

**THE METABOLIC REGULATION OF THE TOR PATHWAY  
AND THE CIRCADIAN RHYTHMICITY OF  
*NEUROSPORA CRASSA***

MARYAM FAYYAZI

A THESIS SUBMITTED TO THE FACULTY OF GRADUATE  
STUDIES IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR  
THE DEGREE OF MASTER OF SCIENCE

GRADUATE PROGRAM IN BIOLOGY

YORK UNIVERSITY

TORONTO, ONTARIO

APRIL 2024

© MARYAM FAYYAZI, 2024

## Abstract

Eukaryotic circadian rhythms are often attributed to transcription-translation feedback loops (TTFLs). However, rhythms can still persist in the absence of functioning TTFLs. Our laboratory has discovered that mutations in the components of the TOR pathway compromise the non-TTFL rhythms, implying the potential involvement of this pathway in regulating the rhythmicity of *Neurospora crassa*.

The TOR pathway is a nutrient-sensing pathway that its modulation in *Neurospora* remains enigmatic. Here, I investigated the metabolic regulation of the TOR pathway to delineate its relationship with circadian rhythmicity. Using the phosphorylation state of S6 ribosomal protein, I found that different metabolites, including amino acids and lipids affect the TOR pathway only after a prolonged glucose-starvation period, suggesting that glucose alone is sufficient to activate the TOR pathway. Interestingly, my findings indicate that factors perturbing the TOR activity also influence the period of conidiation, thereby establishing a connection between the TOR pathway and circadian rhythmicity.

## Dedication

To the kind and humble *Neurospora* community

## Acknowledgement

I would like to express my immense gratitude and appreciation to my supervisor, Dr. Patricia Lakin-Thomas for her dedicated guidance, support, and encouragement during this period. I have benefited both professionally and personally from her mentorship, and I am very grateful to have worked with such an outstanding scientist.

My sincere thanks to my advisor, Dr. Emanuel Rosonina for his valuable comments and questions. His advice and expertise have helped me tremendously.



## Table of Contents

|                                                                                             |           |
|---------------------------------------------------------------------------------------------|-----------|
| Abstract.....                                                                               | ii        |
| Dedication.....                                                                             | iii       |
| Acknowledgement.....                                                                        | iv        |
| Table of Contents.....                                                                      | v         |
| List of Tables.....                                                                         | ix        |
| List of Figures.....                                                                        | x         |
| List of abbreviations.....                                                                  | xiv       |
| <b>CHAPTER 01: Introduction.....</b>                                                        | <b>1</b>  |
| <b>1.1: Circadian Rhythms.....</b>                                                          | <b>1</b>  |
| 1.1.1: Fundamental Properties of Circadian Rhythms .....                                    | 1         |
| 1.1.2: Historical Background.....                                                           | 3         |
| 1.1.3: The Molecular Basis of Circadian Rhythms.....                                        | 4         |
| 1.1.3.1: Transcription-Translation Feedback Loops (TTFLs).....                              | 5         |
| 1.1.4: The Molecular Mechanism of Circadian Rhythms in Cyanobacteria.....                   | 5         |
| 1.1.5: The Molecular Mechanism of Circadian Rhythms in Plants.....                          | 8         |
| 1.1.6: The Molecular Mechanism of Circadian Rhythms in <i>Drosophila melanogaster</i> ..... | 9         |
| 1.1.7: The Molecular Mechanism of Circadian Rhythms in Mammals.....                         | 11        |
| 1.1.7.1: Synchronization of Peripheral Clocks by the SCN.....                               | 13        |
| 1.1.8: The Circadian Clock mechanisms in <i>Neurospora crassa</i> .....                     | 14        |
| 1.1.8.1: <i>Neurospora crassa</i> life cycle.....                                           | 14        |
| 1.1.8.2: The Molecular Mechanism of Circadian Rhythms in <i>Neurospora crassa</i> .....     | 16        |
| 1.1.8.3: The FRQ-less oscillator in <i>Neurospora crassa</i> .....                          | 18        |
| 1.1.9: Circadian Rhythmicity and Metabolic Processes.....                                   | 21        |
| 1.1.9.1: Rhythmicity of Metabolic Processes.....                                            | 21        |
| 1.1.9.2: Crosstalk between Circadian Clock and Metabolic Networks.....                      | 23        |
| 1.1.9.3: Circadian Clock Disruption, Disease, and Adaptive Treatments.....                  | 28        |
| <b>1.2: TOR Signaling Pathway.....</b>                                                      | <b>32</b> |
| 1.2.1: TOR (Target of Rapamycin) pathway.....                                               | 32        |
| 1.2.2: Conserved Domains and Structure of TOR kinases.....                                  | 32        |

