OVEREXPRESSION OF MIRNA-975 INDUCED IMMUNE GENES AND ABNORMAL SPERMATOGENESIS IN *Drosophila melanogaster*

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OVEREXPRESSION OF MIRNA-975 INDUCED IMMUNE GENES AND ABNORMAL SPERMATOGENESIS IN *Drosophila melanogaster*

by

SHARVIN A/L MANICKAM

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ACKNOWLEDGEMENT

'My name is Sharvin, fool of fools. Look upon my work, ye Mighty, and chortle'

The spirited, but jammy claims may just be the memorandum of my years as a Master's graduate. Despite many flower pots thrown at my general direction, I'd like to think that I did an adequate job. And it's the godsends of many names that delivered me safely to TBS, for my journey home and beyond.

Amma + Appa + Sharmila + Rivisya, we have grown so much over the years. It makes me glad even when, I am far away, you'd care for each other; when I come knocking, I know we can be a family five again. Thank you for the blessings and prayers. P.S.: Can we please get a plumber to fix the water pump? I'd like to effectively bathe for 3 minutes.

Dr. Ghows, Sir, I could never believe my luck when you accepted me in to be part of Lab 418! What charms! Thank you for caring for us despite the distance, and being vigilant about our conduct and research. Hat's off for taking a chance with me, and for your infinite patience for every mischief I managed. Dr. Kamarul, Sir, my appreciation for all the guidance, sit downs, chats, and assurances we have had. Thank you for the lit beacon, let's see about the coffee!

My dearest friends, I adore you. To Shallinie, whose words and smile calms my stormy mind; Wen Chen, who is a terrifying force of nature, and the loveliest companion; Kenneth for being absolutely chaos agent and steadfast in your pursuit – like a cat; Florence, for you a sanctuary city and schools of wisdom; Bob from whom I learned to share and give; in Charlie a confidante whom I have shared pain, laughter and drives with; and in Mus, a kindred poet and my fellow journeyman. I have missed you all, and I adore you each at every passing day.

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I not deserve any of you, but I hope I had made you proud. My voice may be all but quiet now, but my love for you endures in hymns and lips, thoughts and dreams.

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LIST OF SYMBOLS

%	Percentage
=	Equal to
±	More or less
×	Multiply
≤	Less than or equal to
℃	Celsius
µg	Microgram
µL	Microliter
µm	Micrometre
bp	Base pair
g	Gram
L	Litre
M	Molar
mL	Millilitre
mm	Millimetre
mM	Millimolar
ng	Nanogram
™	Trademark
V	Volt
α	Alpha

LIST OF ABBREVIATION

%	percentage
°C	degree Celsius
µg	microgram
µL	microliter
Act5C	*Actin5C*
AGO	Argonaute
AMPs	Antimicrobial peptides
Anp	Andropin
APC	Antigen-presenting cells
AttC	Attacin-C
Bam	Bag of marbles
BDSC	Bloomington Drosophila Stock Center
BMP	Bone Morphogenetic Protein
C.	*Caenorhabditis*
cDNA	Complementary DNA
CecA1	Cecropin A1
CO_2	Carbon dioxide
CTPSyn	CTP Synthase
CyO	Curly wings
CySCs	Cyst stem cells
D.	*Drosophila*
DAPI	4′,6-diamidino-2-phenylindole
DAVID	Database for Annotation, Visualization, and Integrated Discovery

DGRC	Kyoto Stock Center
dH_2O	Distilled water
DIAP	*Drosophila* Inhibitor of Apoptosis
DNA	Deoxyribonucleic acid
DSHB	Developmental Studies Hybridoma Bank
EGFR	Epidermal growth factor receptor
eIF4A	Eukaryotic translation-initiation factor 4A
EIf4G	Eukaryotic translation-initiation factor 4G
Fas III	Fasciclin III
FC	Fold change
GB	Gonialblast
Gj	Gap junction
GO	Gene Ontology
GO	Gene ontology
GSC	Germline stem cells
Hh	Hedgehog
HISAT	Hierarchical indexing for spliced alignment of transcripts
HTS	Hu-li tai shao
IL-1	interleukin 1
IL-6	interleukin 6
IMD	Immune deficiency
JAK-STAT	Janus-kinase – signal transducer and activator of transcription
KEGG	Kyoto Encyclopedia of Genes and Genomes
Mb	Megabase
MHC	Major histocompatibility complex

miRNA	microRNA
mL	mililiter
mRNA	messenger ribonucleic acid
mRNPs	Free messenger ribonucleoproteins
NGS	Normal Goat Serum
NK	Natural killer
Nplp2	Neuropeptide-like precursor 2
PAMPs	Pathogen associated molecular patterns
PASPC	Poly(A)-binding protein
PASPC	Poly(A)-binding protein
PCD	Programmed cell death
PFA	Paraformaldehyde
PGRPs	Peptidoglycan receptor proteins
PNG	Portable Network Graphic
pri-miRNA	Primary transcript microRNA
pri-miRNA	Primary transcript microRNA
PRRs	Pattern recognition receptors
qPCR	Qualitative Polymerase Chain Reaction
Rel	Relish
RISC	RNA - induced silencing complex
RNA	Ribonucleic acid
RNAi	RNA Interference
ROS	Reactive oxygen species
Rpm	Revolutions per minute
Sb	Stubbles

TA	Transit amplification
TCR	T-cell receptors
Tj	Traffic jam
TNF	Tumour necrosis factor
UAS	Upstream activating sequence
Upd	Unpaired
UTR	Untranslated region
UV	Ultraviolet
VDRC	Vienna Drosophila Resource Center
Zpg	Zero population growth
α-	Anti-

PENGEKSPRESAN BERLEBIHAN MIRNA-975 MENGARUH GEN KEIMUNAN DAN SPERMATOGENESIS TIDAK NORMAL DALAM *Drosophila melanogaster*

ABSTRAK

Homeostasis adalah sistem penting bagi organisma untuk berevolusi. Maka, kebarangkalian sistem tersebut tergugat, sistem imun organisma tersebut akan bertidak balas. Apabila miR-975 diekspres berlebihan, hujung apikal testis *D. melanogaster* menyerupai penampilan tumor, menunjukkan bahawa homeostasisnya telah 'terganggu'. Maka dengan ini, perhubungan antara miR-975, spermatogenesis, dan imuniti akan disiasat dalam manuskrip ini. Untuk memahami fenotip testis yang sfera, perkembangan spermatogenesis perlu dicirikan, dari permulaan hingga peringkat spermatosit lewat, sementara transkriptomik miR-975 digunakan untuk mengenal pasti gen-gen imuniti *D. melanogaster* yang bertindak balas terhadap homeostasis yang tidak seimbang tersebut. Antibodi-antibodi yang spesifik digunakan untuk melakarkan setiap peringkat spermatogenesis tersebut. Antibodi-antibodi tersebut dipadankan dengan penanda yang sesuai, akan diinkubasi dengan testis dari keturunan kawalan (Actin5C-GAL4 > Oregon R) dan keturunan transgenik (Actin5C-GAL4 > UAS-miR-975) masing-masing, menggunakan mikroskopi konfokal. Untuk menjelaskan data transkriptomik, gen-gen berekspresi tinggi akan disahkan menggunakan qPCR. Hujung apikal testis sfera didapati memiliki amplifikasi transit (TA) yang lebih pendek, lebih banyak sel positif coilin (spermatosit), dan kekurang spermatid. Gen imuniti telah disahkan seperti yang dicadangkan oleh transkriptomik. Model penyelidikan yang disarankan untuk membincangkan hujung apikal yang sfera adalah perbezaan awal, persaingan sel, pembahagian sel asimetri nyah spesifik, penghindaran

apoptosis, dan kecacatan proses meiosis. Interaksi antara spermatogenesis dan imuniti seperti yand dilihat adalah disebabkan oleh pengalihan tenaga dari spermatogenesis ke tindakan imuniti, yang menyumbang kepada kadar fertiliti yang amat rendah.

