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Studying stress-related epigenetic and metabolic changes in fruit flies and mice through monogenic and polygenic models

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vorgelegt von

Anuroop Venkateswaran Venkatasubramani

aus ***Chennai*, Indien**

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Erster Gutachter:	Prof. Dr. rer. nat. Axel Imhof
Zweiter Gutachter:	Prof. Dr. rer. nat. Andreas Ladurner
Dekan:	Prof. Dr. med. Thomas Gudermann
Tag der mündlichen Prüfung:	16 Juli 2024

Affidavit

Affidavit

Venkatasubramani, Anuroop Venkateswaran

Surname, first name

I hereby declare, that the submitted thesis entitled:

Studying stress-related epigenetic and metabolic changes in fruit flies and mice through monogenic and polygenic models.

is my own work. I have only used the sources indicated and have not made unauthorized use of services of a third party. Where the work of others has been quoted or reproduced, the source is always given. I further declare that the dissertation presented here has not been submitted in the same or similar form to any other institution for the purpose of obtaining an academic degree.

Planegg-Martinsreid, 22.11.2023 Anuroop V Venkatasubramani

Place, Date Signature doctoral candidate

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List of abbreviations

αKG – α-ketoglutarate

4-HPO-DAEE – 4-hydroperoxy-2-decenoic acid ethyl ester

5hmC – 5-hydroxymethylcytosine

5mC – 5-methylcytosine

5-mTHF – 5-methyl tetrahydrofolate

5,10-mTHF – 5,10-methylene tetrahydrofolate

5-fTHF – 5-formyl tetrahydrofolate

10HDA – (E)-10-hydroxy-2-decenoic acid

Akh – Adipokinetic hormone

AMP – Adenosine monophosphate

AMPK – AMP-activated protein kinase

ATP – Adenosine triphosphate

bmm – brummer

CBP – CREB-binding protein

CC - Corpus Cardiacum

chm – chameau

CoA – Coenzyme A

DAG – Diacylglycerol

DHF – Dihydrofolate

DNA – Deoxyribonucleic acid

DNMT – DNA methyltransferases

ETC – Electron transport chain

FADH2 – Flavin adenine dinucleotide

FA – Fatty acid

FASN – Fatty acid synthase

FB – Fat body

FFA – Free fatty acid

GxE – Gene-Environment interaction

GSEA – Gene set enrichment analysis

GO – Gene ontology

H2A – Histone 2A

H2B – Histone 2B

H3 – Histone 3

H4 – Histone 4

H4K12ac – Histone 4 Lysine 12 acetylation

HAT – Histone acetyltransferase

HBO1 – Human acetylase binding to ORC1

Hcy – Homocysteine

HDAC – Histone deacetylases

HK – Hexokinase

HMT – Histone methyltransferases

ILPs - Insulin-like peptides

IPCs - Insulin-producing cells

JH – Juvenile hormone

KMT – Lysine methyltransferase

KAT – Lysine acetyltransferases

KDAC – Lysine deacetylases

LDs – Lipid droplets

LC-MS – Liquid chromatography mass spectrometry

MSL-DCC – Male specific lethal dosage compensation complex

NADH – Nicotinamide adenine dinucleotide (NAD) + hydrogen

ncRNAs – non-coding RNAs

OAA – Oxaloacetate

OCM – One-carbon metabolism

OCR – Oxygen consumption rate

OXPHOS – Oxidative phosphorylation

PcG – Polycomb group

PFK – Phosphofructokinase

PK – Pyruvate kinase

PTM – Post-translational modification

qRT-PCR – Quantative real time polymerase chain reaction

RNAi – Ribonucleic acid interference

SAM – S-adenosylmethionine

SAH – S-adenosylhomocysteine

SNP – Single nucleotide polymorphism

TADs – Topologically Associated Domains

TAG – Triacylglycerol

TCA – Tricarboxylic acid

TDG – Thymine DNA glycosylase

TET – Ten-eleven translocases

THF – Tetrahydrofolate

UAS – Upstream activating seqence

UDP-glucose – Uridine diphosphate glucose

List of publications

a. Major publications included from this doctoral study

- ✓ ***Paper-I:***

Venkatasubramani AV, Ichinose T, Kanno M, Forne I, Tanimoto H, Peleg S, Imhof A. The fruit fly acetyltransferase chameau promotes starvation resilience at the expense of longevity. EMBO Rep. 2023 Sep 19:e57023. doi: 10.15252/embr.202357023. Epub ahead of print. PMID: 37724628.

- ✓ ***Paper-II:***

Müller-Eigner A*, Sanz-Moreno A*, de-Diego I*, **Venkatasubramani AV**, Langhammer M, Gerlini R, Rathkolb B, Aguilar-Pimentel A, Klein-Rodewald T, Calzada-Wack J, Becker L, Palma-Vera S, Gille B, Forne I, Imhof A, Meng C, Ludwig C, Koch F, Heiker JT, Kuhla A, Caton V, Brenmoehl J, Reyer H, Schoen J, Fuchs H, Gailus-Durner V, Hoeflich A, de Angelis MH, Peleg S. Dietary intervention improves health metrics and life expectancy of the genetically obese Titan mouse. Commun Biol. 2022 May 3;5(1):408. doi: 10.1038/s42003-022-03339-3. PMID: 35505192; PMCID: PMC9065075.

- ✓ ***Paper-III:***

Dietz LJ, **Venkatasubramani AV**, Müller-Eigner A, Hrabe de Angelis M, Imhof A, Becker L, Peleg S. Measuring and Interpreting Oxygen Consumption Rates in Whole Fly Head Segments. J Vis Exp. 2019 Jan 7;(143). doi: 10.3791/58601. PMID: 30663674.

- ✓ ***Paper-IV:***

Serefidou M*, **Venkatasubramani AV***, Imhof A. The Impact of One Carbon Metabolism on Histone Methylation. Front Genet. 2019 Aug 28;10:764. doi: 10.3389/fgene.2019.00764. PMID: 31555321.

b. Other publications from this doctoral study that are not included

- ✓ Wartewig T, Daniels J, Schulz M, Hameister E, Joshi A, Park J, Morrish E, **Venkatasubramani AV**, Cernilogar FM, van Heijster FHA, Hundshammer C, Schneider H, Konstantinidis F, Gabler JV, Klement C, Kurniawan H, Law C, Lee Y, Choi S, Guitart J, Forne I, Giustinani J, Müschen M, Jain S, Weinstock DM, Rad R, Ortonne N, Schilling F, Schotta G, Imhof A, Brenner D, Choi J, Ruland J. PD-1 instructs a tumor-suppressive metabolic program that restricts glycolysis and restrains AP-1 activity in T cell lymphoma. Nat Cancer. 2023 Sep 18. doi: 10.1038/s43018-023-00635-7. Epub ahead of print. PMID: 37723306.

- ✓ Lukacs A, Thomae AW, Krueger P, Schauer T, **Venkatasubramani AV**, Kochanova NY, Aftab W, Choudhury R, Forne I, Imhof A. The Integrity of the HMR complex is necessary for centromeric binding and reproductive isolation in Drosophila. PLoS Genet. 2021 Aug 23;17(8):e1009744. doi: 10.1371/journal.pgen.1009744. PMID: 34424906.

- ✓ Choudhury R, **Venkatasubramani AV**, Hua J, Borsò M, Franconi C, Kinkley S, Forné I, Imhof A. The role of RNA in the maintenance of chromatin domains as revealed by antibody-mediated proximity labelling coupled to mass spectrometry. Elife. 2024 May 8;13:e95718. doi: 10.7554/eLife.95718. PMID: 38717135; PMCID: PMC11147508.

- ✓ Kiss AE, **Venkatasubramani AV**, Pathirana D, Krause S, Sparr AC, Hasenauer J, Imhof A, Müller M, Becker PB. Processivity and specificity of histone acetylation by the male-specific lethal complex. Nucleic Acids Res. 2024 May 22;52(9):4889-4905. doi: 10.1093/nar/gkae123. PMID: 38407474; PMCID: PMC11109948.

- ✓ **Venkatasubramani AV**, Ichinose T, Forne I, Tanimoto H, Imhof A[#], Peleg S[#]. Temperature changes modify starvation resilience phenotype in fruit flies lacking the acetyltransferase, *chameau* (chm). *In preparation*

***** denotes shared first authorship

[#] denotes shared corresponding authorship

1. Contribution to publications

The thesis is submitted in a cumulative style that reflects some of the major achievements during my doctoral study. It is composed of two main publications and two publications in the Appendix with research conducted in the lab of Prof. Dr. Axel Imhof (Molecular Biology, Biomedical Center Munich). My contribution in each of the manuscripts are given below.

1.1. Contributions to paper-I:

Venkatasubramani AV, Ichinose T, Kanno M, Forne I, Tanimoto H, Peleg S, Imhof A. *The fruit fly acetyltransferase chameau promotes starvation resilience at the expense of longevity*. EMBO Rep. 2023 Sep 19:e57023. doi: 10.15252/embr.202357023. Epub ahead of print. PMID: 37724628

I am the sole first author of this paper, which was conceptualized by Dr. Peleg, Dr. Imhof and me.

Experimental work:

i) I assessed and executed the required experimental design and corresponding statistical tests.

ii) I maintained the all fly lines used in this experiment (*chm*$^{MYST/+}$, *chm*^{RNAi}, all the GAL4 constructs, GeneSwitch systems etc.).

iii) I designed and performed all the crosses, starvation experiments and weight measurements.

iv) I prepared samples for mass spectrometry analysis of histones, proteome and acetylome.

v) I prepared libraries for bulk transcriptomic analysis.

vi) I also carried out the respective data analysis for all the omic experiments.

vii) Dr. Ignasi performed mass spectrometry for histone and acetylome samples, while Dr. Ichinose, Ms. Kanno and Dr. Tanimoto helped with GeneSwitch fly experiments.

Manuscript preparation:

i) I reviewed literature, inferred the results and contributed in the writing of the manuscript.

ii) I wrote figure legends, methods and results section

iii) I prepared all the figures (both supplementary and main)

iv) I was also involved in all the revision process (both writing and executing new experiments)

v) Dr. Peleg and Dr. Imhof were also involved in manuscript preparation

1.2. Contributions to paper-II:

Müller-Eigner A*, Sanz-Moreno A*, de-Diego I*, **Venkatasubramani AV**, Langhammer M, Gerlini R, Rathkolb B, Aguilar-Pimentel A, Klein-Rodewald T, Calzada-Wack J, Becker L, Palma-Vera S, Gille B, Forne I, Imhof A, Meng C, Ludwig C, Koch F, Heiker JT, Kuhla A, Caton V, Brenmoehl J, Reyer H, Schoen J, Fuchs H, Gailus-Durner V, Hoeflich A, de Angelis MH, Peleg S. *Dietary intervention improves health metrics and life expectancy of the genetically obese Titan mouse*. Commun Biol. 2022 May 3;5(1):408. doi: 10.1038/s42003-022-03339-3. PMID: 35505192; PMCID: PMC9065075.

I am the second author of this manuscript.

Experimental work:

i) I assessed and executed the experimental design and corresponding statistical tests for transcriptomic and histone PTM experiments.

ii) I prepared libraries for transcriptomic analysis.

iii) I performed data analysis for mass spectrometry histone PTM using SkyLine and R

iv) I also performed data analysis for transcriptomic and proteomic data.

Manuscript preparation:

v) I inferred the results and contributed partially in the writing of the manuscript.

vi) I wrote figure legends and methods section for the experiments that I performed.

vii) I prepared all the figures (both supplementary and main) for transcriptome and proteome

viii) I was also involved in all the revision process.

1.3. Contribution to paper-III (Appendix A):

Dietz LJ, **Venkatasubramani AV**, Müller-Eigner A, Hrabe de Angelis M, Imhof A, Becker L, Peleg S. *Measuring and Interpreting Oxygen Consumption Rates in Whole Fly Head Segments*. J Vis Exp. 2019 Jan 7;(143). doi: 10.3791/58601. PMID: 30663674.

I am the second author of this manuscript

Experimental work:

i) I was involved in maintenance of fly lines used in this experiment.

Manuscript preparation:

i) I was partially involved in writing of the manuscript

ii) As this was a video journal, I participated in the making of video production in explaining the experimental methodology and the importance of this method.

iii) I also aided in the experimental procedure during the video production.

iv) I also contributed to the manuscript revision process

1.4. Contribution to paper-IV (Appendix B):

Serefidou M*, **Venkatasubramani AV***, Imhof A. *The Impact of One Carbon Metabolism on Histone Methylation*. Front Genet. 2019 Aug 28;10:764. doi: 10.3389/fgene.2019.00764. PMID: 31555321.

I am a co-first author of this publication. There is no experimental work as this is a mini-review aimed at providing substantial background to this thesis.

Manuscript preparation:

i) I was involved in literature review and writing of the manuscript

ii) I also contributed to the revision and submission processes.

iii) Magdalini Serefidou was also equally involved and Dr. Imhof contributed to editing and proofreading.

2. Introduction

2.1 Phenotypic plasticity: Gene and environment interaction

Environmental changes and challenges have been a constant occurrence throughout the course of evolution. To survive during difficult times, an organism has to adapt quickly to such changes. The ability to show differential phenotype by an organism with same genotype when exposed to varying environments, or different genotypes at the same environment can be explained by gene-environment interaction (GxE) (**Ottman, 1996**). The former scenario defined as phenotypic plasticity, can be influenced by changes in nutrition, population density, temperature, etc. One hypothesized model, "norm of reaction", explains the range of phenotypes that are possible under different environmental conditions (**Fig. 1**). Such probable observations can be seen in organisms ranging from plant to animal kingdoms and play an important role in organism's survival, adaptation and evolution, especially under novel and non-ideal conditions (**Manuck, 2010; Via & Lande, 2016; C. H. Yang & Pospisilik, 2019**). A sub-class of phenotypic plasticity called polyphenism, specifically relates to development, i.e. change in development upon environmental stimuli, resulting in diversification of the same organism into different population (**Casasa et al., 2020; C. H. Yang & Pospisilik, 2019**).