|                                                                                                      |           |
|------------------------------------------------------------------------------------------------------|-----------|
| 1.2.3: TOR Complexes and the Maintenance of Cellular Homeostasis in Response to Various Inputs ..... | 34        |
| 1.2.3.1: Composition of TOR complexes.....                                                           | 34        |
| 1.2.3.2: mTORC1 Activation.....                                                                      | 37        |
| 1.2.4: Protein Synthesis downstream of mTORC1.....                                                   | 38        |
| 1.2.5: Regulation of mTORC1: Integration of Metabolic and Autophagic Signals.....                    | 40        |
| 1.2.5.1: Amino acid sensing in the lysosome.....                                                     | 40        |
| 1.2.5.2: Amino acid sensing in the cytoplasm.....                                                    | 42        |
| 1.2.5.3: Amino acid sensing and regulation of autophagy.....                                         | 44        |
| 1.2.5.4: Glucose-Dependent Regulation of mTORC1.....                                                 | 45        |
| 1.2.5.5: mTORC1 Regulation in Response Stress Signals.....                                           | 47        |
| 1.2.5.6: mTORC1 regulation by lipids.....                                                            | 47        |
| 1.2.6: mTORC2 Signaling Network.....                                                                 | 49        |
| 1.2.7: Overview of Regulation of TOR Pathway in Plants.....                                          | 50        |
| 1.2.8: TOR Pathway in <i>Neurospora crassa</i> .....                                                 | 51        |
| 1.3: Hypothesis and Objectives.....                                                                  | 58        |
| <b>CHAPTER 02: Materials and Methods.....</b>                                                        | <b>59</b> |
| 2.1: <i>Neurospora crassa</i> strains.....                                                           | 59        |
| 2.2: <i>Neurospora crassa</i> culture systems.....                                                   | 61        |
| 2.2.1: Amino acid culture systems.....                                                               | 61        |
| 2.2.2: Lipid culture systems.....                                                                    | 63        |
| 2.3. Protein Methods.....                                                                            | 65        |
| 2.3.1. Protein Extraction.....                                                                       | 65        |
| 2.3.2. Bio-Rad DC Protein Assay.....                                                                 | 66        |

