is achieved within 1 day in *Drosophila*, the flies used for sleep measurements were still considered to be young animals. The robust and significant correlation between daily sleep time in young animals and median longevity suggested that sleep loss caused by genetic mutation was prevalent throughout lifetime. This idea was partially demonstrated by monitoring sleep behavior of the RNA-seq flies over two months (**Figure 2.4B**). Daily total sleep amounts for wild type flies and $wake^{D2}$ flies remained relatively constant, and $wake^{D2}$ animals showed significantly reduced levels of sleep over the entire course of the experiment. However, total sleep of inc^{1} flies increased dramatically during aging, which was likely due to survivor bias. In fact, the number of tested inc^{1} animals in the later stage was much smaller than that number in the early stage, because there were only a handful of aged inc^{1} animals available for sleep measurement. Previous study documented sleep behavior of *Hk* mutant flies over lifetime (Bushey et al., 2010). Mutants and control animals were continuously and simultaneously monitored for sleep and survivorship. *Hk* mutant flies exhibited elevated total sleep amount during aging, the extent of which was comparable to the increase of total sleep observed in inc^{1} animals. Results of Hk mutants reiterated the survivor bias, where the flies that lived longer showed longer sleep duration.

Furthermore, from data not shown in this study, I observed that most sleep mutants did not exhibit significant increase in total sleep amount between 7-day-old and 30-day-old, except for inc^{1} and *fmn*. Total sleep per day of *fmn* flies increased by 100% from Day 7 to Day 30 after eclosion. The dramatic improvement in total sleep amount might explain why *fmn* flies did not exhibit robust longevity reduction (Kume et al., 2005). Neuronal knockdown of *inc* using RNA interference (RNAi) reduces sleep but does not alter lifespan (Stavropoulos and Young, 2011), reminiscent of *fmn* phenotypes. One possible explanation could be that transcription suppression via RNAi was not consistent over the entire lifetime of these flies, thus older animals received sufficient amounts of sleep for normal survival. To better support the correlation between sleep and longevity, it is necessary to monitor sleep behavior at multiple stages during aging.

6.2 Circadian rhythm in the relationship between sleep and longevity

Identification of oscillating transcripts in various physiological context has drawn significant attention since the introduction of transcriptome profiling. Claridge-Chang et al. (2001) used microarray assays to study the differences in circadian gene oscillation between wild type animals and arrhythmic circadian mutants; and discovered 158 genes with circadian expression. The application of RNA-sequencing greatly expanded the scale of transcriptome profiling. RNA-seq experiments with JTK_cycle analysis identified 870 and 262 oscillating genes in wild type animals and per^{0} mutant flies, respectively (Hughes et al., 2012). In similar experimental settings, ARSER identified 2036 oscillating genes in 5-day-old wild type flies (Kuintzle et al., 2017). The huge spread between the numbers of oscillating genes identified in different studies not only emphasized greater power and higher coverage of RNA-seq, but also called for extra caution when selecting bioinformatic algorithms. In the present study, I decided to exploit an ensemble approach to analyze the datasets with multiple bioinformatic algorithms. Individual program yielded comparable results to previous studies. For example, when analyzing the control group, JTK_cycle and ARSER identified 977 and 2180 rhythmic genes, respectively (**Figure 3.2**). By selecting genes that were determined to be rhythmic by at least two programs, I was able to boost the robustness of the results without sacrificing too much accuracy. This approach identified 1115 oscillating transcripts in 27-day-old wild type animals.

Although the expression of core clock genes did not show substantial changes, I observed intriguing effects on the rhythmicity of many circadian genes. Perhaps the most striking finding was that 1583 genes were classified as rhythmic in aged wild type flies, about 40% more than the rhythmic genes in control wild type flies. Such a gain of rhythmicity during aging has been reported recently when comparing the circadian gene oscillation between 5-day-old flies and 55-day-old flies raised in LD cycles (Kuintzle et al., 2017); by analyzing the results generated by ARSER, 48 genes were found to be highly rhythmic in young flies but arrhythmic in old flies, while 38 genes were highly rhythmic in old but arrhythmic in young flies. In my thesis study, I used the same criteria in the determination of highly rhythmic and highly arrhythmic genes. My analysis discovered 111 loss of rhythmicity genes and 203 gain of rhythmicity genes during aging. The disparities could be explained by two possibilities: 1) age of the profiled flies, and 2) algorithms used to calculate p-value. The young group in the present study was 27 days old and I used p-values calculated by JTK-cycle. Nevertheless, both studies discovered age-dependent rhythmicity changes. It would be interesting to investigate the functions of these genes and perhaps more importantly, how the circadian gene transcription was modulated during aging.