OVEREXPRESSION OF MIRNA-975 INDUCED IMMUNE GENES AND ABNORMAL SPERMATOGENESIS IN *Drosophila melanogaster*

ABSTRACT

Homeostasis is the measure of an organism's evolutionary adaptation. Any oddities to the organism's phenotype signal a system at disarray, with the full force of immune systems in response. When miR-975 was overexpressed, the apical tip of the *D. melanogaster*'s testis was bulged, mirroring a tumour-like appearance, suggesting its homeostasis were 'knocked off' balance. Thence, the associations between miR-975, spermatogenesis and its innate immunity is to be investigated in this manuscript. To understand the bulged phenotype of the testis's apical tip, the developmental stages of spermatogenesis had to be characterised, from the onset till late spermatocyte stage, whilst miR-975's transcriptomics identifies which of the immune genes were in response to the said imbalanced homeostasis. To characterise the spermatogenesis of the bulged testis, antibodies complimentary to the aforesaid stages, matched to its appropriate markers each were incubated with dissected testes from control (*Actin5C-GAL4 > Oregon R*) and transgenic progenies (*Actin5C-GAL4 > UAS-miR-975*) respectively, using confocal microscopy. On the other hand, to elucidate the transcriptomics' data, genes that were upregulated were singled out and validated using qPCR. The bulges apical tips were found to have shorter transit amplification (TA), more of coilin-positive (spermatocyte) cells' number, and fewer elongating spermatids. Upregulated immune genes were validated as per suggested by the transcriptomics. The research models proposed to discuss the bulged apical tips are early differentiations, cell competition, rogue asymmetrical cell division, apoptosis aversion, and meiosis entry defects. A profound interplay between spermatogenesis

and the innate immunity, due to energy re-direction from spermatogenesis towards innate immunity actions according to the low fertility rate, had been suggested here.

CHAPTER 1

INTRODUCTION

1.1 Overview

MicroRNA 975 is a lonely divinity, bewitched and duty-bound as part of miRNA silencing mechanism – as a mean regulate gene expressions. But therein the short tale falters, and this epic begins – this is a blockbuster of when the gods went mad.

Fruit flies or *Drosophila melanogaster* is truly a modern Prometheus in the modern pantheon of research animal models. *D. melanogaster* has speedy regeneration cycle, small but serendipitous genome make-up, and Promethean genetics tools. The aforementioned tools had made leaps and bounds in cell-, developmental- and molecular biology, especially the latter third with its balancer chromosomes and UAS-GAL4 binary system (Hales et al., 2015; Yamaguchi, 2018).

It was with these, a curious phenomenon at the *D. melanogaster*'s testis is observed – its typically tapered apical tip was bulged. An overexpression of microRNA 975 (miR-975) causes the apical tip to be bulbous-shaped. Found only in *D. melanogaster*'s testes, mir-975's overexpression has had prior attempts to characterise the bulged phenotype (Mohammed et al., 2014; Zhao et al., 2021). *D. melanogaster*'s testes undergo spermatogenesis, for its namesake, is the creation of 64 spermatid haploids, that would ultimately individualize, mature and deposited at seminal vesicles (Demarco et al., 2014). The developmental stages are as follow: spermatogonia, spermatocyte, and spermatid.

Previous attempts characterising miR-975's overexpression in the testes' developmental stage were performed from its early spermatogonia stages till its early spermatocyte stages, (Mohammed et al., 2014; Zhao et al., 2021). This manuscript thenceforth extends the characterisation to its late spermatocyte stage, whilst recording the fertility of the *D. melanogaster*

In lieu of the late-stage spermatocyte characterisation, miR-975 transcriptomics were studied. Transcriptomics are sets of RNA transcripts of specific cell types at any particular developmental stages or physiological condition. Transcriptomics visualizes gene expressions, thus providing insights into molecular mechanisms and specific biological processes (Dong & Chen, 2013). Hereafter, these would lay way to comprehend how the bulged phenotype could have occurred, and possibly, its potential targets.

Any perfect organism has a balanced homeostasis. Disrupted homeostasis would be recalibrated by immunity (Taniguchi et al., 2009). A bulged testis from the miR-975 may not be of an equilibrium homeostasis, thence a warrant for a closer look at an interplay between miR-975 overexpressed - addled spermatogenesis and *D. melanogaster*'s innate immunity. This is the mission of the thesis.

1.2 Objectives

1. To characterize spermatogenesis from spermatogonia till late spermatocyte stage:
2. To identify resultant interplay of miR-975's overexpression and innate immunity.

1.3 Outline

This thesis has five chapters, as listed far below:

Chapter 1 houses brief overview, research objectives, and outline of the dissertation at its entirety.

Chapter 2 details all the elements that are relegated as the focus of the research – such as: i) The introduction and conception of microRNA (miRNA), besides its dogmatic functions and its master of the ceremony, miRNA 975; ii) The animal model used for the research, *D. melanogaster*, is prominently discussed here, for what their advantages may be and their devastating secret weapons that would aid for phenotypic scorings and molecular analyses in the subsequent chapters. A special recognition is written for the spermatogenesis, truly the backbone for the research; iii) Finally, it is a small trip around the topic of insect immunity, with particular in *D. melanogaster*.

Chapter 3 is the cook-chapter, tracing an origin at the fly husbandry before leafing down to dissecting testes out of the male progenies, staining those testes with antibodies for microscopy analyses, complete with a detailed instruction to modify its settings, crushing other supplementary testes for RNA extraction, that would be then spun into complementary DNA (cDNA), and ultimately be verified for its immune-related genes that may have had a hand in spermatogenesis; the immune genes were isolated with relevant software from its preceding transcriptomics.

Chapter 4 is an amalgamation of results consequent for visualization and screening of the spermatogenesis amongst controls (*Actin5C-GAL4>Oregon-R*) and the transgenics (*Actin5C-Gal4>UAS-miR-975)*. Transcriptomic analyses and quantitative polymerase chain reaction (qPCR) would suggest an interplay between miR-975 and innate immunity, encompassing the immune genes.

Chapter 5 discusses every facet of the results reported in regards to the bulged testes and upregulated genes singled out by the transcriptomics, by ways of proposing research models that could best explain the phenomenon.

Chapter 6, after a long last, encapsulates the key findings throughout the manuscript.