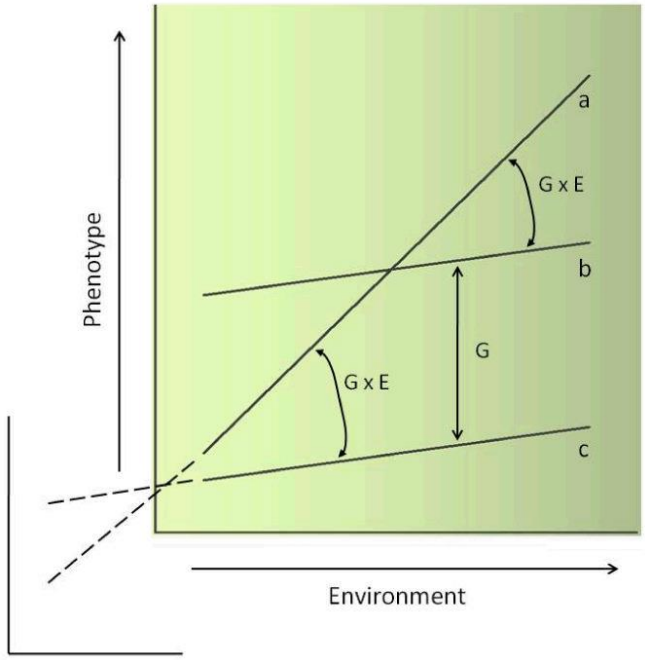

Figure. 1*: Norm of a reaction explained with three hypothetical genotypes, a, b and c (G = genetic effect, G x E = gene-environment interaction, Obtained from* ***Manuck, 2010****)*

In order to understand gene-environment interaction, plants and insects have been the best model organisms for research as they are affected by marginal changes in

environment. Plants for example, undergo a natural process called vernalization that results in early flowering at temperate conditions after exposure to colder temperatures (**Feil & Fraga, 2012**). In the case of insects, the best-known example is honeybee, *Apis mellifera*. Differences in diet can modify the development of a female bee into becoming either a queen or a worker bee, each displaying differences in size and longevity (**Duncan et al., 2020**). Fruit flies of the sibling species show differences in development under same temperature. For example, *Drosophila melanogaster* and *Drosophila simulans* show differences in weight, thorax and wing size, such that the former weighs more and has larger thorax and wing size in the tested temperature ranges (12 to 30˚C) (**Pétavy et al., 1997**). Additional examples and occurrences of phenotypic plasticity can be observed in higher organisms such as reptiles, fish and also in humans (**Kelly et al., 2012; C. H. Yang & Pospisilik, 2019**).

Environmental changes entail organisms to respond with either adaptation, survival or death. These responses can be characterized by changes in behavior and or morphology of the organism. However, the fundamental changes that drive these phenotypic differences could be a function of genetic variation or epigenetic mechanisms. Genetic variation involves long-term random mutations in gene sequences that change the molecular characteristics of genes or a set of genes in terms of expression, activity, localization etc. However, because these are random changes, the output is unpredictable and it could take many generations for a population that is fit and is able to adapt to changes. Therefore, genetic changes could be the preferred driver of adaptation in a stable environment (**Gao et al., 2022; Lehmair et al., 2022; Stajic et al., 2021**).

On the other hand, information can also be inherited independent of the DNA sequence, which are more immediate and reversible. Epigenetics, as defined by Waddington, focuses on the relationship between genes and the products that results in a phenotype and an organism (**Dupont et al., 2009**). These can be inherited via chemical changes that occur on DNA and proteins called histones. DNA is wrapped around an octamer of histone proteins (H2A, H2B, H3 and H4) to form a DNA-protein complex called the nucleosome, which further condense to form a string-like structure called chromatin that is stabilized by histone H1, the linker histone (**Dai et al., 2020; Feil & Fraga, 2012**). These can be modified by chemical changes that

affect the environment of the gene and influence its expression. Most of these processes are enzymatic reactions that require corresponding donors for the enzyme to carry out their activities. These are metabolites, which are intermediates from several metabolic processes, that can act as donors of chemical groups for modifying DNA or proteins. Metabolism is a complex association of many biochemical reactions that cater to the needs of a cell in terms of energy demands. As diet, lifestyle and ambient changes can impact the metabolism directly, they serve as a connection between chromatin and environmental stimuli **(Dai et al., 2020; Katada et al., 2012**).

The following sections will therefore provide a general idea on chemical changes that can occur in a DNA or protein followed by some of the important metabolic processes.

2.2 Overview of chemical changes in DNA and proteins

Both DNA and proteins (histone and non-histone) can be chemically modified to alter their structural and/or molecular characteristics. As mentioned earlier, DNA wraps around the histones to form nucleosomes that are further condensed to form chromatin (**Feil & Fraga, 2012**). The epigenetic profile dictates the type of chromatin. If the region becomes inaccessible to other proteins, it is called a heterochromatin, while the counterpart is referred to as euchromatin. Furthermore, heterochromatin can be either constitutive or facultative. The former is more compact and are always repressed, while the latter is a pseudo-euchromatin that is in a compacted state and can become accessible or active depending on the context, which in most cases is developmentally regulated (**Allis & Jenuwein, 2016; Gilbert et al., 2003**).

Chromatin landscape is generally decided by the type of epigenetic changes that occur within the region. The most common and well-studied are DNA methylation and histone PTMs. Apart from these two, non-histone PTMs, RNA methylation, non-coding RNAs (ncRNAs), enhancers, chromatin remodeling, topologically associated domains (TADs) etc., can determine chromatin accessibility (**Allis & Jenuwein, 2016**). For example, non-histone proteins can be acetylated or ubiquitylated, which would affect its stability, localization, binding and can even signal degradation by the proteasome. Some of the ncRNAs like *roX* in *Drosophila*, *Xist* in mammals or HOTAIR in plants have been shown to cause large scale gene expression changes

(**Maenner et al., 2012; Wei et al., 2017**). Moreover, RNAs can also be modified by methylation, in this case m^6A methylation of mRNA being the most prominent. This modification influences gene expression by affecting the stability of the mRNA. This field of studying chemical changes in mRNA is referred to as epitranscriptomics (**Kan et al., 2022**).

Intriguingly, histones are not just modified by chemical groups but can also be evicted, moved and exchanged for variants. These process are carried out by specialized proteins called remodeling factors, which require ATP to carry out their function **(Allis & Jenuwein, 2016**). Enzymes related to Snf2- or SWI/SNF-related families use the energy from ATP and regulate nucleosome positioning or their complete removal, thereby aiding other proteins to access the DNA/histones for cellular processes (**Hargreaves & Crabtree, 2011; Narlikar et al., 2013**). Finally, a more recent development views chromatin in a three-dimensional architecture due to the existence of TADs. These domains promote long-range interactions within the genome at sub-mega base level which happens by the formation of DNA loops containing cis-regulatory elements with many enhancer-promoter pairs that in turn can control gene expression (**Cavalheiro et al., 2021; Tang et al., 2022**).

However, in the following sub-sections, the focus will primarily be on the two most common epigenetic processes, i.e. DNA methylation and protein post-translational modifications.

2.2.1 DNA methylation

DNA methylation is the addition of a methyl-group to 5^{th} carbon of cytosine (5mC) by enzymes collectively called as DNA methyltransferases (DNMTs). This process occurs with the help of SAM which serves as a methyl donor (**Etchegaray & Mostoslavsky, 2016**). Three DNMTs in mammals include DNMT1, DNMT3a and 3b. Each of them can methylate DNA but functions slightly at different contexts. DNMT1 is generally involved during DNA replication to copy the patterns to the new strand, while the other two are involved in *de novo* methylation. These processes are important for an organism's development and hence is the most researched chemical modification (**Moore et al., 2013; Neri et al., 2015**). Most of the 5mC marks in

mammals are deposited in the CpG dinucleotide and approximately 80% of CpGs are methylated. Furthermore, this modification can cause repression of gene (or genomic regions) as evidenced by genomic imprinting, X-chromosome inactivation and transposon silencing in mammals. Although the mark is more stable than histone PTMs, there are mechanisms to remove these marks, termed DNA demethylation that are of two types. In active demethylation, ten-eleven translocase (TET) and thymine DNA glycosylase (TDG) enzymes remove the methyl group either via oxidation or deamination respectively. The former converts 5mC to 5hmC (5-hydroxymethylcytosine), while the later converts 5mC to thymine, both leading to a repair mechanism converting the base to cytosine. Interestingly, like SAM for DNMTs, TET enzymes also require the presence of a metabolite, α-KG. Alternatively, they can also be removed by passive demethylation through rounds of replication in situations lacking the maintenance methylases (Z. X. Chen & Riggs, 2011; Kohli & Zhang, 2013).

Most of the vertebrates have been shown to possess DNA methylation. Contrary to this, invertebrates like *Caenorhabditis elegans* have been shown to have complete absence of this mark. Studies have often provided conflicting evidence for the prevalence of this mark also in *D. melanogaster* (**Dunwell & Pfeifer, 2014**). For example, it was shown that fruit flies have low but detectable amounts of 5mC at an early embryonic stage and this reduces upon development such that the adult flies do not possess any detectable DNA methylation. Furthermore, a Dnmt2 candidate gene, *Mt2*, was discovered in fruit flies that was developmentally regulated and was found to methylate cytosine at CpT and CpA sites rather than CpG. (**Kunert et al., 2003; Lyko et al., 2000**). However, subsequent studies revealed that *Drosophila* genome do not have any DNA methylation and Mt2 was also found to methylate RNA and aid their biogenesis especially upon stress (**Dunwell & Pfeifer, 2014; Raddatz et al., 2013**). Recently however, cytosine methylation was measured in various *Drosophila* species using a liquid chromatography coupled to mass spectrometry (LC-MS) based technique. This showed that head rather than the body of fruit flies had significantly higher DNA methylation. Furthermore, *D. melanogaster* showed the lowest amount of DNA methylation as compared to 11 other species of *Drosophila* (**Deshmukh et al., 2018**). Additionally, the existence of DNA methylation was also studied using

methylated DNA immunoprecipitation sequencing (MeDIP-Seq) technique. Loss of Mt2 affected the DNA methylation patterns especially for genes involved in cell cycle processes. However, the study has not yet been peer reviewed and hence the results should be considered with reservations (**Deshmukh et al., 2020**).

With all these and many other research providing conflicting evidences, the existence of DNA methylation in *D. melanogaster* is still unclear and will need further investigations with new techniques and strategies (**Dunwell & Pfeifer, 2014**). Therefore, methylation in later sections refers to histones and not DNA.

2.2.2 RNA methylation

While DNA methylation has been a debated topic in *Drosophila*, methylation of RNA and their role in aging and stress has been reported. Further, RNA methylation also regulates sex determination by affecting mRNA splicing in *Drosophila*. In eukaryotes, m6A modification is one of the most observed mRNA modification, which is performed by METTL3 methyltransferase. This modification causes decaying of mRNA, differences in splicing and promotion/inhibition of translation. Interestingly, UV-damage and heat shock result in the regulation of m6A methylation, which aid RNAs to be concentrated in stress granules. In *Drosophila*, knockdown of Mettl3 results in an increased stress resistance, reduction in m6A methylation and upregulation of Hsp70 protein levels (**Perlegos et al., 2022**). In addition, Mettl3 loss, specifically in glia and not in neurons has been shown to increase longevity and stress resilience. However, removal of the same in neurons results in a decreased longevity (**Perlegos et al., 2024**). Furthermore, dTrmt10A is another methyltransferase, whose upregulation results in a decrease of m6A levels, increased resilience to heat stress and Hsp70 protein levels. It was also observed that Hsp70 mRNA loses m6A methylation, which could potentially result in elevated levels of the protein upon heat shock (**Perlegos et al., 2023**).

2.2.3 Post-translational modification of proteins

Besides DNA, histones can also be modified at their tails by post-translational modifications (PTM). In this regard, phosphorylation, acetylation and methylation of

histones have been the most studied and well-documented PTMs. In addition to these, histones can also be glycosylated, SUMOylated, butyrylated, citrullinated, succinylated and much more. Unlike DNA methylation, which is mainly involved in mediating transcriptional repression, histone PTMs are more complex and can result in either transcriptional activation or repression. This depends on the histone, amino acid and PTM that is involved. In addition, the transcriptional output can also be decided by the combinatorial PTMs resulting in multiple permutations and combinations that could occur due to changes at histone tails (**Feil & Fraga, 2012; Lu & Thompson, 2012**). For example, Histone 3 Lysine 9 methylation (H3K9me) is associated with repression, while H3K36me is associated with activation. Acetylation of histones is generally associated with transcriptional activation due to their charge neutralizing property. H4 tail is quite interesting in this regard as it possesses four lysine resides in close proximity, all of which can be acetylated. H4K5ac and H4K8ac have been considered as activating mark, while H4K12ac may result in activation or repression. These modifications are carried out by group of proteins called lysine acetyltransferases (KATs) for acetylation (or in some cases acylation) and lysine methyltransferases (KMTs) for methylation. Additionally, these modifications are also reversible in nature and can be removed by histone deacetylases (HDACs) and demethylases for acetyl- and methyl-marks respectively (**Dai et al., 2020; Dancy & Cole, 2015; Etchegaray & Mostoslavsky, 2016**). A major limitation of these studies is the use of antibodies. As antibodies can cross-react, especially in the case of combinatorial modifications, they are not best suitable for quantification as compared to mass spectrometry.

Compelling evidence suggests *Drosophila* as unique models to study histone modifications due to their key molecular characteristics. The presence of polytene chromosomes (**Johansen et al., 2009**) and the abundance of H4K16ac on the male X chromosome for dosage compensation (**Turner et al., 1992**) are some of the well-known examples. Furthermore, position effect variegation (PEV) provides a unique strategy to assess changes in chromatin status with the help of marker genes. The output is quite simple and are assessed based on the variation in the eye color of the organism (**Girton & Johansen, 2008**). Additionally, chromatin domains in *Drosophila* can be clustered based on the prevalence of certain histone modifications. Repressive regions containing H3K9me2 (pericentric) and H3K27me3 (polycomb-

type) are considered as blue and green chromatin, while regions containing active histone marks such as H3K4me2 and H3K79me3 are red and yellow chromatin. Black chromatin is present in half the genome and exhibits low levels of active marks (**Boros, 2012**).