Rhythmicity changes in sleep mutants inc^{1} and $wake^{D2}$ were predominantly loss of rhythmicity. Most interestingly, more than 50% of the genes with altered rhythmicity overlapped between the two sleep mutants. The circadian clock is believed to be intact in *inc* mutants at least within days after eclosion (Stavropoulos and Young, 2011). WAKE proteins have been shown to be rhythmically expressed in clock neurons and act downstream of CLK to regulate timing of sleep onset (Liu et al., 2014). Since I did not observe substantial changes in the expression of core clock genes, it is likely that the rhythmicity changes resulted from sleep disruption instead of central clock dysfunction. It remains unclear how the two processes of sleep regulation interact with each other.

6.3 Function of *Nplp3* in sleep and longevity regulation

The DGE pipeline used in this study was a combination of established methods and customized measures. DESeq2 and edgeR both have standard parameter settings that can select differentially expressed genes. However, application of DGE algorithms on time series data needs special handling of the programs. RNA-seq experiments with simple design, for example treated vs control, are very different from RNA-seq experiments with multiple factors, for example my thesis study. In DESeq2, the former uses the Wald test and the later uses likelihood ratio test (LRT). Several circadian-related studies averaged the count data of all times and tested the averaged counts for differences or looked for genes that are significantly changed at all timepoints (Gill et al., 2015; Kuintzle et al., 2017). Such approach is problematic because a gene that showed phase shift or rhythmicity change could be missed. In my thesis study, I took a different approach to detect gene expression changes at any timepoint, which undoubtedly raised the false discovery rate. This problem was solved by intersecting the results from multiple conditions and applying filters on expression change. My approach was proved to be effective because all the final candidate genes showed robust and significant changes in the RNA-seq gene plots (**Figure 4.4**).

The DGE analysis led to the identification of candidate gene *Nplp3*. RT-qPCR results demonstrated that expression levels of *Nplp3* decreased in multiple sleep mutants. Furthermore, the reduction in *Nplp3* expression seemed to be age-dependent, present in 30-day-old mutants but absent in 7-day-old mutants. Data from another transcriptome profiling study also showed that expression levels of *Nplp3* are significantly lower in 55-day-old flies than in 5-day-old flies (Kuintzle et al., 2017). It would be interesting to perform a longitudinal study to examine the expression levels of *Nplp3* at multiple stages during development and aging. Meanwhile, RNA-seq data suggested that *Nplp3* expression oscillated in wild type flies. Aging as well as mutations of *inc* and *wake* dampened the oscillation amplitude. If these results could be validated by RT-qPCR, it would provide an entry point to study the interaction between circadian rhythm and sleep.

Involvement of *Nplp3* in longevity regulation was revealed by the hypomorph mutation $Nplp3^{e}$. The mutation caused substantial decrease in *Nplp3* expression and significantly shortened lifespan. Interestingly, the mutant flies showed normal amounts of sleep, indicating that *Nplp3* was not necessary for normal sleep. On the other hand, overexpression of the wild type version of *Nplp3* slightly reduced total sleep but did not shorten longevity. The decoupling of sleep disruption and longevity reduction further indicated that *Nplp3* was not a sleep gene like *inc* and *wake*.

Results of *Nplp3* overexpression also pointed to the importance of the signal peptide of *Nplp3*. Overexpression of truncated form of *Nplp3* without signal peptide showed significantly shortened lifespan compared to overexpression of wild type form of *Nplp3*. This evidence, together with previous neuropeptide profiling study and bioinformatic predictions, hinted that NPLP3 proteins might be processed into neuropeptides (Almagro Armenteros et al., 2019; Baggerman et al., 2002). Although there was no orthologs of *Nplp3* in mammalian species, the sequence of *Nplp3* was highly conserved within the *Drosophila* genus. Homology of neuropeptide signaling pathways has been shown to be more evident in the receptors. For example, PDF does not have a mammalian homolog, but its receptor is believed to share homology with mammalian calcitonin receptor (Mertens et al., 2005). The insights that *Nplp3* could

provide for sleep and longevity relationship in humans awaits the characterization of its molecular identity and potentially its receptor.

With the available genetic tools including *Nplp3-Gal4* and *UAS-Nplp3*, it is now possible to study the properties of *Nplp3* in vivo. Using *Nplp3-Gal4* to drive the expression of fluorescent proteins would reveal the cells that *Nplp3* is expressed. Overexpression of *Nplp3* using brain region or cell type specific drivers could help to understand the requirement of *Nplp3* in various contexts. Most importantly, I am curious about whether it would be possible to improve longevity in sleep mutants by restoring *Nplp3* expression. Such experiments are underway, and the results will contribute to our knowledge about the relationship between sleep ang longevity.