CHAPTER 2

LITERATURE REVIEW

2.1 RNA-lm

A comical portmanteau of RNA and realm, reflecting its vast legacy, with microRNA (miRNA) a brick upon the proverbial empire.

In 1993, the first discovery of miRNA was made to light upon observing the presence of miRNA family, *lin-4* and *let-7*, where a correlation between *lin-4* and *lin-14*, it has been postulated that the former regulates the latter at the post-transcriptional level. This then have since become a modern dogma for miRNA's function - where it downregulates gene expression of a messenger RNA (mRNA) through base-pairing at 3' untranslated regions, or more commonly written, 3' UTRs (Lee et al., 1993; Reinhart et al., 2000; Wightman et al., 1993). Since its public conception, miRNA had been reported amongst plants, animals, and viruses. These indicates miRNAs may have been a regulatory control of gene expressions (Bushati & Cohen, 2007).

MiRNA has an average of 22 nucleotides in length, besides being associated with the Argonaute (AGO) family proteins (O'Brien et al., 2018). MiRNA is brought forth with Drosha and Dicer. There have been reports of miRNA interacting with 5' UTR and gene promoters, alongside their typical interactions with 3' UTR (Broughton et al., 2016). Recent reports had shown that miRNA is capable of activation gene expression at certain conditions, besides having a deft hand in controlling translation and transcription rate within the realm of subcellular compartments (Makarova et al., 2016; Vasudevan, 2012).

miRNA has the proverbial keys to the kingdom; for plants and animals' development, for proliferation and differentiation engines within cells, besides apoptosis and metabolism's action myriads (Fu et al., 2013; Krol et al., 2010; Voinnet, 2009). Any dysregulations of the miRNA have been referenced with human diseases, including cancer or neurodevelopmental disorders (Paul et al., 2018; Peng & Croce, 2016). Excreted miRNA in extracellular fluids can be seen as biomarkers to track diseases apart from being a signalling molecule for cell-cell communications (Hayes et al., 2014; Huang, 2017; Wang et al., 2016). The campaign of which the proverbial keys were endowed, are the subject of great interest in Section 2.1.3.

2.1.1 miRNA biogenesis

Unto the object of interest's biogenesis; **1)** miRNA is first transcribed by RNA polymerase II and is called primary transcript (pri-miRNA); **2)** pri-miRNA forms a complex with Drosha to cleave the 5' Cap and 3' poly (A), in which it will become pre-miRNA; **3)** The pre-miRNA will be transported into the cytoplasm with the assistance of Exportin5; **4)** Once there, the pre-miRNA cleaved to a duplex miRNA strand will be with a Dicer complex; **5)** After the duplex had unravelled, one of the strand would be elected to be a guide, thus a mature miRNA, while the other would degrade away; **6)** The remaining strand stays loaded on an Argonaute-1 (AGO1) complex and now be known to be RNA-induced Silencing Complex (RISC) (Ha & Kim, 2014). Thence, the hunt begins.

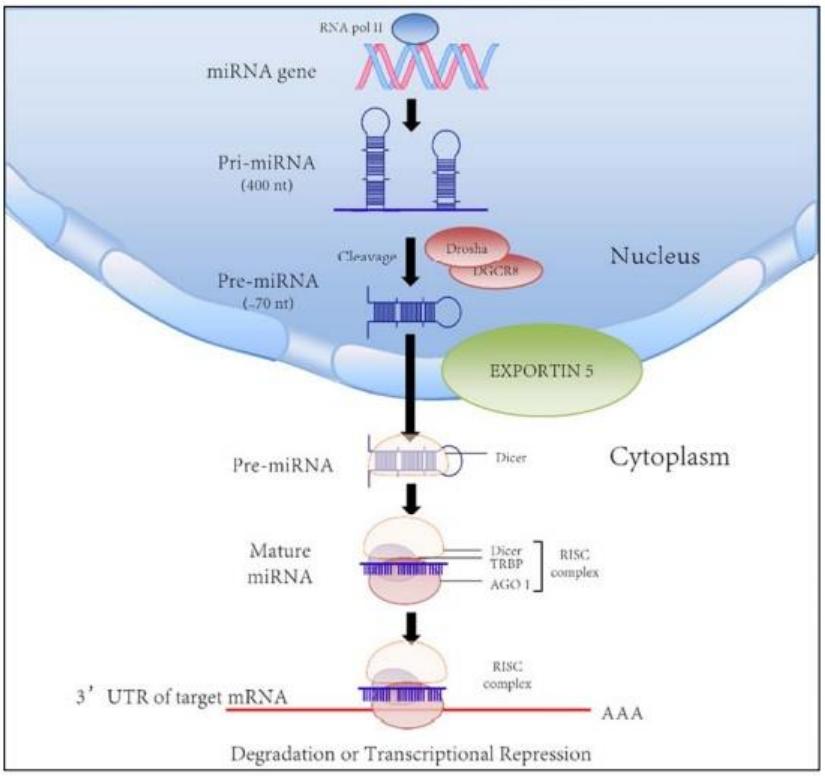

Figure 2.1 An illustration of miRNA biogenesis. The RNA polymerase II transcribes the mRNA into pri-miRNA, where it would be cleaved into pre-miRNA, whence it would be guided into the cytoplasm to be cleaved by a Dicer complex to form a miRNA-miRNA duplex. One of the strands would be selected as a mature miRNA to be uploaded onto an AGO1, and thus be known now collectively as RISC. RISC complex would target specific mRNAs based on its complementary seed region. Adapted from (Pisarello et al., 2015)

2.1.2 miRNA silencing

miRNA had since been defined by its function – to be a critical posttranscriptional regulators of gene expression by promoting mRNA degradation and translational repression (Fu et al., 2013). The miRNA silencing mechanisms - either by inducing translational repression, mRNA deadenylation, or mRNA decay will be decided by its sheer complementary base pairing between a miRNA and its target mRNA. Should the complementary percentage be high (or sufficient), then mRNA cleavage would take place, however, if the complementary percentage be low (or insufficient), the target mRNA would be degraded and translationally repressed (Guo et al., 2005; Hutvágner & Zamore, 2002)

The major players deployed into the field for a miRNA-mediated silencing are: Argonaute protein (AGO), GW182 trinucleotide-repeat-containing protein, cytoplasmic poly(A)-binding protein (PASPC), deadenylase complex's components (DCP1, EDC4, and DDX6), decapping enzyme DCP2, and decapping activators, such as DCP1, EDC4, and DDX6 (Huntzinger & Izaurralde, 2011; Ipsaro & Joshua-Tor, 2015).

2.1.2(a) Translational repression

Translation is a doctrinaire process amongst cells, with unchanging ribosomal subunits, and a strict procedure of initiation, elongation, and termination. It is an irrefutable fact that an mRNA worthy of translation would be those with an intact 5' cap structure and a 3'-poly(A) tail.

Translational repression is a reversible process, for complex's binding to its target does not eliminate it completely from the cellular system. Iwakawa and Tomari (2015), three major methods were thought to repress the mRNA translation: **(i)** GW182-mediated PABP displacement; **(ii)** translational repressors' recruitment through GW182; **(iii)** and dissociation of eukaryotic translation-initiation factor 4A (eIF4A) from eIF4F complex, made up entirely of eIF4E, eIF4G, and eIF4A. GW182 protein networks with C-terminal MLLE domain of PABP through PAM2 motif located in the C-terminal silencing domain.