Apart from histones, other proteins can also be modified by different chemical modifications offering varying functional output. For instance, phosphorylation affects signaling, ubiquitylation indicates protein degradation, glycosylation affects stability and structure while acetylation can affect activity, localization etc. (**Leutert et al., 2021**). It is also possible for the enzymes carrying out these modifications to get modified by themselves or by other proteins. For instance, acetyltransferase MOF, which is a part of MSL-DCC involved in H4K16ac of X-chromosome in *Drosophila* is ubiquitylated by another protein in the complex, MSL3. Lack of ubiquitylation in some of the lysines affects complex formation, localization and consequently affects dosage compensation (**Schunter et al., 2017**). This further indicates the complexity of protein modifications as there are many confounding factors resulting in a final output. Similarly, another well-known MYST acetyltransferase, p300/CBP can be modified post-translationally. Phosphorylation of the protein can affect its activity, interaction and stability depending on the site of phosphorylation. Moreover, it can be SUMOylated and acetylated (auto-acetylation) which can also modify its characteristics. p300/CBP has been shown to acetylate a number of proteins including histone H4. They have been shown to play a role in stress response and are capable of acetylating p53 and other DNA damage response proteins. The efficacy and regulation of these functions are dependent on the type of modification, which in some cases could be combinatorial. For example, SUMOylation of p300/CBP inhibits the acetylation of the same residue and hence the protein's activity (**Dancy & Cole, 2015; Drazic et al., 2016; Narita et al., 2019**). All these make protein PTMs extremely complex with a number of permutations and combinations. Moreover, the complexity is further increased by the availability of corresponding metabolites and the energy context of the cell (or organism) which can further alter the levels of metabolic intermediates such as ATP (phosphorylation), acetyl-CoA (acetylation), SAM (methylation), acyl-CoA (for acylations like butyrylation, succinylation, etc.) and others (**Dai et al., 2020**). The inter-communications between the metabolite and

enzymes with regards to histone (or protein) PTMs will be discussed in the following section.

2.3 Overview of metabolic processes

Within a cell, most of the metabolic reactions occurs in the cytosol. Mitochondria are the primary hub for some of these biochemical reactions, especially for the production of ATP. The structure of mitochondria is quite unique as it possesses an inner and outer membrane. Most of the metabolic control occurs on the inner membrane, called cristae, that hosts the Electron Transport Chain (ETC) complexes and Adenosine triphosphate (ATP) synthases, such that any changes in its morphology can affect metabolism and respiration. Furthermore, mitochondria also possess their own DNA (mtDNA), and mutations in these could influence the ETC and affect oxygen consumption (**McBride et al., 2006**). In addition, they are also important for regulating adaptation to cellular stress such as DNA damage and oxidative stress **(Martínez-Reyes & Chandel, 2020**).

In *Drosophila*, fat body (FB) is one of the major tissues that play an important role in both absorption and storage of nutrients. It is the equivalent of liver and adipose tissue in mammals. The FBs are involved in the exchange and transport of metabolites and nutrients upon varying environmental conditions. They store excess energy for growth and development thereby regulating the organisms' behavior. In addition, these tissues also function as sites for immunity and detoxification. Apart from fat body, another specialized group of cells called oenocytes are required during periods of starvation to mobilize fat for energy production, similar to hepatocytes in mammals (**Chatterjee & Perrimon, 2021; S. Li et al., 2019; Mattila & Hietakangas, 2017**).

For most metabolic processes, nutrients in simple form such as, carbohydrate, amino acids and fatty acids in the cells are converted to adenosine triphosphate (ATP) for energy in the mitochondria **(Spinelli & Haigis, 2018**). During these processes, several other intermediary metabolites are produced, which along with ATP serve as donors for epigenetic processes. The following sub-sections will give a brief overview of carbohydrate metabolism, citric acid cycle, lipid metabolism and one-carbon cycle.

Although majority of the review is based on *Drosophila*, most of the processes are conserved in higher organisms as well.

2.3.1 Carbohydrate metabolism

Glucose is one of the major carbohydrates that is simple and readily available as a source of energy (**Miyamoto & Amrein, 2019**). Three major process are key for glucose oxidation: Glycolysis in aerobic and anaerobic conditions and pentose phosphate pathway, which is involved in synthesis of nucleotides (**X. B. Li et al., 2015**). Glucose undergoing glycolysis gets converted to smaller molecules that can be used as a source for producing high energy compounds that are required for most cellular processes. However, when excess glucose is available, they have to be converted into suitable storage form for use in times of reduced food availability. Therefore, glucose is converted to glycogen (and trehalose in insects) and fat via glycogenesis and lipogenesis respectively. Similarly, upon lack of energy source, these should be converted back to glucose, which occurs via glycogenolysis or gluconeogenesis **(Alfarouk et al., 2014; Miyamoto & Amrein, 2019; Yamada et al., 2019**).

Glycolysis is a series of reactions, where, a 6-carbon glucose is broken down to produce 3-carbon pyruvate or lactate (**Fig. 2**). The obtained pyruvate can also enter the TCA cycle under aerobic conditions to undergo complete oxidation resulting in ATP and the reduced from of nicotinamide adenine dinucleotide (NAD), called NADH. Under anaerobic conditions, pyruvate is converted to lactate, which later results in producing ATP (**Alfarouk et al., 2014; X. B. Li et al., 2015**). Glycolysis relies on three important enzymes or key steps: Hexokinase (HK), phosphofructokinase (PFK) and pyruvate kinase (PK). Production of glucose-6-phosphate by hexokinase is one of the most important steps of glycolysis. This intermediate can be used in multiple ways in not just

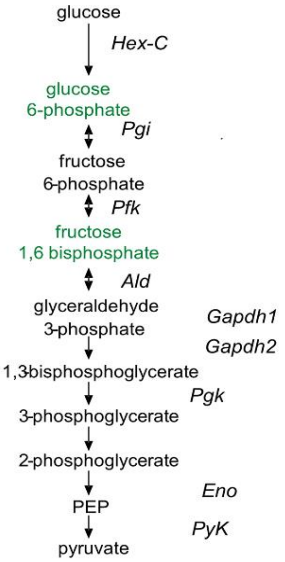

Figure 2: *Glycolysis, its intermediates and enzymes in D. melanogaster (Obtained and modified from (**Wangler et al., 2017**)*

glycolysis, but can also enter pentose phosphate pathway, glycogenesis, and in insects, trehaloneogenesis. The rate-limiting step in glycolysis however is the synthesis of fructose-1,6-biphosphate carried out by PFK. Finally, PK converts phophoenolpyruvate to pyruvate and ATP. This is an important and irreversible reaction as the latter can be further oxidized to acetyl-CoA and ATP, which can serve as donors for acetylation and phosphorylation of proteins respectively (**X. B. Li et al., 2015; Pietrocola et al., 2015**). Under conditions of low glucose, organisms can also synthesize glucose via multiple pathways. Gluconeogenesis is one such process and also the reverse of glycolysis. Most of the enzymes are shared between the pathways, except for three additional enzymes: phosphoenolpyruvate carboxykinase (rate-limiting enzyme), fructose-1,6-bisphosphatase and glucose-6-phosphatase. Precisely, the process starts from TCA cycle in mitochondria and continues in the cytoplasm, where those three enzymes aid in the synthesis of glucose (**Chatterjee & Perrimon, 2021; Miyamoto & Amrein, 2017, 2019; Spinelli & Haigis, 2018**). In addition to glucose, insects and other smaller species have an additional carbohydrate called trehalose. The synthesis of trehalose occurs in FB from glucose-6-phosphate and UDP-glucose with the help of the bi-functional enzyme, *Tps1*. Synthesis of trehalose from non-glucose sources is identical to gluconeogenesis until glucose-6-phophate (**Fig. 3**). Furthermore, synthesized trehalose can be broken down by the enzyme trehalase (*Treh*) to produce glucose that can again enter glycolysis. Trehalose is an important sugar in *Drosophila* metabolism and is in fact the primary circulating sugar in insects. It serves as a source of glucose in the brain, provide the required energy for flight muscle and protect the organism against environmental stresses. This is due to its unique property of being a non-toxic and nonreductive sugar thereby allowing it to be present at high concentrations in the organism. Furthermore, trehalose is unaffected by dietary sugar levels but are present in varying concentrations during development (**Arrese & Soulages, 2010; Chatterjee & Perrimon, 2021; Miyamoto & Amrein, 2017, 2019; Tellis et al., 2023; Yamada et al., 2019**).

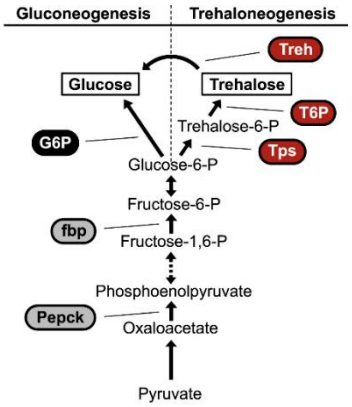

Figure 3: *Gluconeogenesis and trehaloneogenesis (Modified from* ***Miyamoto & Amrein, 2017)***

Excess glucose produced or taken up by the organism from diet can be stored as glycogen, a branched polysaccharide of glucose. This occurs by a process called glycogenesis. They are synthesized with the help of glycogen synthase using UDP-glucose, which is an intermediate of glycolysis that is also required for trehalose synthesis (**Fig. 4**) (**Arrese & Soulages, 2010; Yamada et al., 2019**). Hence, glycogenesis occurs only upon the inhibition of trehaloneogenesis or when the organism has enough trehalose. Furthermore, the stored glycogen can also be used for glucose or trehalose synthesis in times of energy demands or environmental challenges via glycogenolysis. This requires the enzyme glycogen phosphorylase that aids in the mobilization of glycogen and converts glycogen to glucose-1-phosphate (**Arrese & Soulages, 2010; Tellis et al., 2023**). Although glycogen is stored for energy needs in the FB, they are also an important make up in flight muscles and are generally used by the organisms during flight (**Mattila & Hietakangas, 2017**). Furthermore, change in glycogen metabolism has been shown to affect longevity in flies and worms (**Yamada et al., 2019**).

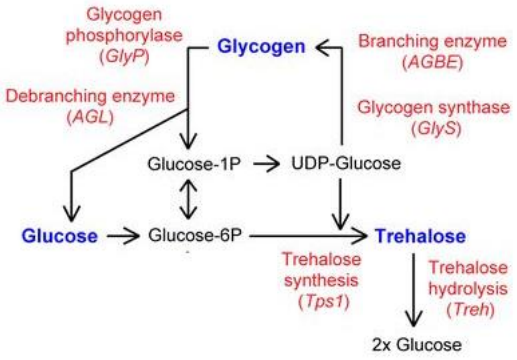

Figure 4: *Interaction between glycogenesis, glycogenolysis and trehaloneogenesis (Obtained and modified from* ***Yamada et al., 2019)***

2.3.2. Citric acid cycle

Citric acid cycle (or TCA cycle or Krebs cycle) plays a key role in energy and respiration by serving as the fueling compartment in mitochondria. It is also the point where three pathways, glycolysis, fatty acid beta-oxidation and amino acid breakdown, meet via the central metabolite, acetyl-CoA (**Jacobs et al., 2020**). Its intermediates serve as important constituents for the synthesis of nucleotides, lipids and proteins and also controls chromatin signaling via post-translational modification of proteins and DNA demethylation. They consist of both reversible and irreversible biochemical reactions such that there is a requirement for constant regeneration of each metabolite (**Cheng et al., 2013; Chinopoulos, 2013; Martínez-Reyes & Chandel, 2020**).

The process starts with acetyl-CoA to produce citric acid by combining with oxaloacetate (OAA), which is the final product of TCA cycle (**Fig. 5**). Citric acid is then converted to alpha-ketoglutarate (αKG) and subsequently to succinate and fumarate, which can serve as donors/inhibitors of DNA (or histone) demethylation and histone (or protein) succinylation, thereby regulating gene expression. Succinate and fumarate being structurally similar to αKG inhibits demethylation of both DNA and proteins. Finally, the cycle ends with the conversion of fumarate to OAA via malate, with OAA being used for next cycle. (**Chinopoulos, 2013; Dai et al., 2020; Martínez-Reyes & Chandel, 2020; Teperino et al., 2010**). Recently, another module of TCA cycle was discovered, which partially occurs in cytosol. In this case, citrate is transported to cytosol, where it is converted to malate through a series of reactions and is then transported back to mitochondria to complete the cycle. This was reported to occur during cell state transitions, although it remains to be understood if this is a driver or a requirement (**Arnold et al., 2022; Doan & Teitell, 2022**).

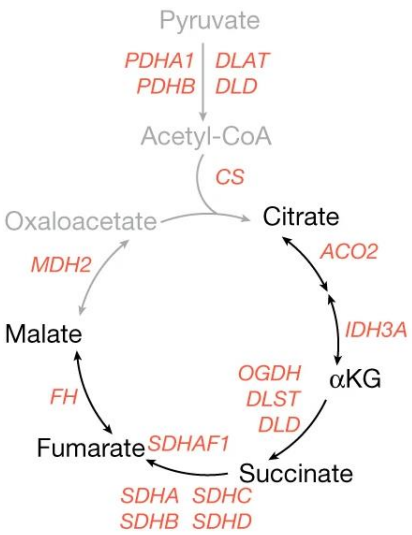

Figure 5: *TCA cycle showing major metabolites and the corresponding enzymes (Obtained from* ***Arnold et al., 2022****); Note: enzymes are Mus musculus nomenclature)*

Interestingly, TCA cycle is involved in both breakdown and biosynthetic reactions. For instance, citrate produced in mitochondria gets transported to cytosol that is converted to acetyl-CoA for lipid and amino acid synthesis. Similarly, many primary intermediates of TCA are transported to cytosol and nucleus for various chromatin and signaling activities. This makes TCA cycle an interesting process as there needs to be a constant replenishment of these metabolites for the cycle to proceed, which is called anaplerosis. On the other hand, the removal of those metabolites that cannot be oxidized in the TCA cycle is called cataplerosis (**Inigo et al., 2021; Martínez-Reyes & Chandel, 2020; Owen et al., 2002**). A full cycle of TCA therefore generates multiple primary intermediates and final byproducts, ATP, NADH and FADH2. The latter two are further transferred to ETC and the inner mitochondrial membrane components for generating electrochemical gradient. This is then used by ATP-

synthase to produce ATP in mitochondria via oxidative phosphorylation (OXPHOS). This indicates that the functioning of TCA cycle and OXPHOS are inter-dependent (**Fernie et al., 2004; Martínez-Reyes & Chandel, 2020; Shen et al., 2022; Spinelli & Haigis, 2018**).