|                                                                                                                   |           |
|-------------------------------------------------------------------------------------------------------------------|-----------|
| 2.3.3: Protein Electrophoresis by SDS-PAGE.....                                                                   | 66        |
| 2.3.4: Western Blotting.....                                                                                      | 67        |
| 2.3.5: Immunodetection.....                                                                                       | 67        |
| 2.3.6: Staining of PVDF membrane with Colloidal Gold.....                                                         | 68        |
| 2.4: Race Tube Assay.....                                                                                         | 69        |
| 2.5: Statistical Analysis.....                                                                                    | 70        |
| <b>CHAPTER 03: Results and Discussion.....</b>                                                                    | <b>71</b> |
| 3.1: Amino acid Regulation of TOR Pathway Activity in <i>Neurospora crassa</i> .....                              | 71        |
| 3.1.1: Introduction and Objectives.....                                                                           | 71        |
| 3.1.2: Results & Discussion.....                                                                                  | 73        |
| 3.1.2.1: Characterization and optimization of the culture conditions.....                                         | 73        |
| 3.1.2.2.: Effects of Various Amino acids on the TOR Pathway Activity of <i>Neurospora Crassa</i> .....            | 73        |
| 3.1.2.3: Amino acid Regulation of <i>Neurospora crassa</i> ’s TOR Pathway via GTR2-VTA Signaling.....             | 80        |
| 3.1.2.4: Effects of Amino Acid Starvation on <i>Neurospora crassa</i> ’s TOR Pathway.....                         | 86        |
| 3.2: Lipid Regulation of TOR Pathway Activity in <i>Neurospora crassa</i> .....                                   | 92        |
| 3.2.1: Introduction and Objectives.....                                                                           | 92        |
| 3.2.3: Results & Discussion.....                                                                                  | 92        |
| 3.2.3.1: Effects of Choline Starvation on the TOR Pathway Activity of <i>Neurospora crassa</i> .....              | 93        |
| 3.2.3.2: Effects of Increased Diacylglycerol levels on the TOR Pathway Activity of <i>Neurospora crassa</i> ..... | 100       |
| 3.3: TOR Pathway Perturbation and Its Effects on the Circadian Rhythmicity of <i>Neurospora crassa</i> .....      | 107       |

|                                                                                                                             |            |
|-----------------------------------------------------------------------------------------------------------------------------|------------|
| 3.3.1: Introduction and objectives.....                                                                                     | 107        |
| 3.3.2: Results & Discussion.....                                                                                            | 110        |
| 3.3.2.1: Effects of TOR Pathway Inhibitors on Rhythmicity in <i>chol-1</i> Strain of <i>Neurospora crassa</i> .....         | 110        |
| 3.3.2.2: Effects of Elevated Diacylglycerol levels on Rhythmicity of <i>chol-1</i> Strain of <i>Neurospora crassa</i> ..... | 117        |
| 3.3.2.3: Role of the VTA Protein in Regulating the Rhythmicity of <i>Neurospora crassa</i> .....                            | 121        |
| <b>CHAPTER 04: Discussion.....</b>                                                                                          | <b>125</b> |
| 4.1: Summary & Conclusion.....                                                                                              | 125        |
| 4.2: Future Directions.....                                                                                                 | 129        |
| <b>References.....</b>                                                                                                      | <b>131</b> |
| <b>Appendices.....</b>                                                                                                      | <b>149</b> |
| Appendix A- Sample Immunoblot.....                                                                                          | 149        |
| Appendix B- Statistical analysis.....                                                                                       | 150        |
| Appendix C- Growth media, buffers and solutions.....                                                                        | 164        |

## List of Tables

|                                                                                                                                                                                                               |     |
|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| Table 1.1: Circadian clock-driven rhythmic TOR pathway activity in various tissues and organisms measured by activation status of downstream effectors of the TOR signaling network.....                      | 56  |
| Table 2.1: The genotype of <i>Neurospora crassa</i> strains used in the thesis.....                                                                                                                           | 59  |
| Table 2.2: The full description of <i>Neurospora crassa</i> strains used in the thesis.....                                                                                                                   | 60  |
| Table 3.1: Growth rate and period of <i>chol-1</i> strain growing on different choline concentrations.....                                                                                                    | 113 |
| Table 3.2: Growth rate and period of <i>chol-1</i> strain growing on different choline concentrations supplemented with 500 nM concentration of Torin II.....                                                 | 115 |
| Table 3.3: Growth rate and period of <i>chol-1</i> strain growing on choline-deficient cultures supplemented with 250 $\mu$ M DOG and 500 nM Torin II.....                                                    | 119 |
| Table 3.4: Growth rate and period of <i>chol-1</i> and $\Delta$ <i>vta</i> strain growing on different choline concentrations (0,10, and 100 $\mu$ M) supplemented with 500 nM concentration of Torin II..... | 123 |