MATERIALS AND METHODS

Drosophila Strains and Culture

Drosophila melanogaster stocks were raised on standard media (cornmeal/yeast/ molasses/agar) at 25°C under 12-hour light/12-hour dark (LD) cycles. Wild type isogenic strain *Canton-S* w^{1118} *(iso1CJ)* (Li et al., 2013) was used as controls for behavioral experiments and for RNA-seq experiments and referred to as *WT*. Sleep mutant strain inc^{1} (Stavropoulos and Young, 2011) was a standing stock of the laboratory. Sleep mutants sss^{P1} (Koh et al., 2008), *fmn* (Kume et al., 2005), and Hk^{Y} (Bushey et al., 2007) were kindly provided by Dr. Amita Sehgal. Sleep mutants $wake^{D1}$ and $wake^{D2}$ (Liu et al., 2014) were kindly provided by Dr. Mark Wu. Sh^{5} (Bloomington #111), Hk^{1} (Bloomington #3562), and $Nplp3^{e}$ (Bloomington #17988) were obtained from Bloomington *Drosophila* Stock Center. *Nplp3-Gal4*, *UAS-Nplp3*, and $UAS\text{-}Nplp3^{\Delta}$ strains were generated with methods detailed below. All fly strains used in this study were backcrossed to the wild type strain *Canton-S* w^{1118} *(iso1CJ)* for at least 5 generations. For sleep measurements and longevity assays in this study, male animals were used.

For sleep mutants without selectable markers, including *fmn*, Hk^{1}, Hk^{Y}, and Sh^{5}, backcrossing was carried out in two steps. Fly strains with selectable markers inserted in adjacent to the sleep gene mutations were first backcrossed to *WT* strain and subsequently used to backcross the sleep mutants. For *fmn*, $PBac\{WH\}CG4945^{f02115}$ was used; for Hk^{1} and Hk^{Y}, $PBac\{WH\}Hk^{f00239}$ was used; for Sh^{5}, $PBac\{WH\}Sh^{f04502}$ was used. $PBac\{WH\}CG4945^{f02115}$ (Bloomington #18515) was obtained from Bloomington *Drosophila* Stock Center. $PBac\{WH\}Hk^{f00239}$ (Exelixis f00239) and $PBac\{WH\}Sh^{f04502}$ (Exelixis f04502) were obtained from Exelixis *Drosophila* Collection.

Sleep Measurement and Analysis

Locomotor activity of flies was collected using the *Drosophila* Activity Monitor (DAM) system (TriKinetics, Waltham, MA). Flies were singly housed in glass tubes containing fly culture food and assayed at 25°C under LD cycles. Activity counts were collected at 1-min bins for 5 LD cycles immediately after the loading day. DAM monitor files were processed with pySolo software (Gilestro and Cirelli, 2009). Sleep was determined as at least 5 minutes of inactivity. Sleep profiles were analyzed with a customized R script. For each fly, data from 5 LD cycles was averaged to generate sleep profile and calculate daily total sleep, daytime sleep, and nighttime sleep.

Longevity Assay

Parent flies were raised on standard media for 2-3 days and then removed. Offspring were collected within 24 hours after eclosion and were raised in LD cycles at 25°C for 2 days. Subsequently, 15 male animals were placed into each individual food vial, and the vials were placed in LD cycles at 25°C. Animals were transferred to new food vials every 1-3 days and the number of dead animals was recorded in parallel. dLife software was used to analyze longevity data (Linford et al., 2013). Log-rank tests were performed to determine statistical significance. Flies escaped during transfer were included in the analysis as right-censored events.

RNA Extraction

Heads of about 200 flies were collected at designated age and time. For RNA-seq experiments, four groups of samples were collected, *WT* at Day 27 as control group, inc^{1}

at Day 27, *wake*^{D2} at Day 27, and *WT_old* at Day 49. Separately for RT-qPCR experiments, samples were collected from 7-day-old or 28-day-old flies, as indicated in main text. Total RNA was extracted using TRIzol reagents and homogenized using a BeadBug microtube homogenizer (Benchmark Scientific). Samples were further extracted using chloroform and the aqueous phase containing nucleic acids was harvested. RNeasy Mini Kit (Qiagen) was used to remove DNA with DNase and further purify the samples, according to the manufacturer's protocol.

RNA Sequencing

RNA-seq was conducted at the Genomic Resources Center of the Rockefeller University. Sequencing libraries were prepared with the Illumina TruSeq stranded mRNA LT kit. 100 nanogram of total RNA for each sample was used. Libraries were multiplexed and sequenced on the Illumina NextSeq 500 sequencer using high output V2 reagents and NextSeq Control Software v1.4 to generate 75bp single reads, following manufacturer's protocol. The sequencing depth was about 25 million reads per sample. Reads were aligned to the *D. melanogaster* reference genome assembly (Release 6.13) (dos Santos et al., 2015) with STAR (Dobin et al., 2013) and read counts were generated with featureCounts (Liao et al., 2014). Genes with cpm values less than 1 in more than 28 samples in the entire dataset were considered as low expression and were removed from further analysis. Pearson's correlations between samples were calculated using package in R. PCA was performed using module implemented in DESeq2 (Love et al., 2014).