2.3.3 Fatty acid metabolism

Fats are another source of stored energy that can be used by the organism when food supply is limited or unavailable. They also serve as signaling molecules and are required for the formation of membrane lipids. Excess fat in flies are stored in FB as neutral triacylglycerol (TAG) and are the highest energy compounds that yield more calorie upon complete oxidation as compared to carbohydrates. Furthermore, TAGs constitute the major component of specialized storage structures called lipid droplets (LDs) which is also conserved in higher organisms (**Heier & Kühnlein, 2018; Kühnlein, 2012**). Apart from these, TAGs also make-up a part of few other tissues in *Drosophila* such as oenocytes, brain, gut and ovary (**Chatterjee & Perrimon, 2021**).

Like carbohydrate metabolism, lipid metabolism also comprises of synthesis and breakdown referred to as lipogenesis and lipolysis respectively. TAG metabolism and its mobilization is controlled by endocrine signals that influence the activity of corresponding proteins and enzymes. Some of the key hormones that control TAG metabolism include insulin, adipokinetic hormome (Akh), juvenile hormone (JH) and ecdysone signaling. While insulin, Akh and ecdysone are lipolytic, JH is a lipogenic hormone (**Heier & Kühnlein, 2018; Toprak et al., 2020**).

The synthesis of TAG requires the conversion of acetyl-CoA obtained from carbohydrates (and proteins) to fatty acids (FAs) CoA in the cytosol. Lipogenesis starts in the endoplasmic reticulum with the acylation of glycerol-3-phosphate using FA-CoA and ends with the conversion of diacylglycerol (DAG) to TAG or phosphatidylcholine (**Fig. 6**). It is four-step enzymatic process involving different types of acyltransferases that use FA-CoA for acylation (**Kühnlein, 2012; Thanh et al., 2020; Toprak et al., 2020**). Recently, it was identified that the concentration of acetyl-CoA can serve as a switch for lipogenesis via acetylation of proteins that occur independently of acetyltransferases. Furthermore, biosynthesis of TAG is especially

important during *Drosophila* development as th ey are key for survival during metamorphosis (**Miao et al., 2022**).

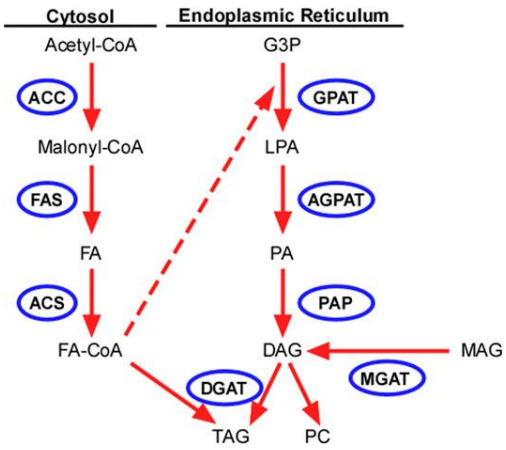

***Figure 6**: Simplistic flowchart showing TAG synthesis in Drosophila melanogaster (Obtained from **Toprak et al., 2020**)*

On the other hand, lipolysis is the breakdown of TAGs into shorter fatty acids for energy production. This process is important for organism's survival upon metabolically challenging environments (**Heier & Kühnlein, 2018; Houten & Wanders, 2010**). In flies, two types of lipolysis occur, basal and stimulated lipolysis. The lipase brummer (bmm) plays a key role in both types along with LSD1/2. Basal lipolysis functions more to provide a balance with lipogenesis, while stimulated lipolysis occurs during reduced energy status and involves TAG mobilization. During basal mode, LSD1 is not phosphorylated and hence inhibits bmm to act on TAGs, while upon stimulated environments, LSD1 gets phosphorylated by kinases and endocrine signals and recruits' lipases, including bmm and facilitates TAG breakdown in lipid droplets (**Kühnlein, 2012; Thanh et al., 2020**). This results in the production of DAGs and free fatty acids (FFA), where the former can also be hydrolyzed further to FAs, while the latter can enter β-oxidation in mitochondria to produce ATP (**Chatterjee & Perrimon, 2021; Heier & Kühnlein, 2018**). In addition to mitochondria, peroxisomes also aid in β-oxidation, especially in the case of long chain FFAs that cannot be processed by mitochondria. Approximately, the 20 different proteins involved in β-oxidation aid in reducing the length of FAs to produce acetyl-CoA in the mitochondria, which can enter the TCA cycle for energy production or gets transported to nucleus and cytoplasm via citrate conversion to be used for protein acetylation (**Houten & Wanders, 2010; Tiwari et al., 2020; Wangler et al., 2017**).

2.3.4 One-carbon metabolism

One-carbon metabolism (OCM) is primarily the combination of two cycles that comprises of folate and methionine metabolic pathways generating one-carbon units for most cellular activities. The process is also key in the generation of S-

adenosylmethionine (SAM), which serves as a donor of methyl groups for all types of methyltransferases (**Clare et al., 2019**). Some of the principal cofactors or donors of OCM include methionine, folate, choline, betaine and vitamin B_{12}. Folate, for example, cannot be synthesized by animals and hence should be included in the dietary intake, as opposed to smaller organisms like plants, yeast and bacteria. Similarly, methionine is an essential amino acid that can only be obtained from food or gut microbiome in humans (**Ducker & Rabinowitz, 2017; Korsmo & Jiang, 2021; Parkhitko et al., 2019**). These examples further bolster the view that this process can be directly influenced by environmental changes as differences in diet or lifestyle can affect the levels of folate or methionine and hence downstream intermediates (**Mentch & Locasale, 2016**).

Folate can be obtained naturally from the diet or via its synthetic form as folic acid. Dietary sources of folates are converted to glutamated forms before they enter folate cycle whereas folic acid, is reduced to tetrahydrofolate (THF). Interestingly, folate cycle occurs in multiple compartments such as mitochondria, nucleus and cytoplasm and are well connected (**Fig. 7**). In nucleus and cytoplasm, folic acid is oxidized to monoglutamted folate, which is converted to tetrahydrofolate (THF) via dihydrofolate (DHF). In mitochondria, the folate cycle starts with either monoglutamate THF or 5-fTHF (5-formyl-THF) (**Lionaki et al., 2022; Shuvalov et al., 2017; Zarou et al., 2021**). In all these cases, serine serves as a donor of carbon, that is used by enzyme isoforms in different compartments. It must be noted that serine can also be obtained from dietary sources or can be synthesized by the body from intermediates of glycolysis, i.e. 3-phosphoglycerate (**Ducker & Rabinowitz, 2017**). THF is then converted to 5,10-methylene-tetrahydrofolate (5,10-mTHF) and this enzymatic conversion requires vitamin B6 as a cofactor, with serine as donor of carbon group. This transfer converts serine to glycine. This is followed by a reduction to 5-mTHF with vitamin B2 as cofactor, which is an irreversible reaction. In some cases, 5,10-mTHF, can be converted to 10-fTHF (10-formyl-tetrahydrofolate) or to DHF, both of which can also be converted back to THF. The final step combines folate cycle to methionine cycle via transfer of methyl-group (or one-carbon) from 5-mTHF to result in THF. The first step of methionine cycle starts with the last step of folate cycle (**Clare et al., 2019; Parkhitko et al., 2019; Shuvalov et al., 2017**). The transfer of methyl-group from 5-mTHF converts to homocysteine (Hcy) to methionine.

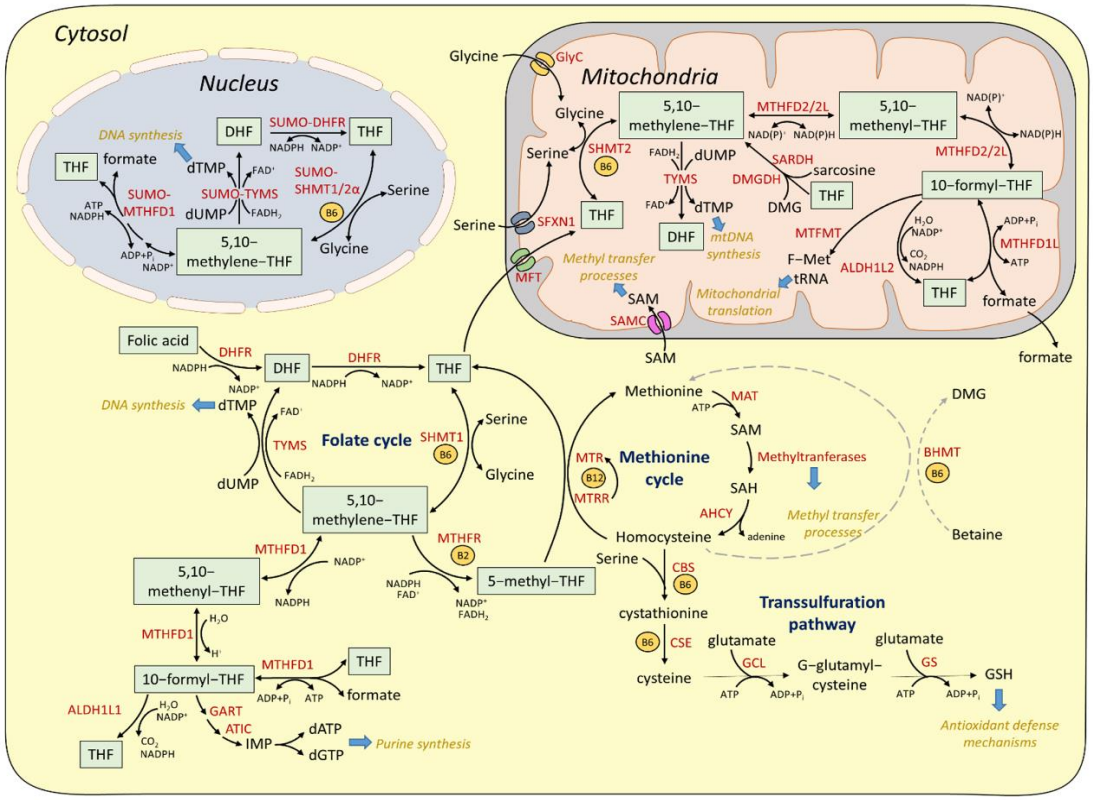

Figure 7**: Overview of one carbon metabolism and the corresponding enzymes involved* (Obtained from Lionaki et al., 2022***)

The latter can then be converted to SAM, which is used as a methyl-group donor. The transfer of methyl group from SAM results in S-adenosylhomocysteine (SAH), which can again be converted to Hcy and the cycle continues. Alternatively in some tissues, Hcy can be converted to methionine with vitamin B6 and betaine as methyl donor, the later can be obtained from diet or choline degradation (**Lionaki et al., 2022**).

OCM can branch into multiple biosynthesis pathway via the generated intermediates depending on the environment and cellular context. When SAM levels are high, the remethylation of Hcy is inhibited and Hcy gets used up for synthesis of cystathionine and cysteine, which are precursors for glutathione that protects the cells from oxidative stress (**Lauinger & Kaiser, 2021**). In addition, cystathionine can end up in propionate pathway for the production of propionyl-CoA that can be converted to succinyl-CoA to enter into the TCA cycle for energy production (**Clare et al., 2019**). Furthermore, the conversion of 5,10-mTHF to 10-fTHF or DHF, also results in inosine monophosphate or deoxythymidylate respectively, both of which can be used for purine synthesis (**Froese et al., 2019**). SAM, apart from its use as methyl-donor, is

also important for polyamine biosynthesis, which are important for aging, cellular growth and DNA repair related processes. Decarboxylated SAM generated from this process can be converted back to methionine via the salvage pathway and can enter methionine cycle (**Lionaki et al., 2022**). Finally, SAM is also used for conversion of phosphotidylcholine to phosphotidylethanolamine that are constituent of lipoproteins involved in lipid transport. Alternatively, glycine can serve as a sink or a buffer to maintain SAM homeostasis by being converted to sarcosine (methyl-glycine) (**Clare et al., 2019**).

2.4 Interplay of metabolism and epigenetics upon environmental challenges

Cellular fates are determined by the status of external environment that affects the molecular response of a cell. These can be interpreted by epigenetic components and are conveyed into the epigenome with the help of various enzymes that use corresponding donor metabolites. Further, environmental conditions can also affect the metabolic response, which can subsequently be translated to gene expression changes via changes in epigenetic mechanisms (**Fig. 8**) (**Katada et al., 2012; Martínez-Reyes & Chandel, 2020**). For instance, ATP is the simplest molecule that provides energy to most reactions occurring within the cell. The concentration of ATP is sensed by the protein, AMP-activated protein kinase (AMPK) that is activated under low ATP or high AMP levels to phosphorylate proteins that promote ATP generation and energy availability (**Shen et al., 2022**). Ironically, ATP also serves as a donor for phosphorylation of histone and non-histone proteins. Histone phosphorylation triggers a number of key cellular processes such as DNA damage, mitosis, apoptosis, cell cycle checkpoint, meiosis, etc. (**Alaskhar Alhamwe et al., 2018; Banerjee & Chakravarti, 2011; Rossetto et al., 2012**). However, an overall increase or decrease in ATP does not change the phosphorylation status of all proteins correspondingly. A study in *Drosophila* lacking the bcl-2 gene, *buffy*, was observed to have reduced levels of cellular ATP under *ad libitum* conditions. In spite of that, the protein S6K showed increased phosphorylation, which lead to increased basal TOR signaling and differential energy metabolism. Subsequently, these flies were unable to adapt to changes in nutrient status and were hence susceptible to starvation (**Monserrate et al., 2012**). Such observations could be explained either by spatial compartmentalization of specific metabolites and the corresponding enzymes or the

varying ATP affinities between enzymes carrying out the same activity. In either case, the effect could be local or global depending on the external influence, metabolic changes and enzymes/transcription factors involved (**Katada et al., 2012**).