## List of Figures

|                                                                                                                                                                                                                                      |           |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------|
| Figure 1.1: The schematic diagram representing the circadian rhythms in the form of sinusoidal waves when tested under entrainment conditions (LD: light-dark cycles) and free running conditions (LL: constant light) .....         | 3         |
| Figure 1.2: The schematic diagram representing the core post-translational oscillator (PTO) in the cyanobacterium <i>Synechococcus elongatus</i> circadian clock.....                                                                | 7         |
| Figure 1.3: The schematic diagram representing the multiple interlocked transcriptional-translational feedback loops (TTFLs) involved in the circadian network of <i>Arabidopsis thaliana</i> .....                                  | 9         |
| Figure 1.4: The schematic diagram representing the molecular components of transcriptional-translational feedback loops (TTFLs) in the circadian clockwork of <i>Drosophila melanogaster</i> .....                                   | 11        |
| Figure 1.5: The schematic diagram representing the molecular circuitry involved in the regulation of the mammalian circadian clock.....                                                                                              | 13        |
| Figure 1.6: The schematic diagram representing the molecular component of FWO (FRQ-WCC-Oscillator) as the core transcriptional-translational feedback loop (TTFL) involved in the circadian system of <i>Neurospora crassa</i> ..... | 18        |
| Figure 1.7: The schematic diagram representing the hierarchy of mammalian circadian clock and its molecular circuitry.....                                                                                                           | 27        |
| Figure 1.8: The schematic diagram representing the conserved domain structures of TOR kinase in mammalian cells.....                                                                                                                 | 34        |
| Figure 1.9: The schematic diagram representing the major regulators and downstream effectors of mTORC1 and mTORC2.....                                                                                                               | 36        |
| Figure 1.10: Hypothetical diagram representing the TOR (Target of Rapamycin) pathway in <i>Neurospora crassa</i> .....                                                                                                               | 54        |
| Figure 1.11: Comparative analysis of the S6 ribosomal protein in various eukaryotic organisms.....                                                                                                                                   | 55        |
| Figure 2.1: Sample western blot analysis of <i>Neurospora crassa</i> S6 ribosomal protein separated on 12.5% acrylamide gel.....                                                                                                     | 69        |
| <b>Figure 3.1: Effects of various amino acids on the TOR pathway activity of <i>Neurospora crassa</i>.....</b>                                                                                                                       | <b>77</b> |

3.1.1: When glucose is available as a carbon source, amino acids (AAs) have no effect on the phosphorylation levels of S6.....77

3.1.2: When maltose is available as a carbon source, AAs have no effect on the phosphorylation levels of S6.....77

3.1.3: In low glucose-cultures with short-term glucose-starvation period, AAs have no effect on the phosphorylation levels of S6.....77

3.1.4: In low glucose-cultures with long-term glucose starvation period, AAs have significant effects on the phosphorylation levels of S6.....77

**Figure 3.2: Amino acid regulation of *Neurospora crassa*'s TOR pathway via GTR2-VTA signaling.....83**

3.2.1: When glucose is available as a carbon source, the deletion mutations of *vta* and *gtr2* have no effect on the phosphorylation levels of S6 in response to amino acids (AAs).....83

3.2.2: In low-glucose cultures with a short-term (20 and 60 minutes) glucose-starvation period, the deletion mutations of *vta* and *gtr2* have no overall effect on the phosphorylation levels of S6 in response to AAs.....83

3.2.3: In low-glucose cultures with long-term (24 hours) glucose-starvation, the deletion mutation of the *vta* significantly diminishes S6 phosphorylation in response to metabolites while the, *gtr2* deletion exhibits less pronounced effects.....83

**Figure 3.3: Effects of amino acid starvation on *Neurospora crassa*'s TOR pathway.....90**

3.3.1: When glucose is available, overall, no dramatic changes in P-S6 levels between the auxotrophic mutants and the wild-type strains growing on both the inhibitory and rescue AA concentrations were found.....90