There were suggestions that GW182-mediated displacement of PABP from poly(A) tail would unwound the closed-loop structure, created by eIF4G and PABP's interaction, henceforth repressing the translation initiation. Onto the second method, GW182 could repress translations of nonpolyadenylated mRNA through an interaction

with carbon catabolite repressor 4 – negative on TATA (CCR4-NOT) complex (Iwakawa & Tomari, 2015).

Finally, the third method centres in cytoplasm, where 3'-poly(A) tail is associated with poly(A)-binding protein (PASPC), while 5' cap plays a hand with eukaryotic translation-initiation factor 4G (eIF4G), alongside cap-binding protein, elF4E. This would lead to a circular mRNA that can be translated with no obstruction, thence protected from degradation. It has been since suggested that miRNA interferes the assembly of eIF4G complex by binding at the target transcript's seed sequence, henceforth rendering translation's initiation process defunct (Fukao et al., 2014; Huntzinger & Izaurralde, 2011).

Evidence of translational repression at the post-translational phase was that the translation of *lin-14* and *lin-28* mRNAs were repressed by lin-4 miRNA, for of *lin-14* and *lin-28* mRNAs were found in polysomes, hence a conclusion that translation repression happened after translation had occurred (Olsen & Ambros, 1999; Seggerson et al., 2002). Besides that, miRNA could potentially curb translation initiated autonomously of the cap structure through an internal ribosome entry sites (IRES) (Petersen et al., 2006). As for translational repression early at initiation, it was postulated that the miRNA – mRNA dyads were not pooled in at the sucrose gradient, alongside the polysomes. Rather, it seemingly favours a faction with fewer ribosomes or free messenger ribonucleoproteins (mRNPs) (Pillai et al., 2005).

2.1.2(b) mRNA target degradation decay

In a review by Huntzinger and Izaurralde (2011), evidences of target degradations are shown when the miRNA target counts are inversely proportional to the level of miRNA itself. Besides that, when specific miRNAs are swirled into a

cultured cell, the transcript with the specific seed sequence would show a small count, as well as depriving key components in miRNA pathways with such actors as Dicer or AGO, the miRNA's target reign supreme still.

The mechanism that brings forth this pathway are when miRNAs shepherd their target 5' to 3' mRNA decay pathway. Here, the mRNA would be deadenylated by CAF1-CCR4-NOT deadenylase complex, after which the transcript would be decapped by DCP2. The decapped mRNA will be degraded by XRN1, a cytoplasmic 5' to 3' exonuclease.

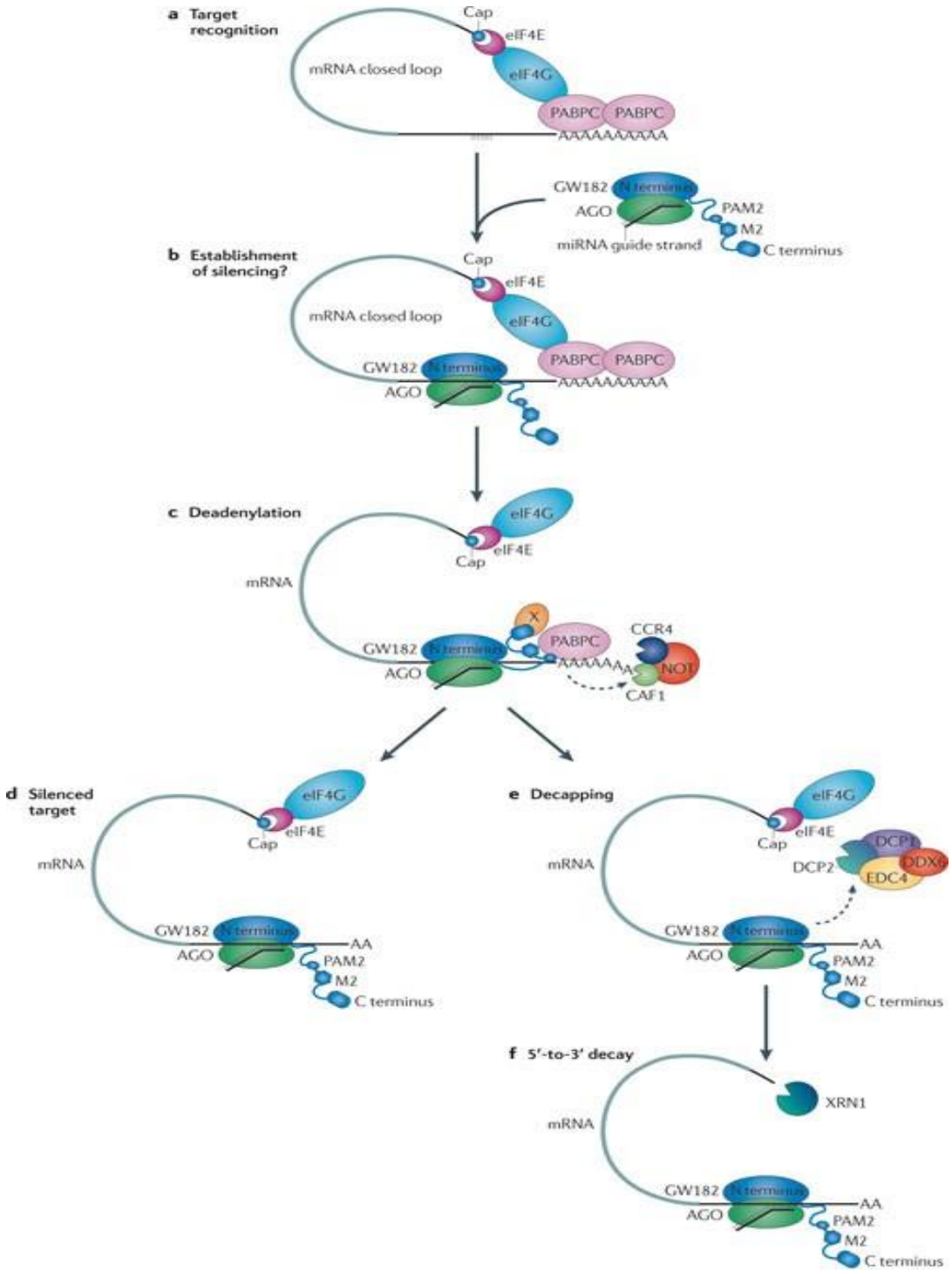

Figure 2.2 An illustration of miRNA-mediated gene silencing. Figure (a) depicts mRNA's target in a looped form where PABPC interacts with the poly-A tail, while elF4G is bound to elF4E, a cap-binding protein. In figures (b) and (c), the miRNA bound to AGO searches for their target, while AGO interacts with GW182 and PABPC. Figure (d) paints an image of translational repression, while Figure (e) and (f) shows the eventual mRNA decay, through decapping by exonuclease XRN1. Adapted from (Huntzinger & Izaurralde, 2011).