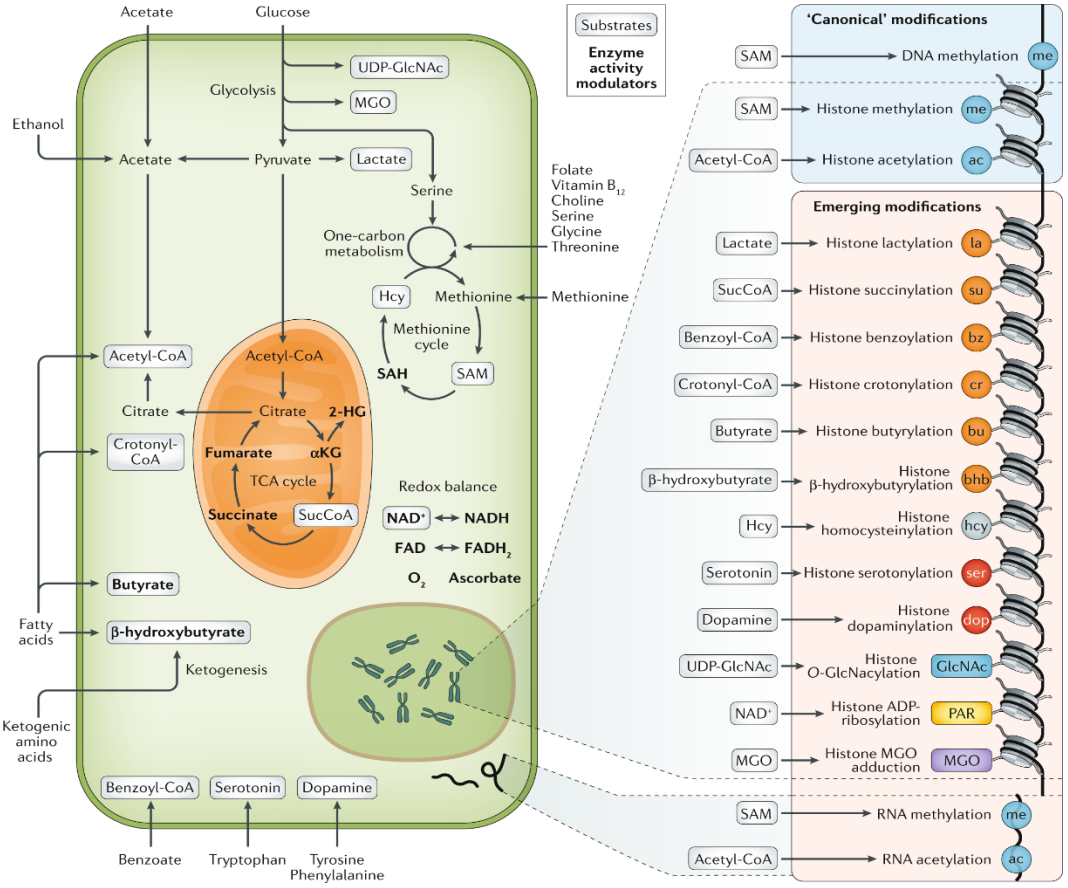

Figure 8: *An overview of metabolism and its products' function in epigenetic modifications (Obtained from **Dai et al., 2020**)*

Studies have also shown that intermediary metabolites that are important for cofactor synthesis can also cause changes in the epigenome, e.g. one-carbon metabolites and SAM. Surprisingly, studies that alter the levels of one-carbon metabolites (except SAM) and its influence on histone methylation in *Drosophila* are extremely limited. Nevertheless, it was shown that genetic ablation of *dAhcy* (*Drosophila* adenosylhomocysteinase), which converts SAH to Hcy, extended the life- and health-span of the organism. In addition, it also reduced Hcy, increased SAH and consequently reduced H3K4me3, all of which phenocopies the effects of methionine restriction (**Parkhitko et al., 2016**). Intriguingly, flies fed with spermidine, a polyamine synthesized with the help of SAM, show an increase in longevity. (**Clare et al., 2019**). However, spermidine here was found to act as an inhibitor of HAT

activity, causing H3 hypo-acetylation and promoting autophagy (**Eisenberg et al., 2009**). In fact, flies show increased histone acetylation and acetyl-CoA levels upon aging (**Peleg, Feller, Forne, et al., 2016**). Moreover, the effect of dietary folate intake has been to shown to alter SAM/SAH ratio and DNA methylation in other organisms (**Dai et al., 2020; Feil & Fraga, 2012**). These observations also indicate that either genetically manipulating the metabolic enzymes or environmentally induced change in metabolite levels can affect the downstream epigenetic mechanisms.

In addition, neuroendocrine response plays an important role in stress regulation and abrogation of these components will impact resilience to stress. Stress response by neuroendocrine system is composed of insulin signaling pathway, Akh, JH, ecdysone signaling and biogenic amines such as dopamine and octopamine. All these together control various aspects of the organism from development to stress response (**Bobrovskikh & Gruntenko, 2023**). For example, IIS pathway (Insulin/insulin-like growth factor signaling) plays an important role in thermoregulation even in poikilothermic species such as *Drosophila*. While mammals show a reduction in body temperature upon hunger, fruit flies prefer a reduced ambient temperature due to their poikilothermic nature. This preferential behaviour is controlled by anterior cells and insulin-signaling, a process that is also conserved in mammals (**Umezaki et al., 2018**). Furthermore, differences in longevity between reproductive and non-reproductive members of ants occurs as a result of differential insulin signaling (**Yan et al., 2022**).

In *Drosophila*, insulin-producing cells (IPCs) and corpus cardiacum (CC) serve as the equivalent of beta- and alpha-cells of pancreas, producing insulin and Akh (similar to glucagon in). The latter regulates lipid and trehalose mobilization in response to stress, while the former is produced upon hyperglycemia to balance glucose levels. JH and 20-hydroxyecdysone are primarily involved in growth and development, while dopamine and octopamine are neurotransmitters that control organismal behaviour and endocrine glands. Interestingly, all these are interconnected in a complex network for efficient stress response. For instance, JH regulates the synthesis of insulin-like peptides (ILPs) from IPC and is important for trehalose metabolism. ILPs are hypotrehalsemic hormone which aid in the maintenance of trehalose in hemolymph. They increase transcription of genes to modulate trehalose accumulation. Apart from JH, 20-hydroxyecdysone affects the activity of proteins involved in trehalose

metabolism and is important for starvation response. On the other hand, Akh, which is a hypertrehalsosemic hormone, enhances trehalose synthesis by inhibiting glycolysis and reducing the substrate competition. Octopamine enhances lipid mobilization upon acute stress response, thus serving as an equivalent to noradrenaline (**Arrese & Soulages, 2010; Bobrovskikh & Gruntenko, 2023**).

2.4.1 Impact of genetic variation

Adaptation in non-ideal environments could require long-term changes that can occur via genetic mutations. It is also possible that such mutations can coordinate with epigenetic machinery in conferring to organisms' survival in novel conditions. In *Drosophila*, the gene *for*, that aids in regulation of behavior and physiology, contains a naturally occurring SNP (A to C) that results in rover (for^R) and sitter (for^S) populations respectively. This genetic change along with differences in H3K9me2 by G9a methyltransferase results in molecular and behavioral differences including starvation resilience. In addition, rovers were found to be more adaptable to changes in nutrition (**Anreiter et al., 2017; Kent et al., 2009**). Similar observations were also seen in ants at the *Egfr* gene locus. This quantative trait loci (QTL) shows differential DNA methylation causing corresponding variation in size of worker colony (**Alvarado et al., 2015**). Long-term effects can also be observed in higher organisms such as the Dutch famine in humans. Even after generations of exposure to famine in 1940s, the off-springs of mothers exposed to the famine pose higher risks of developing metabolic diseases such diabetes and obesity. Incidentally, those individuals also show DNA hypo-methylation of *IGF2* gene, which is maternally imprinted and plays an important role in growth and development (**Heijmans et al., 2008; Katada et al., 2012**).

2.4.2 Impact of nutrition

One of the most important metabolite, acetyl-CoA, is often referred to as the central metabolite. It is the precursor of anabolic reactions and determinant of catabolic process and links glycolysis, TCA cycle and lipid metabolism and is the sole donor for acetylation of histones and non-histone proteins (**Pietrocola et al., 2015**). Correspondingly, acetylation can also affect the energy metabolism directly as most

metabolic proteins involved in catabolic processes are acetylated. In addition, HATs can also be acetylated, either by themselves or other proteins, which improves their activity by preventing degradation (**Katada et al., 2012**). Moreover, the communication between different HATs and acetyl-CoA can be affected based on the environmental contexts. Moderation of acetyl-CoA (or the ratio of acetyl-CoA and CoA) affects the affinity and specificity of acetyltransferases. Such changes in the metabolite concertation can occur specifically on certain subcellular or membrane-less compartments which could be translated to proteins acetylation (**Pietrocola et al., 2015**).

The interplay of acetylation and physiological/behavioral changes can be observed in eusocial insects such as ants and honey bees, both displaying phenotypic plasticity. Queen and worker bees are genetically identical organisms but they differ in physiology, longevity and reproductive capacity. In addition, the two sub-type of bees also differ in their dietary intake. While queen bee feeds on royal jelly, worker bees' diet is mostly restricted to honey and pollen (or worker jelly). One of the major component of royal jelly is a small-molecule deacetylase inhibitor, (E)-10-hydroxy-2-decenoic acid (10HDA). Both the molecule and royal jelly administration inhibits transcriptional repression and increases histone acetylation when tested using a cell-based reporter assay showing similarities to sodium butyrate. Moreover, a derivative of 10HDA, 4-hydroperoxy-2-decenoic acid ethyl ester (4-HPO-DAEE), increased H4 acetylation at the promoters of genes involved in oxidative stress response. These observations indicate that changes in nutritional status has direct effect on histone and possibly protein acetylome (**Makino et al., 2016; Spannhoff et al., 2011**). On the other hand, diet differences in ants during development has not yet been observed but differences exhibited by worker and queen bees are also be observed in genetically identical ants of different sub-groups. Notably, major and minor workers of *Camponotus floridanus* (or carpenter ants) show morphological differences and behavioral variation in foraging and scouting. These differences were primarily influenced by differential localization of CBP, a histone acetyltransferase, and consequently distinctive H3K27ac depositions. Moreover, loss of Rpd3, a protein that removes H3K27ac, was able to diminish the differences in foraging behavior between majors and minors (**Simola et al., 2013, 2016**). Of course, these changes could be a function of both genetic and epigenetic component, like the ones observed in

Drosophila for rovers and sitters (**Anreiter et al., 2017**). Nevertheless, while there are studies showing the effect of nutrition or environment on behavior and physiology of such eusocial insects, its direct effect on cellular metabolism and metabolic intermediates and the link with downstream acetylation especially of non-histone proteins has not yet been explored in depth.

2.4.3 Impact of temperature changes

Apart from nutrition and genetics, change in ambient temperatures, especially in poikilothermic species, could also lead to differences in metabolism and possibly downstream signaling process (**Mołoń et al., 2020**). *Drosophila*, in this regard, has been studies quite extensively to one of the extreme forms of temperature adaptation. Increase in chaperone proteins (or heat shock proteins (HSPs) is one of the most conserved response to temperature regulations. In fruit flies, heat shock response results in the appearance of chromosomal puffs, which are regions of active transcriptions observed especially in polytene chromosomes. These chaperone proteins mediate proper protein folding, prevent the formation of aggregates and regulate protein assembly and trafficking. Reports suggest a strong correlation between their expression based on population distribution and climatic conditions. This differential or plastic nature of HSPs are instrumental to cope with temperature changes at different altitudes. In general, HSPs are named and differentiated based on their molecular weight, with different *Drosophila* species possessing varying number of HSP genes (**B. Chen et al., 2018; Storey & Storey, 2023**).

Studies from *Bicyclus anynana* (African butterfly) live in both dry and wet seasons, each resulting massive differences in survival, behavior and reproduction. One interesting aspect was the plasticity in response to starvation. It was observed that butterflies that developed at lower temperatures showed reduced starvation resistance possibly due to higher resting metabolic rate (RMR) that could lead to rapid depletion of energy and hence reduced resistance to stress (**Pijpe et al., 2007**). Similar studies in *Drosophila* have also implicated that exposure to colder temperatures early in development could result in increased metabolic rates. In fact, fruit flies grown at 18˚C and starved at 23˚C showed reduced resistance to starvation than those grown and starved at 23˚C (**Jang & Lee, 2018**). In another study, it was observed that

metabolic rate in *Drosophila* does not affect the resistance to stress and was hypothesized that energy accumulation, in terms of lipids and carbohydrates could be a more determining factor. Indeed, metabolic rate and temperature in *Drosophila* show a linear relationship between 13˚C-33˚C temperature range. This was also explained by an inverse relationship between mean survival upon starvation and different temperatures and was also suggested that under non-optimal conditions, improper energy utilization could affect energy storage (**Djawdan et al., 1997; Klepsatel et al., 2019**). However, these studies were performed at constant temperature and one non-optimal condition could be the varying temperatures during development and stress. Although these studies focused on the effect of temperature on metabolism, its implication on downstream signaling such as acetylation was not explored. Nevertheless, one study reported reduced expression of proteins involved in energy production but enrichment of proteosomal proteins when comparing flies grown at 31˚C and 25˚C, indicating a preference in removal of damaged proteins at the loss of basal metabolic functions at higher temperatures (**Kristensen et al., 2016**). Furthermore, studies in *C. elegans* have shown that temperature changes result in downstream transcriptomic and epigenetic alterations. It was observed that exposure to higher temperatures only during development was found to increase both immune responsive genes and longevity. An acetyltransferase, CBP-1 was required to translate the early stress exposure to histone acetylation and consequent transcriptomic changes. The early exposure to heat stress resulted in a lasting activation of stress-related genes thereby promoting longevity (**Zhou et al., 2019**).

2.4.4 Non-enzymatic acetylation of proteins

An interesting aspect of acetylation and the influence of metabolism is the role of non-enzymatic protein acetylation (or acylation). One of the earliest instances of this observation was in the 70s, where histones were observed to be acetylated non-enzymatically. The efficiency of this reaction was dependent on pH, number of lysine/arginine residues and time of incubation (**Paik et al., 1970**). Recent studies suggest that change in metabolism or metabolite levels either by stress or environment could contribute to such reactions. Smaller organisms such as *Escherichia coli* (*E. coli*) show instances of acetylation independent of acetyltransferase enzymes. Such reactions are dependent on the metabolic intermediates, in this case acetyl-phosphate.