3.3.2: When auxotrophic mutants, especially arginine auxotrophs, are starved for their required amino acid for an extended period of time, S6 phosphorylation levels are likely to increase....90

3.3.3: When the auxotrophic mutants are starved for their required amino acid, the linear growth rate and biomass accumulation are significantly lower than those of the wild-type.....90

Figure 3.4: Effects of choline starvation on the TOR pathway activity of *chol-1* mutant strain of *Neurospora crassa*.....96

**Figure 3.5: Effects of choline starvation on the TOR pathway activity of *chol-1* mutant strain of *Neurospora crassa*.....97**

3.5.1: When glucose is available, the choline starvation of *chol-1* strain does not overall affect S6 phosphorylation levels.....97

|                                                                                                                                                                                                                                                 |            |
|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------|
| 3.5.2: In low-glucose cultures with short-term (20 and 60 minutes) glucose-starvation periods, choline-depletion of <i>chol-1</i> does not affect S6 phosphorylation levels.....                                                                | 97         |
| 3.5.3: In low-glucose cultures with long-term (24h) glucose-starvation period, choline-depletion of <i>chol-1</i> significantly increases S6 phosphorylation levels.....                                                                        | 97         |
| 3.5.4: Choline starvation of <i>chol-1</i> significantly increases S6 phosphorylation levels in cultures grown on solid agar culture long-term.....                                                                                             | 97         |
| <b>Figure 3.6: Effects of increased diacylglycerol levels on the TOR pathway activity of <i>chol-1</i> mutant strain of <i>Neurospora crassa</i>.....</b>                                                                                       | <b>104</b> |
| 3.6.1: When DOG is added directly to the growth media, it does not overall alter S6 phosphorylation levels in both choline sufficient or deprived cultures.....                                                                                 | 104        |
| 3.6.2: When cultures are grown on high glucose media, DOG supplementation does not change S6 phosphorylation levels.....                                                                                                                        | 104        |
| 3.6.3: In low-glucose media, DOG treatment significantly lowers S6 phosphorylation levels only in choline-deprived cultures.....                                                                                                                | 104        |
| Figure 3.7: The schematic diagram representing the race-tube assay used to study the circadian rhythms of conidiation of <i>Neurospora crassa</i> under laboratory conditions.....                                                              | 109        |
| <b>Figure 3.8: Effects of TOR pathway inhibitors on the period of conidiation rhythms and the linear growth rate of <i>chol-1</i> strain of <i>Neurospora crassa</i>.....</b>                                                                   | <b>113</b> |
| 3.8.A: A 200 nM concentration of Torin II significantly lengthens the period of conidiation rhythms at low choline concentrations, but it is ineffective at high choline concentrations...                                                      | 113        |
| 3.8.B: At all of the tested choline concentrations, both Torin I and Torin II significantly diminish the linear growth rate.....                                                                                                                | 113        |
| <b>Figure 3.9: Effects of 500 nM concentration of Torin II concentrations on the period of conidiation rhythms and the linear growth rate of <i>chol-1</i> strain of <i>Neurospora crassa</i> growing different choline concentrations.....</b> | <b>115</b> |
| 3.9.A: A 500 nM concentration of Torin II significantly lengthens the period of conidiation rhythm at both low and high choline concentrations.....                                                                                             | 115        |
| 3.9.B: A 500 nM concentration of Torin II significantly diminishes the growth rate at all tested choline concentrations.....                                                                                                                    | 115        |
| <b>Figure 3.10: Effects of Elevated Diacylglycerol levels on Rhythmicity of <i>chol-1</i> strain of <i>Neurospora crassa</i>.....</b>                                                                                                           | <b>119</b> |



3.10.A: In choline-deficient cultures, DOG significantly lengthens the period of conidiation, but in combination with Torin II, the observed effects do not exhibit an additive increase.....119

3.10.B: In choline-deficient cultures, DOG significantly diminishes the growth rate, but in combination with Torin II, the inhibitory effects are not additive.....119