2.2 *Drosophila melanogaster*: Wonder bug of science

Within the walls of the Fly Room in University of Columbia, Thomas Hunt Morgan and his band of prodigious scientists, they have made *Drosophila melanogaster* a perennial celebrity in modern science (Morgan, 1910). Though named as a common fruit fly (or interchangeably, a vinegar fly), these winged insectoids have put in the builder stones in cell biology, population genetics, developmental and molecular biology, besides improving a wide array of analysis techniques through gene cloning, hybridization of P-element-based transformation, and clonal analyses in search for the knowledge, besides as a research model for studying human diseases, such as tumour, Alzheimer's disease, and Parkinson's disease – to name a few (Masamitsu Yamaguchi & Hideki Yoshida, 2018). It has since shot our understanding of genes, chromosomes, and the inheritance of genetic information (Ashburner & Bergman, 2005; Hales et al., 2015).

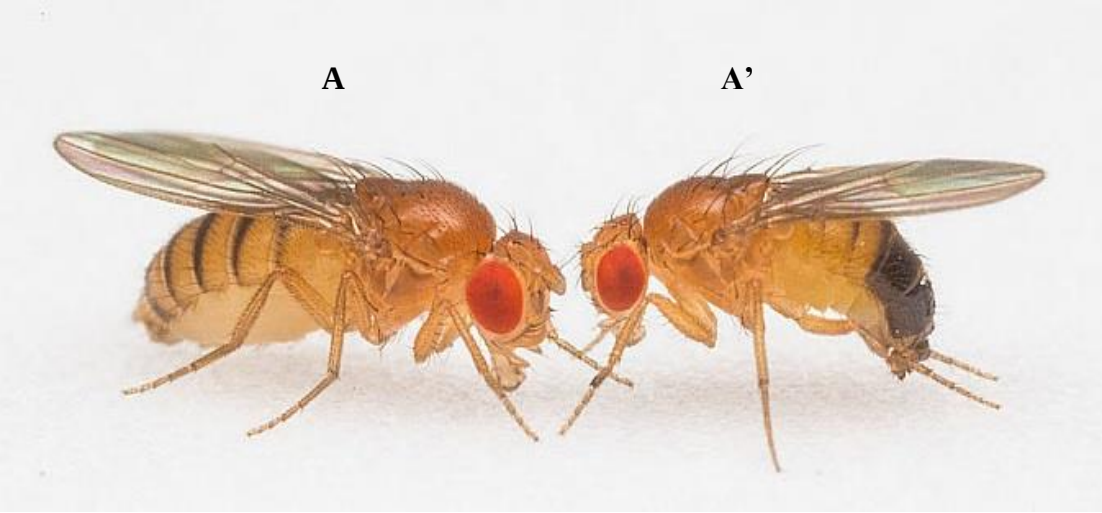

Figure 2.3 *Drosophila melanogaster* portrait of the wild types. A depicts a female fruit fly, while A’ depicts the male fruit fly. The latter can be differentiated from the former with a darkened abdomen, a more diminutive size and presence of sex combs on its pair of foremost legs. Adapted from (Floris van Breugel, 2017. Nature Picture Library)

2.2.1 Advantages of *D. melanogaster* as a research model

The miracles afforded by *D. melanogaster* are; it has short life cycle, enabling rapid generation, and a single fertile mating pair could pour out genetically identical offspring in a mere 10-day incubation at 25 °C. Having a small genome set at 180 Mb in size, with only four sets of chromosomes, does not hurt its chances to be a mantle of perfection. Since the completion of its genome project, *D. melanogaster* had since shown a high homology between human’s genome set – be it on its human disease functional orthologues, or its amino acid sequences, or even the functional protein domains (Adams et al., 2000; Yamaguchi, 2018). Its developments are external, hence an easy handbook to chart and record it with a simple dissecting microscope. The flies have a vast selection of online databases and community to map experimental designs, stock identifications, with the likes of FlyBase (Larkin et al., 2021), and purchases from Bloomington Drosophila Stock Center (BDSC), Kyoto Stock Center (DGRC),

and Vienna Drosophila Resource Center (VDRC) (M. Yamaguchi & H. Yoshida, 2018).

The infamous of all are its genetic tools – known as balancer chromosome and UAS-GAL4 binary system, as described in Section 2.2.3.

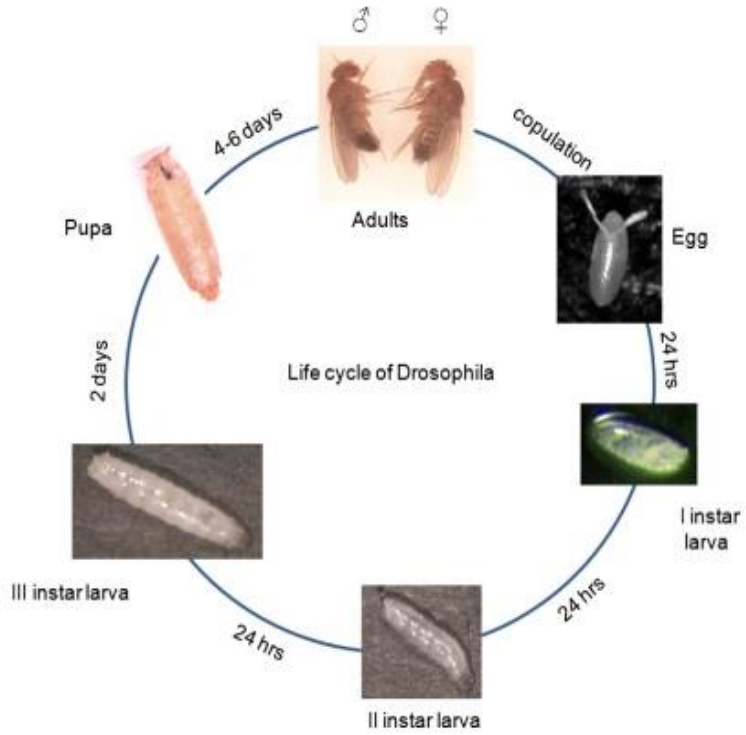

Figure 2.4 A complete life cycle *D. melanogaster*. It would often take 8 to 10 days for the eggs to mature into an adult fly, hitherto surviving for another 20 to 50 days. The eggs hatches after 20 hours at 25 °C, where the larvae grow over the span of 3 days, and molting twice at every 24-hour interval. The 3rd instar larvae would then be encapsulated in a puparium for a 4-day long metamorphosis, before emerging out as an adult fly. Adapted from (Ram & Chowdhuri, 2014).