In fact, *in vitro* assays with the intermediate show an almost complete acetylation of bacterial proteins. Further, some of these acetyl marks, although added non-enzymatically, can only be removed enzymatically with the help of sirtuins, NAD+-dependent protein deacetylases (**Wagner & Hirschey, 2014; Weinert et al., 2013**). A more recent study observed a functional role for these reactions in *E. coli.* Of the many proteins acetylated, acetylation of two proteins, GapA and GmpA, both of which were involved in glycolysis, inhibited their activity. This loss of function was observed under physiological conditions and aided the production of acetyl-phosphate and reduced the glycolytic flux. Moreover, acetylation was not observed when bacteria were cultured under reduced acetate or with non-fermentable galactose, indicating the importance of this non-enzymatic reaction in organism's growth (**Schastnaya et al., 2023**). Furthermore, organisms such as *C. elegans* and *D. melanogaster* also possesses non-enzymatic protein acylation. Nematodes show an age-dependent increase of protein acylation (succinylation) that was also associated with a decrease in oxygen consumption and consequently reduced activity of those mitochondrial proteins. Fruit flies also show similar molecular characteristics, but a more recent study explained the importance of this biochemical reaction in development. One of the well-studied lipid metabolic enzyme, fatty acid synthase (FASN), was observed to be acetylated non-enzymatically. The regulation of this reaction primarily depends on the flux of metabolite intermediate, acetyl-CoA and an alkaline pH. The K813 auto-acetylation of FASN increased its activity resulting in proper development and *de-novo* lipogenesis. This increase of TAGs was associated with increased activity of FASN but not that of protein expression. As observed in *E. coli*, removal of this acetyl mark is mediated by one of the sirtuins, Sirt1, that consequently brings the FASN activity to basal level. Intriguingly, the non-enzymatic acylation of mitochondrial proteins was not observed higher organisms such as rats (**Hong et al., 2016; Miao et al., 2022**). Environmental changes such as temperature fluctuations can affect the flux and metabolic status of smaller organisms, whose internal temperature is highly dependent on external influence. In such scenario, a rapid adaptation might be necessary and therefore it can be speculated that such enzyme-independent reactions could be evolutionarily observed in smaller organisms to cope with such environmental stressors (**James et al., 2018**).

2.4.5 Metabolism and epigenetics of stress response

As acetylation and metabolism are highly interlinked, lack or abnormality of one or the other can result in perceived molecular changes as explained before. These changes result in the regulation of many biological processes, one of which is stress response. In smaller organisms such as *S. cerevisiae*, glucose starvation reduces both the levels of acetyl-CoA and bulk H3K9 acetylation. However, the metabolic stress resulted in a shift of H3K9ac to growth-promoting genes, especially those involved in carbohydrate and fat metabolism via components of SAGA complex (**Hsieh et al., 2022**). In fact, studies in mammalian cells have shown that ATP-citrate lyase, the enzyme responsible for acetyl-CoA production, is important for histone acetylation that is dependent on glucose availability, further indicating the connection between acetylation and metabolism (**Wellen et al., 2009**). Moreover, metabolomic studies in *Drosophila* have shown that nutrient stress can alter the levels of various metabolites and in a tissue-specific manner. This does make sense as the corresponding major tissues, neurons and fatbody can communicate via neuro-endocrine signaling (**Wilinski et al., 2019**). In this regard, one of the acyl-CoA synthetase in *Drosophila*, pudgy, plays a significant role in sleep, aging and starvation. Lack of this enzyme reduces the TAG concentration and make the flies prone to starvation stress. Furthermore, these flies also show increased aging and lack of sleep and these effects could be due to reduced expression of few genes (**Thimgan et al., 2018**), which are likely be mediated by changes in histone acetylation. On the other hand, depletion of Ada2b, a subunit of SAGA complex that is required by Gcn5 acetyltransferase, affects organism's viability possibly due to reduced DNA damage response upon global H3 hypoacetylation. Moreover, yeast and plants lacking this enzyme show high susceptibility to genotoxic and abiotic stress respectively (**Qi et al., 2004; Vlachonasios et al., 2011**). A more interesting aspect is tissue-dependency of enzyme's functional role. Such an example is the fatbody-specific knockdown of NAA40, a member of less explored family of N-terminal acetyltransferases. This loss increases TAG levels in fruit flies by enhancing lipid metabolism (**Charidemou et al., 2022**). Although no role in stress response has been identified in *Drosophila,* it would be logical to speculate that the enzyme could function in stress-related processes. Apart from acetyltransferases, deacetylases such as HDAC1 in *Drosophila* also show similar effects on metabolism and stress-response. Flies lacking HDAC1 were found

to live longer, have increased energy storage and consequently improved starvation resistance, all of this due to reduced insulin signaling (**Woods et al., 2016**). This could likely be mediated via changes in histone or protein acetylation, although these were not explored in this study. For example, mutants of Sir2 were shown to remove acetylation of autophagy-related factors and induce autophagy upon nutrient stress (**Jacomin et al., 2020**). Finally, acetylation of the proteins can also influence other post-translational modifications. Nejire (nej) another acetyltransferase in *Drosophila* acetylates members of AP-1 transcription factors, specifically Jra, upon osmotic stress. However, phosphorylation of Jra that is also induced upon osmotic stress prevents acetylation and improves the stability of the protein. This indicates a tight regulation of Jra stability based on two different post-translational modifications that can be context or tissue dependent (**Zhang et al., 2013**).

Despite the understanding of connections between metabolism and epigenetics explained in these studies, there is lack of information on how the communication occurs upon environmental challenges and long-term genetic selection.

2.5 Aims of the thesis

During the course of this doctoral study, we decided to understand metabolic stress via interaction of genetic, epigenetic and metabolic components associated with environmental changes and phenotypic selection. To this end, we studied the evolutionary importance of an acetyltransferase in fruit flies *(Drosophila melanogaster)* called chameau (chm) and characterized an obese mouse model (*Mus musculus*) named Titan, which has been selected for over 180 generations based on its physiology, and proposed it as a model for metabolic syndrome that closely resembles human condition.

chm is a MYST-domain containing acetyltransferase in *Drosophila* that has been shown to increase aging and associated phenotypes (**Peleg, Feller, Forne, et al., 2016; Peleg, Feller, Ladurner, et al., 2016**). It was initially identified as an enzyme involved in gene silencing by suppressing position effect variegation. In fact, chm along with PcG proteins represses Hox gene loci in fruit flies (**Grienenberger et al., 2002**). Furthermore, chm was also found to act as a coactivator of AP-1 transcription factors in a JNK-dependent manner to regulating the genes upon osmotic stress

(**Miotto et al., 2006**). Apart from this, chm also has roles in nervous system development (**Hainaut et al., 2012**), can bind to amplicon origins to regulate origin amplification via H4K12 acetylation (**McConnell et al., 2012**), and phenocopy loss of fatty acid oxidation in hematocyte progenitors (**Tiwari et al., 2020**). More recently, lack of chm increased the levels of TAG at larval E3 stage further implying its role in metabolic regulation of *Drosophila* (**Miao et al., 2022**). Interestingly, its mammalian orthologue, HBO1, shows 75% similarity in the MYST domain and is important for origin amplification and AP-1 transcriptional activity (**Miotto & Struhl, 2006**). HBO1 was also shown to be essential for stem cell maintenance (**Y. Yang et al., 2022**) and extending life span in mouse (**Wang et al., 2021**). Intriguingly, chm was predicted as one of the four chromatin-remodelling loci important for adaptation to novel environments (**Levine & Begun, 2008**) and is under balancing selection in both European and African population (**Croze et al., 2017**). The potential explanation is evidenced by the advantage in chm MYST domain hemizygous fruit flies which have increased benefits as mentioned earlier. As chm is an essential enzyme and is evolutionary present in spite of its negative effects, we decided to explore if chm could have other significant functions that supports its evolutionary presence.

Although using a mutant organism aids in studying the functionality of the protein, naturally occurring changes via selection or genetic mutations in an outbred population resemble more with what happens in nature (**Lehmair et al., 2022; Via & Lande, 2016**). One of the well-known, naturally occurring genetic mutation that is that of rovers and sitters in *Drosophila*. The allelic difference in foraging gene region (*for*) results a phenotypic difference in activity and feeding behavior. In the presence of food, larvae with rover allele (for^R) eat less but move more than those with sitter allele (for^S) (**Kent et al., 2009**). Examples of such selection in laboratory settings are common in smaller organisms such as fruit flies. For instance, fruit flies were selected for starvation resistance from an outbred population by starving them until only 15% are alive and then reintroducing those to food for collecting eggs for the next generation such that these flies have been selected for 120-130 generations. As a result, starvation selected flies were found to be bigger in size, have increased duration of development and sleep, decreased metabolic rate, and increased feeding, all of which could explain in their improved starvation resistance (**Brown et al., 2019; Clark & Gibbs, 2023**). In this regard, selection for an outbred mouse line based on

size was started in 1970s by combining four outbred and four inbred mouse lines. One of the lines were selected based on body mass at six years of age (**Schüler, 1985**). These mice showed higher body weight and fat content than the control unselected outbred line. Hormonal levels, such as, insulin, leptin and IGF-1, were higher in these mice at generation 70 (**Bünger et al., 1998; Timtchenko et al., 1999**). In a follow-up study at 100 generations, microarray analysis was carried out to assess transcriptional differences. Some of the differentially expressed genes were involved in fat/carbohydrate metabolism and immune response. Mutations that occurred naturally as a result of selection could have contributed to this altered transcriptional response (**Aksu et al., 2007**). Furthermore, these selected mouse consumed more food at 6 weeks of age and at generation 146 and did not develop glucose intolerance upon aging or when fed with high fat diet (**Renne et al., 2013**). More recently, at around 180 generations, first ever whole genome sequencing was carried out to assess the differences between the selected and unselected lines. Interestingly, genes such as *Hcrt, Wdr27, Atp11b* that are involved in feeding and energy metabolism showed regions of distinct genetic differentiation (**Palma-Vera et al., 2022**). We decided to use this mouse line to further understand the complexities of natural selection, by characterizing this mouse line (referred to as Titan) especially at the proteome and epigenome level and used transcriptomic analysis to getter a better idea on the changes as compared to earlier used microarray analysis.

3. Summary

Epigenetic factors and metabolic intermediates have been at the forefront of chromatin research in the past few years. The communication between metabolic process and epigenetic factors with the genetic component is especially important to translate environmental changes to the organisms to either improve its survival in short term or provide the ability to adapt in the longer run. We studied these aspects using a poikilothermic species such as *Drosophila melanogaster* (Publication-1) and a higher organism, *Mus musculus*, which are more closely related to humans (Publication-2).

In the first study, **Venkatasubramani et al., 2023**, we aimed to understand the evolutionary importance of *chameau* (chm), a MYST-domain acetyltransferase. We have previously shown that absence of this protein improves both physical activity and longevity in *Drosophila melanogaster* (**Peleg, Feller, Forne, et al., 2016**). We employed *UAS-GAL4* system to knockdown the gene both ubiquitously and tissue-specifically. Upon observing massive physiological changes with ubiquitous, neuronal and fatbody loss of chm, we also confirmed the importance of MYST-domain, i.e. acetyltransferase activity, using a heterozygous mutant with a deletion of MYST genomic region that also showed similar observation. Following this, we used a multi-omics strategy to assess the effect of mutation (or knockdown) on transcriptome, histone acetylome, proteome and non-histone acetylome. Observations from these approaches pointed towards misregulated metabolism. We therefore tested the ability of organisms lacking the protein to respond to metabolic stress by administering wet-starvation and as expected, these flies showed reduced starvation resilience. Interestingly, this was independent of chm's role in development as assessed using GeneSwitch system, where *chm* knockdown is induced in an adult-specific manner. Further, we also validated the perceived role of *chm* in metabolism and stress response by over-expressing the protein ubiquitously and in different tissues via the *UAS-GAL4* system. All these experiments resulted in an improved survival upon metabolic stress and specifically upon over-expression in tissues such as neurons and fatbody. In addition, we have evidence that support this putative evolutionary role of *chm* in stress response is limited only to certain temperature ranges, which could be

considered non-ideal for the organism. This is also supported by evidence of sequence variation and balancing selection in different climatic populations (**Levine & Begun, 2008; Croze et al., 2017)**. This is being addressed in a follow-up study that is in preparation.

The second study was a collaborative effort with Peleg lab. In this study, **(Müller-Eigner et al., 2022)**, we characterized a unique and novel mouse model named Titan that has been selected for over 180 generations based on their high body mass. After assessing their genetic, biochemical and physiological status, we perceived that these mouse show similarities to metabolic syndrome in humans. We further compared Titan mice to unselected mice and characterized histone PTMs and subsequently proteome and transcriptome, all of which pointed towards metabolic differences. All these were assessed in two ages in unselected and Titan mouse as the latter also showed a steep decline in lifespan. Intriguingly, genetic analysis showed variation in acetyltransferase genes and accordingly, acetylation of H4 was altered between the mouse models. Finally, we employed dietary intervention, an intervention that may result in extended life span to test if these mice can display improved survival and rescue associated molecular changes. This study characterized and provided an additional animal model for studying metabolic diseases like obesity and showed the importance of having a non-inbred model that displays genetic variability. The study also provided an interesting take on reversing the possible effects of genetic differences via changes in lifestyle.

Altogether, these studies provide an understanding of metabolic dysregulation in organisms, either upon mutation or phenotypic selection. In particular, the evolutionary importance and a previously unknown role of chm in metabolism of *Drosophila melanogaster* and the characterization of a long-term phenotypically selected *Mus musculus* model, Titan mice, as a novel and unique resource for studying metabolic disorders that closely reflects conditions in human.