Differential Gene Expression Analysis

DGE analysis was performed with DESeq2 (Love et al., 2014) and edgeR (McCarthy et al., 2012; Robinson et al., 2010) with recommended parameter settings for time series analysis. Specifically, likelihood ratio test (LRT) was used in DESeq2 and generalized linear model (GLM) was used in edgeR. Differentially expressed genes were selected with significance cutoffs of adjusted $p < 0.05$ for DESeq2 and $p < 0.05$ for edgeR. Gene ontology (GO) analysis was performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) (Huang da et al., 2009a, b).

Circadian Analysis

Rhythmically expressed genes were identified using ARSER (Yang and Su, 2010), JTK_cycle (Hughes et al., 2010), and GeneCycle (Ahdesmaki et al., 2007; Ahdesmaki et al., 2005; Wichert et al., 2004). For all three programs, input data was formatted as a series of two consecutive cycles. Genes that were detected to be oscillating in at least two programs were considered as oscillating (Koike et al., 2012). For phase distribution, peak expression times calculated by JTK_cycle were used. For change of rhythmicity analysis, highly rhythmic genes were defined as adjusted $p < 0.01$ and highly arrhythmic genes as adjusted $p > 0.5$, with adjusted p values calculated by JTK_cycle.

Generation of Transgenic Flies

For *Nplp3-Gal4* construct, genomic DNA was isolated with standard methods and a ~1.7 kilobase (kb) genomic region directly upstream of the *Nplp3* start codon was amplified by PCR using primers zw195 and zw196. PCR products were subcloned into pBPGUw (Pfeiffer et al., 2008) using Gateway Cloning (Thermo Fisher Scientific). The *Nplp3-*

Gal4 construct was inserted into the *attP2* landing site on the third chromosome using PhiC31 (Rianbow Transgenic Flies). pBPGUw was a gift from Gerald Rubin (Addgene plasmid # 17575).

For *UAS-Nplp3* and *UAS-Nplp3$^{\Delta}$*, cDNA was synthesized using iScript cDNA Synthesis Kit (Bio-Rad), following manufacture's protocol. *Nplp3* cDNA was amplified by PCR using primers zw197 and zw198, and *Nplp3$^{\Delta}$* cDNA was amplified using primers zw198 and zw212. PCR products were subcloned into pJFRC7-20XUAS-IVS-mCD8::GFP with XhoI and XbaI (New England Biolabs). The constructs were inserted into the *attP40* landing site on the second chromosome using PhiC31 (BestGene). pJFRC7-20XUAS-IVS-mCD8::GFP was a gift from Gerald Rubin (Addgene plasmid # 26220).

RT-qPCR

cDNA was synthesized using iScript cDNA Synthesis Kit (Bio-Rad) following manufacture's protocol. RT-qPCR was performed using SYBR Green Fast (Applied Biosystems) on a 7500 Fast Real-Time PCR system (Applied Biosystems). The primers for *Nplp3* were zw109 and zw110. Housekeeping gene *Glycerol-3-phosphate dehydrogenase 1* (*Gpdh1*) was used as endogenous control with primers zw91 and zw92 (Kidd et al., 2015; Ozturk et al., 2013).

Primers

zw91	5'-CCACTGCCGAGGAGGTCAACTAC-3'
zw92	5'-ATGCTCAGGGTGATTGCGTATGC-3'
zw109	5'-GCCATTTCTCATGCTGCCATC-3'
zw110	5'-CAGGGCACAGAACTTGTGGTTG-3'
zw195	5'-CACCACTATTAGGCTTGGTTCGCTGAC-3'
zw196	5'-TTTGTTTTGTTGATGGTTTGCAACG-3'
zw197	5'-ACGTCTCGAGCGTTGCAAACCATCAACAAAACAAA-3'
zw198	5'-ACGTTCTAGAGGATCTCCAATGGGTAACTGGT-3'
zw212	5'-ACGTCTCGAGATGGCCCCAGCTCCCGCTCCTGC-3'

Statistical Analysis

Statistical analysis for sleep experiments was performed using GraphPad Prism 8 with specific statistical tests described in figure legends. Statistical analysis for longevity experiments was performed using dLife. RNA-seq experiments were analyzed in R.

Data and Code Availability

The RNA-Seq datasets generated in this study have been deposited in NCBI's Gene Expression Omnibus (GEO) (Edgar et al., 2002) and are accessible through GEO series accession number GSE148136. All code used to process data are available upon request.

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