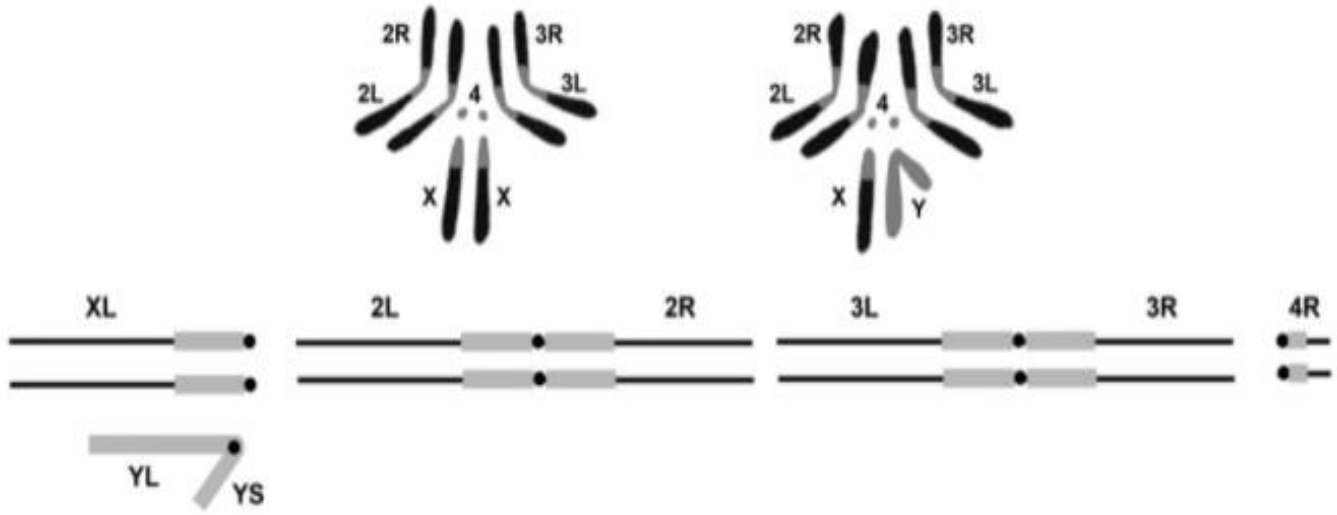

Figure 2.5 Chromosomal comparison between a male and a female *D. melanogaster*. A and A' depicts karyotypic representation of a female and male respectively; B depicts diagrammatic representation of the fly's genome, with its corresponding names on the arms as it did in A and A'. In summation, there are X and Y, in place as the sex chromosomes, two large autosomal elements (chromosomes 2 and 3), and a small dot for chromosome 4. Females would have two X chromosomes as seen in A, while the male would possess an X and a Y, as seen in A'. In B, chromosomes 2 and 3 are classified as metacentric, while the fourth and the sex chromosomes are known to be acrocentric. YL stands for long arm, while YS stands for short arm. Small X (XS) and chromosome 4's left arm (4L) is not depicted here. Adapted from (Kaufman, 2017).

2.2.2 Genetic tools in *D. melanogaster*

2.2.2(a) Balancer Chromosome

Balancer chromosome is a cherry for the icings as they are incredibly valuable two reasons. The foremost, is the maintenance of lethal and sterile mutations within the stock without any selection. The second, it can be used for mutation screening by maintaining the linear integrity of a mutagenized homolog. These balancers have either one or more inversion buried deep within the genome in relative to a typical chromosome, and this prevents the recovery of exchange events, hence preserving the sequences in the balancer and the balanced chromosome.

These will lead to true breeding stocks that stays the lethal mutations, where only doubly heterozygous adult flies with the balancer and the lethal-bearing homologs will survive (Greenspan, 2004). It too, is quite possible for either visible dominant or recessive mutations to keep an eye on for crossing schemes, designing an effective screening, and distinguishing genotypes. Some of the widely used balancers are curly wings (CyO), stubbles (Sb), and TM3. A detailed list can be found in Bloomington Drosophila Stock Centre (BDSC) (Kaufman, 2017).

2.2.2(b) UAS-GAL4 Targeted Expression System.

First described 1993, the system's application had since been used for screening, through expressing and knockdowns of specific choosing genes in *D. melanogaster* (Brand & Perrimon, 1993). GAL4 hails as a yeast transcription factor that commands spatial and temporal expression of the target genes, which henceforth will direct the gene activity at any developmental stage, and at any specific cells or tissues. Simply put, one strain has a promoter region for desired gene and another with

GAL4-binding upstream-activating system (UAS) before the trans gene. Once this two strains mate, it will express the transgene that directs the specific gene's action, all driven by the system.

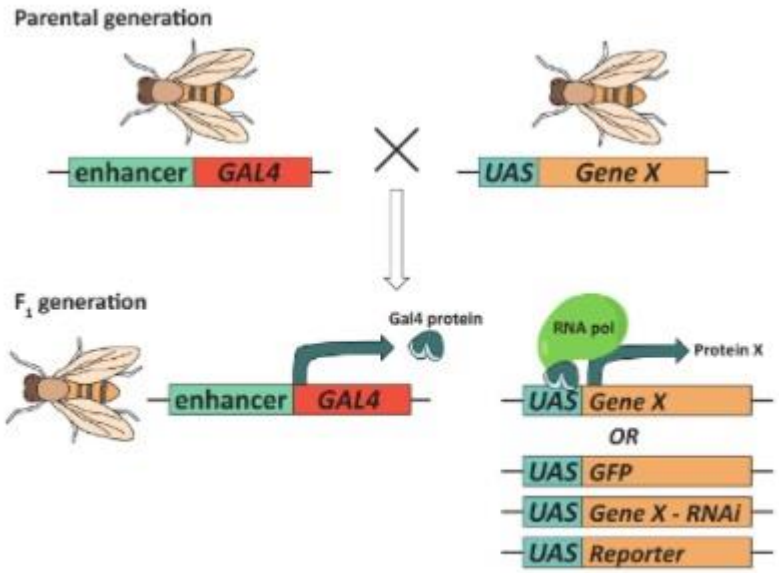

Figure 2.6 Illustration of UAS-GAL4 system. When two individual parental strains carrying the GAL4 sequence and gene of interest downstream of a UAS sequence were genetically crossed respectively, the progeny in the F_1 generation will express the transgene, be it a gene of interest X or RNAi gene, in specific tissues. Adapted from (Kelly et al., 2017).

2.3 Sex Organ: Testis

Adult males have each a pair of testes; both a long dead-end tube snaked around a seminal vesicle, with the stem cell niche found at the dead-end apical tip. At this locale, germline stem cells (GSC) would halve asymmetrically into gonialblast, that would then leave the niche to be differentiate further into matured spermatids, while the other remains a stem cell (De Cuevas & Matunis, 2011). Stem cell niche houses the hub cells, which anchors GSC, and cyst stem cells (CySCs), as represented in Figure 2 (Singh et al., 2016). The subsequent differentiation is well reported in 2.3.2.