4. Zusammenfassung

Epigenetische Faktoren und metabolische Zwischenprodukte standen in den letzten Jahren im Mittelpunkt der Chromatinforschung. Die Anpassungsfähigkeit eines jeden Organismus liegt in der Kommunikation zwischen den epigentischen Faktoren sowie der involvierten Stoffwechselwege. Diese Schlüsselkommunikation ist maßgeblich entscheidend für die Verbesserung des kurzfristigen Überlebens oder dient längerfristig für die Anpassung an die sich verändernden Umweltbedingungen. Wir haben diese Aspekte anhand einer poikilothermen Spezies wie *Drosophila melanogaster* (Publikation-1) und eines höheren Organismus, *Mus musculus*, welcher dem Menschen ähnlicher ist (Publikation-2), untersucht.

In der ersten Studie (**Venkatasubramani et al., 2023**) wollten wir die evolutionäre Bedeutung von Chameau (chm), einer MYST-Domäne-Acetyltransferase, verstehen. Wir haben zuvor gezeigt, dass das Fehlen dieses Proteins sowohl die körperliche Aktivität als auch die Langlebigkeit in *Drosophila melanogaster* verbessert (**Peleg, Feller, Forne, et al., 2016**). Wir verwendeten das UAS-GAL4-System, um das Gen sowohl ubiquitär als auch gewebespezifisch auszuschalten. Wir konnten massive physiologische Veränderungen mit ubiquitärem, neuronalem und fettkörperspezifischem Bezug durch den Verlust von chm beobachten. Die überaus wichtige Bedeutung der MYST-Domäne in chm, welche die Acetyltransferase-Aktivität steuert, wurde durch eine heterozygote Deletionsmutante der MYST-Genomregion untersucht und hierbei wurden ähnliche Phenotypen ersichtlich. Anschließend haben wir eine Multi-omics-Strategie angewandt, um die Auswirkungen der Mutation (oder des Knockdowns) auf das Transkriptom, das Histon-Acetylom, das Proteom und das Nicht-Histon-Acetylom zu bewerten. Die Beobachtungen aus diesen Ansätzen deuteten auf einen fehlregulierten Stoffwechsel hin. Wir testeten daher die Fähigkeit von Organismen, denen das Protein fehlt, auf Stoffwechselstress zu reagieren. Hierbei verabreichten wir eine spezielle Fütterungstechniken (wet-starvation) und wie erwartet zeigten diese Fliegen eine geringere Widerstandsfähigkeit gegen Hunger. Interessanterweise stellte sich heraus, dass die reduzierte Widerstandsfähigkeit gegen Hunger unabhängig von chms Rolle in der Entwicklung ablaufen kann. Dies wurde mit Hilfe des Gene-Switch-Systems,

welches die Ausschaltung von chm auf adultspezifische Weise induziert, bestätigt. Darüber hinaus haben wir die wahrgenommene Rolle von chm im Stoffwechsel und bei der Stressreaktion bestätigt, indem wir das Protein ubiquitär und in verschiedenen Geweben mit dem UAS-GAL4-System überexprimiert haben. All diese Experimente ergaben eine verbesserte Überlebensrate bei metabolischem Stress und insbesondere bei Überexpression in Geweben wie Neuronen und Fettgewebe. Darüber hinaus gibt es Belege dafür, dass diese mutmaßliche evolutionäre Rolle von chm bei der Stressreaktion nur auf bestimmte Temperaturbereiche beschränkt ist, die für den Organismus als nicht ideal angesehen werden könnten. Dies wird auch durch Belege für Sequenzvariation und ausgleichende Selektion in verschiedenen klimatischen Populationen unterstützt (**Croze et al., 2017; Levine & Begun, 2008**). Dies wird in einer Folgestudie untersucht, die sich in Vorbereitung befindet.

Die zweite Studie wurde in Zusammenarbeit mit dem Peleg-Labor durchgeführt. In dieser Studie (**Müller-Eigner et al., 2022**) charakterisierten wir ein einzigartiges und neuartiges Mausmodell mit dem Namen Titan, das seit über 180 Generationen aufgrund seines hohen Körpergewichts selektiert wurde. Nachdem wir ihren genetischen, biochemischen und physiologischen Status untersucht hatten, stellten wir fest, dass diese Mäuse Ähnlichkeiten mit Stoffwechselstörungen beim Menschen aufweisen. Darüber hinaus haben wir die Veränderungen der Histon-PTMs und anschließend des Proteoms und Transkriptoms gemessen, die alle auf eine Fehlregulierung des Stoffwechsels hinweisen. All dies wurde bei alten und jungen Kontroll- und Titanmäusen untersucht, da letztere ebenfalls einen starken Rückgang der Lebenserwartung aufwiesen. Interessanterweise zeigte die genetische Analyse Variationen in Acetyltransferase-Genen massive Änderungen in der Acetylierung von H4 zwischen den Mausmodellen. Schließlich haben wir durch eine diätetische Intervention herausgefunden, dass diese Mäuse eine verbesserte Überlebensrate aufweisen und die damit verbundenen molekularen Veränderungen beheben können. Das resultierende Mausmodell Titan aus dieser Studie bietet nun einen neuen Anhaltspunkt zur Untersuchung von Stoffwechselkrankheiten wie beispielsweise Fettleibigkeit, welches nicht durch Inzucht generiert wurde und auch genetische Variabilität abbildet. Die Studie lieferte auch einen interessanten Ansatz für die Umkehrung der möglichen Auswirkungen genetischer Unterschiede durch Änderungen des Lebensstils.

Zusammenfassend vermitteln die genannten Studien ein besseres Verständnis zum Thema Dysregulation des Stoffwechsels in Organismen, sein diese durch Mutationen oder phänotypische Selektionen hervorgerufen. Ein besonderes Augenmerk kann auf die evolutionäre Bedeutung, aber auch durch die bisher unbekannte Rolle von chm im Stoffwechsel des Modelorganismus *Drosophila melanogaster* gerichtet werden. Weiterhin durch die Charakterisierung eines längerfristig phänotypischen selektierten Mausmodells (Titan) erlangt man neue Einblicke in die Stoffwechselvorgänge, welche dem Menschen sehr ähnlich sind.

5. Discussion

During this doctoral study, we tried to address the molecular and physiological effects metabolic defects in a monogenic and polygenic model using *Drosophila melanogaster* and *Mus musculus* respectively. In the first study using fruit flies, we achieve this using knockdown and mutation approaches, while in the second, we used a phenotypically selected mouse model. Both these studies provided important novel insights, but also raised many questions that could be addressed in future studies.

Chameau (chm) is important for physiology (Publication-1)

chm as an acetyltransferase was shown to reduce both health- and lifespan under ad libitum conditions (**Peleg, Feller, Forne, et al., 2016**). In spites of its disadvantages, the enzyme still exists in the organism. In order to understand its evolutionary importance, we first decided to test if flies with knockdown of chm using UAS-GAL4 system were viable, as the null mutants of chm show pupal lethality. Interestingly, knocking down chm did not result in its lethality, however, these flies showed developmental delay (only upon ubiquitous RNAi) and severe effects on physiology suggesting that chm has a role in development and possibly physiology as well. Moreover, we found that heterozygous acetyltransferase mutant and fatbody-specific RNAi of chm resulted in reduced size, albeit the effects were milder than ubiquitous RNAi. However, neither of these showed any effect on development. This could suggest that either, i) a single functional allele of chm is sufficient to rescue any developmental delay, or ii) the acetyltransferase activity is not important for development, and/or iii) chm in fatbody does not play a role in development.

chm modulates transcriptome, proteome and acetylome of metabolic components (Publication-1)

To further understand the molecular effects of chm loss, we undertook a multi-omic approach to assess the transcriptome, proteome and acetylome. Our omic approaches showed an overarching differential regulation of factors involved in metabolism. This observation was in line with the above mentioned effect of chm's loss on organism's physiology and the importance of chm in fatbody, the tissue which is the equivalent of liver and adipose tissue in mammals.

chm is both detrimental and beneficial depending on food availability (Publication-1)

Based on our omic analysis, we wanted to assess if chm has any effect on metabolic stress. As predicted, ubiquitous- and fatbody-specific RNAi and heterozygous mutants of *chm* showed a strong susceptibility to starvation. Although unpublished, chm mutants were unable to adequately respond to other stress environments such as high temperature, high humidity and high sugar. This suggested that loss of chm results in an overall impaired response to stress and more specifically metabolic stress environments. Furthermore, this is probably the first evidence of an enzyme having opposing effects on longevity and stress response.

Developmental role of chm is indispensable for starvation resilience (Publication-1)

Altered development can lead to indirect effect on starvation resilience (**Brown et al., 2019**). Therefore, we used GeneSwitch approach enable us to knockdown of *chm* in restricted time frame in adult flies. We induced the knockdown of *chm* two and four days prior to starvation. Two days' induction of RNAi was not sufficient to cause any visible effect on starvation resilience. However, upon four-days treatment, a distinct difference in starvation susceptibility was observed suggesting that development does not play a role in the impaired response to nutrient limitation in chm mutants.

In spite of these findings, there are still some unanswered questions, that can be speculated with the available preliminary and unpublished data, but would require further assessment for mechanistic insights.

i) Does the regulation of aging and stress resilience by chm differ in mechanism?

Based on some of our unpublished data, we propose that aging and stress resilience might have independent mechanisms. For instance, both H4K12ac and total H4 mono-acetylation showed no changes upon starvation in the wild-type flies. In addition, histone acetylation profiles from starvation selected and unselected population also do not show any differences. Moreover, preliminary analysis of histone acetylation profiles from *chm* overexpressed flies using *arm-GAL4* does not show any increase in histone acetylation when compared to flies

without *chm* overexpression. However, bulk H4K12 acetylation is an important contributing factor to aging as evidenced by increased H4K12ac in old flies (**Peleg, Feller, Forne, et al., 2016**). Furthermore, *chm* overexpressed flies do not show reduced lifespan but showed improved survival upon starvation as compared to non-overexpressed flies. Nevertheless, it is possible that rather than bulk, histone acetylation at specific regions might be important for starvation resilience. These results suggest that the role of chm in starvation susceptibility and longevity might occur via distinct mechanisms.

ii) Since chm is an acetyltransferase, does it auto-acetylate or is there a feedback mechanism? Further, how does chm acetylate metabolic proteins being primarily localized in the nucleus?

Our acetylome data indicated that chm is acetylated at two lysine residues. However, we were unable to ascertain if chm can acetylate itself as we could not identify chm in the proteome to measure and correlate with the acetylome. Since chm is a nuclear protein, we also have unpublished data where we performed nuclear acetylome of *chm* RNAi and GST- RNAi using extracts from insect cells. Interestingly, we identified numerous acetyltransferases whose acetylation were affected by loss of chm. This led us to hypothesize two possibilities, i) chm could be acetylated by other proteins and could function in a feedback loop, ii) the change in acetylation of metabolic proteins could be an indirect effect via post-translational regulation of other acetyltransferases by chm.

iii) Genetic studies predict that chm is under genetic selection. Would that be important for the observed phenotype?

Genetic studies have shown that *chm* gene region possess many SNPs depending on the population from which fruit flies were collected, i.e. temperate vs tropical (**Levine & Begun, 2008**). These studies further suggest that chm might be important for adaptation to novel environments. Interestingly, our unpublished data indicates that the reduced starvation resilience in *chm* mutants is only required at certain temperature ranges. In fact, we can hypothesize that nutrient limitation and temperature shows an additive effect. This temperature dependence was observed in both male and female flies albeit at different

temperature ranges. Since chm can get acetylated and temperature changes can alter the organism's metabolic profile, we speculate that the acetylation status of chm could be influenced by temperature, which could further modify the enzyme's molecular characteristics.

iv) What are the interacting partners of chm?

One of the questions which we were unable to address is to characterize chm's interactome. Yeast-2-hybrid and co-immunoprecipitation/western blot studies have indicated some interacting partners but we did not observe any significant differential expression of these (at least the ones identified) upon the loss of chm or its acetyltransferase activity. Probably, techniques such as IP-MS could provide a more direct and stronger evidence of chm's interacting partners.

Phenotypic selection resulted in an altered genome, epigenome, transcriptome and proteome that correlates with physiological differences (Publication-2)

First, we assessed the epigenomic profiles between unselected and Titan mice. We measured histone acetylation at H4 and found that Titan mice displayed reduced monoacetylation, more specifically H4K16ac, which is the most abundant histone modification in mammals. This change in acetylation could impact the transcription of genes and consequently, we observed reduced expression of genes involved in metabolic processes from our transcriptomic data. Furthermore, these transcriptomic changes also correlated with proteome.

Interestingly, we also observed changes in the genome as a result of naturally occurring mutations upon selection. Several of the genomic regions that showed variability were related to the observed phenotype of the organism, such as the size and body weight. For instance, *Hcrt* gene, which is important for feeding and metabolic homeostasis, showed regions of distinct genetic differentiation. These mice are larger, heavier and contain higher fat. Moreover, Titan mice also showed high cholesterol and triglycerides, have higher BMI and thick skin. These molecular phenotypes also correlate with genes that belong to regions of distinct genetic differentiation. Furthermore, GO terms such as lipid metabolism and biosynthesis were upregulated at both transcriptome and proteome level in Titan mice as compared

to unselected mice. In summary, these results suggested that Titan mice have misregulated metabolism that affects the physiological characteristics.

Altered metabolism affects lifespan of Titan mice (Publication-2)

As we observed metabolic changes associated with metabolic syndrome, we hypothesized that this affects longevity of the mouse model. Indeed, these mice had a shorter lifespan than the corresponding control unselected mouse, i.e. 325 days vs 645 days median lifespan respectively. This along with increased cholesterol, lipid, insulin, leptin and FGF21, indicated a hallmark of obesity and metabolic syndrome in Titan mice.

Dietary intervention partially rescues the phenotype and molecular characteristics (Publication-2)

Titan mice show dysregulated metabolism, an obese phenotype and consequently faster aging. This led us to wonder whether dietary changes can at least partially rescue the observed effects at both physiological and molecular level. To test this, we replaced the diet of the mouse with energy reduced food starting at 12 weeks of age. Physiological and molecular differences such as body weight, levels of cholesterol, HDL, Glucose, leptin and fat were reduced upon the dietary changes in Titan mice fed with energy reduced food. At the epigenome level, change in diet increased poly-acetylation of H4 to the levels of unselected mice. This increase in acetylation could have contributed to the rescue in metabolic gene expression in Titan mice fed with energy reduced food. Further, intervention with energy reduced food increased the lifespan independent of the genotypes when compared to those fed with standard diet.