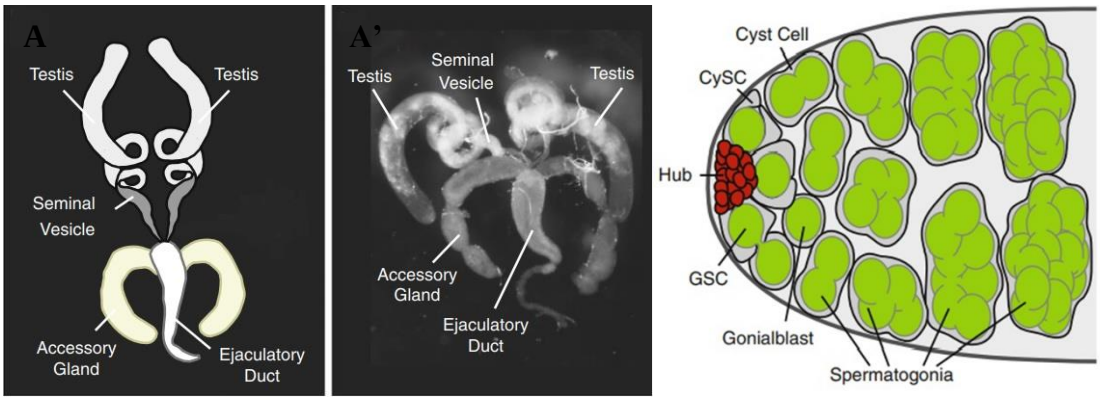

Figure 2.7 *Drosophila melanogaster*'s testis and its stem cell niche. Figure A shows a graphical representation of a dissected testis in A', while Figure B illustrates the graphical representation of the apical tip – has perennially guarded the niche. The red boxes highlight the testes' apical tips. Adapted from (Greenspan & Matunis, 2017).

2.3.1 Spermatogenesis

There are to the fact two different types of cells to be manipulated by any fly's testes to bring upon the spermatogenesis process – somatic cells (soma) and germ cells (germline). Delving deeper, spermatogenesis is forked into three distinct steps: **i)** spermatogonial stage, characterised by mitotic germ cells, with markers Vasa, Bag of Marbles (Bam), hu-li tai shao (HTS) and Fasciclin III (Fas III); **ii)** spermatocyte stage, paints for meiotic stages, where the markers are Coilin and Boule; **iii)** spermatid stage, for spermatozoa formation with the markers, Boule and Don Juan. DAPI is utilized to highlight nuclei (Fairchild et al., 2017).

At the spermatogonial stage, the hub is denoted by 12 non-dividing somatic cells and is tied to the six or nine GSCs and the CySCs through cell-adhesion molecules. The hub, being a signalling centre commands a variety of cell signalling pathways, such as Hedgehog (Hh), through Janus-kinase – signal transducer and activator of transcription (JAK-STAT), and Bone Morphogenetic Protein (BMP) (Amoyel & Bach, 2012; Fairchild et al., 2017; Ma & Xie, 2011; Zhang et al., 2013). JAK-STAT regulates GSCs and CySCs, where it cascades from Unpaired (Upd)

growth factor's expression for self-renewals, and through JAK-STAT pathway regulation manipulations, the ratio of GSCs to CySCs in a wild-type is kept at 1:2 (Singh et al., 2016).

During mitotic divisions, the GSCs and CySCs would position their respective centrosomes perpendicular to the hub, henceforth the subsequent asymmetrical divisions – where both could either be displaced to differentiate, while another remains in contact with the hub (Cheng et al., 2011). Two somatic cyst cells risen from CySCs will encase the gonialblasts to form a 'spermatocyst', and the latter would then undergo a quadruplet mitosis to produce a sixteen-celled spermatogonia (collectively known as spermatocyte). The division is also known as transit-amplifying divisions, where the cytokines were incomplete and the cells were held together by ring canals, an intracellular bridge. The differentiation will continue through the S phase, at which point, the spermatocyte programme for growth and gene expression will kick in, hence heralding the second stage. At this particular point, gene products that are vital for the spermatocytes' and spermatids' developments are translated (Fairchild et al., 2017; White-Cooper, 2010).

Now at the spermatocyte stage then will undergo two rounds of meiosis to produce 64 haploid cells, with genomes thusly halved to haploid. Here, there would be a phase dark mitochondrion (nebenkern) and a phase light nucleus, and is aptly named as onion-shaped cell (White-Cooper, 2004).

Finally, to the third stage, the spermatids' development would undergo dramatic morphological tinkers: the genome was boxed into a minute, high-density nucleus, while their flagellar axonemes extends as a cellular machinery for fertilization, and motility. The cyst cells engulfing the gonialblast would eventually

develop into head and tail cyst cells, with the head cyst cell attaching itself to the terminal epithelium. The mature sperms would then be unleashed from a testis's open end into the seminal vesicle and stored there till its required (Fairchild et al., 2017).

This truly proves without doubt that a *D. melanogaster*'s testis is a wonderful avenue to study a stem cell's niche interactions at both cellular and molecular stage, for it maps the developmental stages extremely well - first from the stem cells to its ultimate stage, mature sperms at its basal end (de Cuevas & Matunis, 2011).

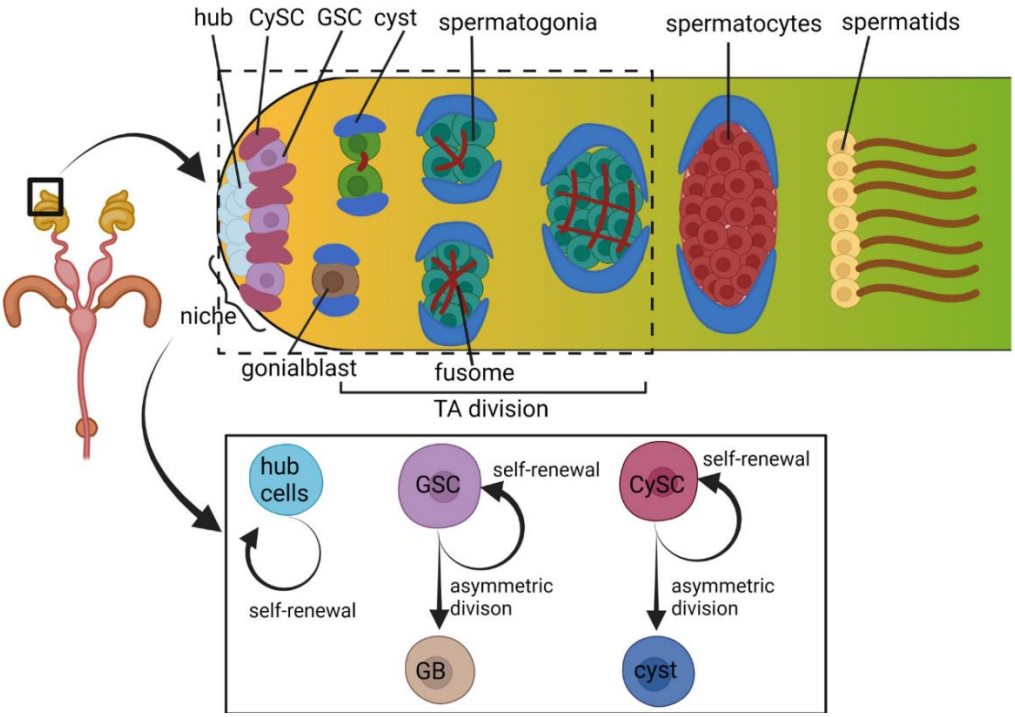

Figure 2.8 A graphical representation of spermatogenesis in *D. melanogaster*'s testis. At the apical tip with GSCs where it'd be encapsulated with two cyst cells from CySCs. Both GSCs and CySCs would self-renew while it rose to gonialblast (GB) and cyst cells respectively. GB will undergo four rounds of mitosis to be a 16-cell spermatocyte, a process transit amplification (TA) division. The spermatocyte would then undergo meiosis twice to produce a 64-haploid cell, before finally the early spermatids will take shape for condensed genome and motility, where mature sperms would then be released or stored into the seminal vesicle. The broken-line box denotes early spermatogenesis stage. Adapted from (Thangadurai et al., 2022).