Titan as a model for metabolic syndrome (Publication-2)

One of the novel observations from this study is the advantage of using Titan as a model for metabolic syndrome. As the mouse model is outbred and possesses polygenic characteristics, they may better resemble the conditions of metabolic syndrome in humans more than other existing models. For examples, mouse models for obesity are generally based on disruption of leptin signaling, i.e. via mutational approach. Metabolic syndrome in humans are complex and interconnected and in some cases do not affect leptin signaling. Further, approaches such as dietary intervention can be evaluated in shorter timespan than other mouse models. This

makes Titan mouse a unique and novel tool to study the molecular and physiological effects of obesity and metabolic syndromes.

As with most studies, these results gave rise to more questions that we have tried addressing but still needs more focus in future studies.

i) Is Titan's characteristics dependent on maternal contribution?

Maternal effects can contribute to changes in the phenotype of the offsprings, specifically because the entirety of mitochondrial DNA is maternal. We therefore wanted to assess if the observed differences in physiology of Titan mice is a consequence of mother's genetics or environmental exposure. Based on our analysis, the observed characteristics in Titan mice occurs independent of mother's genotype. Interestingly, the male F1 hybrids from the cross between Titan and unselected mice show intermediate phenotypes. Their body weight and fat content, histone acetylation levels and lifespan were between that of Titan and unselected mice. Future studies could focus on F2 hybrids to identify specific gene(s) that results in the observed differences between Titan and unselected mice.

ii) Do female show similar changes as male mice?

In this study, almost all the experiments were performed with male mice. However, we did observe that female mouse were large and showed higher levels of leptin and an obese phenotype. Preliminary analysis suggests that there could be sexual dimorphism in Titan mouse model. This is plausible as in humans, metabolism and hormonal characteristics of men and women are markedly different, with obesity being more prevalent in women than in men further validating the novelty of this model for metabolic syndrome that better resembles humans.

iii) What are the metabolic changes associated with phenotypic selection?

One of the less explored aspects of this study is the comprehensive analysis of control and Titan metabolome. Although measurement of fat content, cholesterol, HDL, glucose, glycerol was carried out, other metabolites such as SAM, acetyl-CoA and TCA cycle intermediates were not quantified. This

would be especially important as the levels of genes such as *Accs2* and *Pdhx* were reduced in Titan mice with corresponding changes in overall acetylation. On a similar note, genes involved in folate cycle and one-carbon metabolism were also reduced in Titan mice. Such comprehensive assessment would aid in understanding the interplay of genetic variation and metabolic changes, which could be another facet for future research.

iv) How does the genetic variation influence the characteristics of epigenetic factors?

Another prospect of this study could involve the effect of genetic variation, such as SNPs, and its contribution to the observed epigenetic difference between selected and Titan mice. This could be similar to the rovers and sitters SNP variation of *foraging* (*for*) gene in *Drosophila*, which affects the binding of a methyltransferase, G9a that leads to differential enrichment of H3K9me2 between the two genotypes. One of the interesting candidate in this regard would be *Kat2a*. The expression of this gene does not change between the genotypes but this gene region is a part of distinct genetic differentiation. Moreover, *Kat2a* is involved in the acetylation of H4K5 that is increased in Titan mice. Identification of other candidate regions and finding its association to molecular or phenotypic characteristics would aid in understanding the intercommunication between genetic variation and epigenome changes.

Overall in this doctoral study, the aim was to approach metabolic defects using different genetic models and diverse organisms. We used a mutant version *Drosophila melanogaster* that does not properly express the protein *chameau* and higher model organism, *Mus musculus* that was phenotypically selected for body size. The first study used a monogenic perspective to functionally characterize the protein chameau and its role in metabolic stress response, while the second study used a polygenic approach to characterize the organism as a tool to study metabolic syndrome. In spite of the above mentioned differences, the were some fascinating similarities as well (**Fig. 9**).

A monogenic model such as mutant *chm* flies shows a smaller physiology and increased lifespan, while the Titan mice show increased size and reduced lifespan.

Interestingly, these observed changes irrespective of the model organism could be, at least partially, the effect of histone acetylation. Intriguingly, lack of H4K12ac, the most abundant H4 modification in adult male *Drosophila*, improves longevity, while lack of H4K16ac, which in mouse is the most abundant H4 modification, results in a reduced lifespan. This consequently changes the expression of genes and proteins (and its PTMs). Genes/proteins involved in lipid and carbohydrate metabolism were misregulated in both monogenic and polygenic models. Further, in both cases we observe characteristics of metabolic defects with detrimental effects of obesity and metabolic syndrome in Titan mice and the inability to cope with metabolic stress such as starvation, high temperature, high humidity and high sugar in *chm* mutants.

In summary, one could hypothesize that alteration in protein acetylation (or epigenome) either as a result of genetic variation (polygenic) or mutation (mutation) causes an altered metabolism and physiology of the organisms. This speculation seems to be conserved in smaller organisms like *Escherichia coli* and higher organisms such as humans as well (**Castaño - Cerezo et al., 2014; Iyer et al., 2012; Pessoa Rodrigues et al., 2021**).

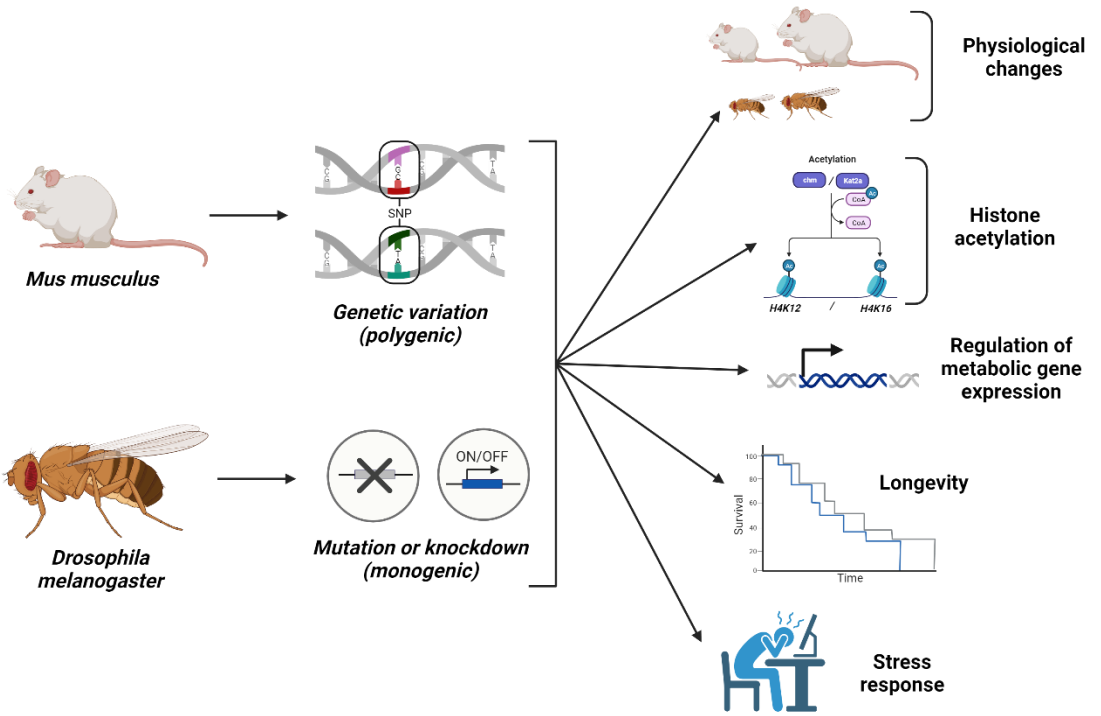

Figure 9: *Summary detailing the key findings that shows commonality between the two studies independent of the model system or the initial approach*

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The contributions of my dear friends and colleagues in the lab/department are immeasurable. From the beginning of my PhD journey, I felt welcomed and included. I was fortunate to have met Magdalini and Andrea Lukacs, both exceptional colleagues and even better friends, on whom I could rely for both professional and personal assistance. They knowingly and sometimes unknowingly provided calming and empathetic support through various challenges. The suggestions, opinions, and advice I received from them were invaluable throughout my PhD, even if I didn't

always heed them. While it was difficult to see them leave the lab, I was excited and happy for their progress. I was also grateful to get to know Vera, Beyza, Marco, and Rupam, who consistently stood by me during the remainder of my PhD. Supervising Beyza and collaborating with Marco and Rupam not only strengthened our professional ties but also allowed us to forge personal connections. Vera, with her guidance and support, helped me navigate challenges arising from my limited knowledge of the German language and offered me communicative advice with others in times of distress. Meeting and getting to know Swathi, Andrea Terrasi, Vio, and Chon was also a highlight, as we shared some of the best times during lunch and coffee breaks, alongside Beyza, Marco, Rupam, and Hua. Our conversations ranged from scientific discussions to sharing lab-related issues and humorous anecdotes, creating enduring memories. Attempts at playful pranks were met with sportive and easy-going responses. Numerous gatherings within the department and beyond, including dinners, home visits, Christmas markets, Oktoberfest, pubs/bars, hikes, and occasional club outings, added to the richness of my experience in Munich.

In the Becker lab, an unexpected scientific collaboration with Anna and Zisis has proven to be an incredibly enriching experience. Learning extensively from both individuals, what began as a scientific partnership has seamlessly transitioned into a friendship. I am grateful for the opportunity to contribute to their research work and appreciate the freedom they've given me to express suggestions and opinions without any constraints. The openness and mutual respect within our collaboration have created a conducive environment for creativity and innovation. Getting to know Anna and Zisis on a personal level has further strengthened our working dynamic, making the overall experience both enjoyable and productive.

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I am profoundly grateful to each of these individuals as my time in Munich would not have been as memorable without them and I will definitely miss every one of them. I also wish to extend my gratitude to everyone in the Imhof lab and Becker department

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I have been incredibly fortunate to encounter numerous individuals beyond the confines of the lab and department, sharing a plethora of memories with them. One of the paramount sources of mental solace for me was engaging in volleyball almost every evening. This activity provided a respite from the world of science and work, serving as a means to release pent-up stress. Moreover, it motivated me to leave the lab early, preventing me from spending unnecessarily extended hours despite not being particularly productive. My heartfelt gratitude goes out to all the members of my volleyball club at TSV Grosshadern and Freundeskreis von Eichenkreuzsport München e.V. Their warm welcome and inclusivity were particularly commendable, considering my limited proficiency in German.

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I'd also like to express my gratitude to friends and family back in India, many of whom played a crucial role in supporting me throughout my doctoral journey. When I relocated to Munich for my PhD, I was thrilled to learn that Neeru would also be pursuing her Masters in Germany, specifically in Tuebingen. I've always seen her as a little kid, and taking care of her has brought me a sense of calm and happiness. Whether I visit her in Tuebingen or she comes to Munich, the time spent together is always fulfilling. Neeru has been a pillar of support, especially during my challenging personal moments, consistently checking in on my well-being. Siva and Loki have been incredible companions during my visits to India, making it a point to connect almost every day, engaging in sports, engaging in lively conversations, and having a great time. Additionally, I had the pleasure of hosting two friends, Preeth and Pranav, in Munich along with their infant, Kichu. Knowing Preeth since our undergraduate days, I was genuinely excited about her visit. Our meet-ups, frequent orders from "too good to go," and explorations in and around Munich made those weeks truly delightful. Despite the demands of my thesis and manuscript work, that period stands out as one of the most serene months. The sight of Kichu has a magical effect, washing away my stress and tension, leaving me in a state of peace. I eagerly anticipate their return to Munich in winter for more quality time and adventures.

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As I transition to this section of acknowledgment, emotions may surface, as I talk about my pillars of support all through my life and career, my **ஆத்தா** ;) Neelu and my girlfriend Sindu (Chanus). They have been the most important people in my life and have never thought for a second to anything that might benefit and help me in any possible way.

Neelu has been the most understanding person, who had to unlearn a lot of her learnings and change a lot of her preferences and liking so that I can do my studies peacefully. She has been so protective and caring that I cannot ask her for anything more. She curbed her interests and what she wanted for me, understood what I wanted to convey, listened to me whenever I needed to talk and much more. There have been situations where sometimes I couldn't talk with her properly or talk at all but she never showed her sadness to make sure I do not get hurt. I get angry at her and we fight a lot for any and every reason but she always forgave me even if the fault was completely mine. I usually never tell her because she is quite sensitive but I miss sleeping on her lap, making fun of her, hanging out and cooking with her, and just spending time. In short, I just miss her whenever I am in Germany. Because of my situation in PhD, I have found it difficult to visit India often but she has understood the situation and never showed the disappointment. She always puts me first and then her, something which I wish she changes. I can obviously keep on writing but all I want to say is that I just love her the most and there's absolutely no question about it ever and I will do anything for her anytime.

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need to explain her anything that I am going through and all she needs is to just see me, and she can understand what I need instantly. She actually knows me more than I do and in some cases, more than Neelu. The past 6 years of our lives have really been a big rollercoaster, from long distance relationship of seeing each other for once in 6 months, to a short distance seeing each other once in 2 months, to a break and now where we are together the best versions of ourselves. She has been my emotional support for a long time now and I cannot think of anyone else who can make me smile with her quirky and goofy nature, which I have always loved the most. She took complete care of me during and after my ACL surgery, both emotionally and physically and she was extremely adjustable despite a lot of things not going her way. I cannot think of a single instance where she has never been there for me, especially emotionally. Whatever I am now, either personally or professionally, a lot of credit has to go to her as I am probably the best version of myself. She always says that I had to thank her specifically for this reason that I was able to finish my PhD because we weren't living together and if not, I would take years to finish because of my priority towards her than other things. But I guess I would probably even have better memories and a lot of happiness and fun, when she is around me. She is curious, always excited, and wants to try something new in every single aspect and most of these stick to me when she is around. She takes care of all my silly and tiny fears. I get teased by her a lot for all my routine, orderliness, schedule bound, fear of change and rule book attitude but that's probably what keeps me grounded and I do enjoy that most times ;). I can keep writing pages and pages but I really cannot thank her enough. Gummi loves you and will always love you more than you can ever think of. I honestly cannot wait more to see the kind of adventure that I am going to experience with you beside me always.

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