2.3.1(a) miR-975

In *D. melanogaster*'s genome, miRNA-975 is part of a tri-miRNA cluster, with miR-976 and miR-977 accounting for the trifecta. Furthermore, this cluster is associated with miR-4966 upstream, and the duo miR-978 and miR-979 genes upstream (Marco et al., 2013). Figure 2.9 illustrates the position of miR-975 amongst the cluster.

Overexpression of miR-975 were discovered to have consistently lengthen cytoophidia, as does CTPSyn. Consequently, the overexpression of both miR-975 and CTPSyn shows a bulging phenotype at the testis's apical tip. Quantitative PCR (qPCR) had reported differential expression of myriad of cancer-related genes, such as, Ack, Myc, and CTPS (Woo et al., 2019). Overexpression of miR-975 affects CTPSyn and cytoophidia indirectly from the actions of an unnamed middling actor, besides enigmatically suggesting traits of tumourigenesis and cancer formation (Dzaki et al., 2019).

On another note, miR-975 is insect and testis exclusive in *D. melanogaster*'s testis (Lyu et al., 2021; Mohammed et al., 2014); Besides that, heeding Red Queen Hypothesis – at which de novo genes were determine for its ability to adapt in changing environment, miR-975 in *D. melanogaster* were found to have lower expression and with lesser ability for target repression, when compared to its expression amongst its sister species (Zhao et al., 2021).

Of the subject miR-975, there has been a scarcity of researches done. This manuscript attempts to shed more light on the miRNA, whilst informing more on its places amongst the RNA-lm.

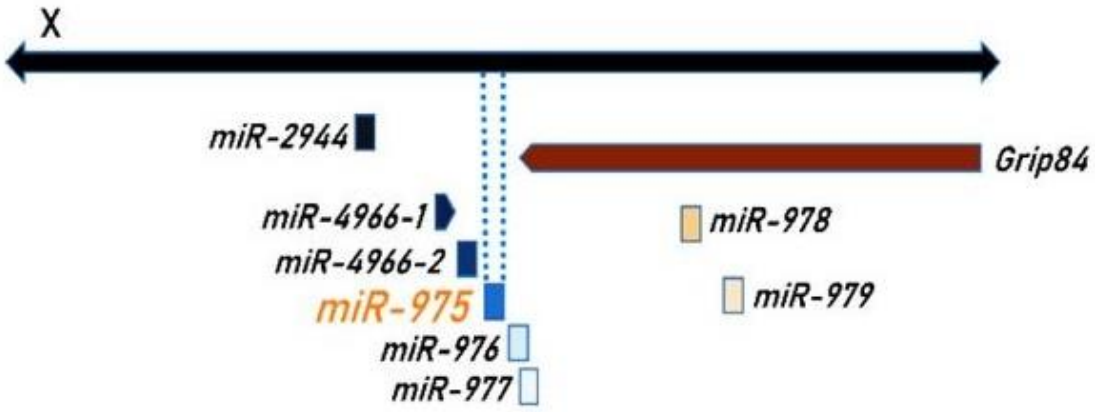

Figure 2.9 Graphical representation of miR-975 and its clusters. miR-975 is grouped with miR-976 and miR-977, whilst being flanked by miR-978 and miR-979 downstream, and miR-2944 upstream. Adapted from (Dzaki et al., 2019).

2.4 Immunity

No organisms are complete without an immune system. It is a thousand scimitars that keep rude trespassers away; should the efforts fail, blades would rust, winds whisper hollow, shadows never multiply. All else will be prelude.

The dramatic overture was in part to impart an impression on immunity's importance – its precise and prompt (Medina, 2016). The system is an amalgamation of cells, chemicals, and processes tasked for the defences of the outer epithelial layer, and intestinal-respiratory tracts from the mentioned rude trespassers – cancer cells, toxins, microbes, and viruses through successions of innate and adaptive immunity. The different arms of the system are activated in respect to their respective speed and specificity (Parkin & Cohen, 2001).

2.4.1 Innate immunity

As the first arm of defence, it's a non-specific defence mechanism deployed either immediately or hours after a foreign entity was registered. This system, while early and rapid, lacks immunologic memories, thusly its inability to memorize nor remember similar foreign entities should it attack again. Its defensive barriers entail: anatomical (skins and mucous membrane); physiological (temperature, irregular pH and chemical mediators); endocytic-phagocytic nexus; and finally, inflammation. Macrophages, neutrophils, eosinophils, basophils, mast cells, lymphocytes, monocytes, and natural killer (NK) cells form the ranks of immune cells (Murphy & Weaver, 2016; Turvey & Broide, 2010).

Innate immunity against pathogens is reliant on pattern recognition receptors (PRRs), that which hunts previously catalogued but other variation of pathogens with pathogen associated molecular patterns (PAMPs). Other programmes activated here are production of cytokines and chemokines. The dyads recruit immune cells to infection and inflammation sites, henceforth sweeping away pathogens, whilst developing fever. Popular cytokines are tumour necrosis factor (TNF), interleukin 1 (IL-1) and interleukin 6 (IL-6).

Another programme within the arm is complement system, a cascading biochemical event that indexes and opsonizes bacteria and other rogue agents. This would designate pathogens (rogue agents) the host's foremost public enemy, hence eligible for phagocytosis. Like John H. Dillinger, pathogens would be vacated after from organs, tissues, blood and lymph. This would eventually activate adaptive immune response.

2.4.2 Adaptive immune system

It is the left hand of the God, the power to be when all else failed, the miracle from desperate prayer. They seek specific 'non-self' antigens (rude invaders), generate pathogen-specific effector pathway that ravages the specific pathogens or its' cell-infect (Bonilla & Oettgen, 2010). Antigen-specific T-cells proliferates from antigen-presenting cells (APC) while B cells differentiates into plasma cells for antibodies' manufacture.

Eying T cells, these units first migrate into thymus to mature from hematopoietic stem cell, where antigen presenting cells (APC) would advice proliferation and differentiation to release cytokines, by introducing pathogen molecules (either phagocytized foreign proteins or antigen fragments from infected cells) in an effort to police immune response justly. T-cells and APC are indivisible couplet; the pathogen molecules were presented on major histocompatibility complex (MHC) found at the surface of APC, to T-cell receptors (TCR) found atop T-cells' membrane. APCs comprises of dendritic cells, macrophages, B-cells, fibroblasts and epithelial cells – each and all involved variedly in wound healing and adaptive immunity initiation.

Counting down, T-cells would soon differentiate into cytotoxic T-cells (CD8+ cells) or T helper cells (CD4+). The former seeks destruction of cells infected by the invading foreign agents, while the latter mediates immune response to marshal a killing field of infected cells and its clearance afterwards. Once the infections were resolved, effector cells decommissioned and dismissed by phagocytes, some would retain still as memory cells. Come another similar blitz, the memory cells will differentiate at light speed and combat it.