**Figure 3.11: role of the VTA protein in regulating Rhythmicity and growth rate of *Neurospora crassa*.....123**

3.11.A. At 100  $\mu$ M choline, the  $\Delta vta$  strain is initially rhythmic but the rhythm dampens out after a few cycles; knocking out VTA blocks Torin II-induced effects on the period of conidiation.....123

3.11.B. Torin II treatment diminishes the linear growth rate in the  $\Delta vta$  strain but not to the same extent as in the *chol-1* strain.....123

## List of Abbreviations

AA - Amino Acids

AMPK - Adenosine Monophosphate-Activated Protein Kinase

APRRs - Arabidopsis Pseudoresponse Regulators

Arf1 - ADP-Ribosylation Factor 1

AGC Family (PKA/PKG/PKC Family)

ATM - Ataxia Telangiectasia Mutated

BCAA - Branched-Chain Amino Acids

BMAL1 - Brain Muscle ARNT-Like 1

BSA - Bovine Serum Albumin

bHLH - Basic-Helix-Loop-Helix

CCA1 - Circadian Clock Associated 1

CK1 Family (Casein Kinase 1 Family)

CLK - CLOCK

COP1 - Constitutive Photomorphogenic 1

Cry - Cytochrome

Cwo - Clockwork Orange

CYC - CYCLE

CikA - Circadian Input Kinase A

DAG - Diacylglycerol

DD - Constant Darkness

DDT - Dithiothreitol

DEPTOR - DEP Domain Containing mTOR Interacting Protein

ddH<sub>2</sub>O - Double Distilled Water

EDTA - Ethylenediaminetetraacetic Acid

ELF - Early Flowering

eIF4E - Eukaryotic Translation Initiation Factor 4E

FAT - FRAP-ATM-TRAPP

FATC - FAT C-Terminus

FLCN - Tumor Suppressor Folliculin

FLO - FRQ-Less Oscillator

FRB - FKBP-Rapamycin Binding

FRQ - FREQUENCY

FWO - FRQ-WCC Oscillator

GAP - GTPase-Activating Proteins

GAP Activity Towards the Rags (GATOR)

GEF - Guanine Nucleotide Exchange Factors

GLUT - Glucose Transporter

GRE - Glucocorticoid-Response Elements

HIF1 - Hypoxia-Inducible Factor 1

HEAT-Huntington-EF3-PP2A-TOR1

IC - Inhibitory Concentration

IPMS1 - Isopropylmalate Synthase 1

LARS/LeuRS - Leucyl-tRNA Synthetase

LD - Light-Dark Cycle

LHY - Late Elongated Hypocotyl

LPAAT - Lysophosphatidic Acid Acyltransferase

L-VGCC - L-Type Voltage-Gated Calcium Channels

LUX - LUX Arrhythmia

Me<sub>2</sub>SO, DMSO - Dimethyl Sulfoxide

mLST8 - Mammalian Lethal with SEC13 Protein 8

mTOR - Mammalian Target of Rapamycin

NAMPT - Nicotinamide Phosphoribosyltransferase

PA - Phosphatidic Acid

PAS - Period-Arnt-Single-minded

Pdp1 - Par Domain Protein 1

PEB - Protein Extraction Buffer

PKD1 - Phosphoinositide-Dependent Kinase-1

Per - Period

PH - Pleckstrin Homology

PI3K - Phosphoinositide 3-Kinase

PI3P - Phosphatidylinositol 3-Phosphate

PKA - Protein Kinase A

PKC- $\alpha$  - Protein Kinase C-Alpha

PLD1 - Phospholipase D1

Prd - Period

Protor1/2 - Protein Observed with Rictor1 and 2

PtdCho - Phosphatidylcholine

PtdEtn - Phosphatidylethanolamine

PTO - Post-Translational Oscillator

RAGs - Ras Family of Small GTPases

RAPTOR - Regulatory-Associated Protein of mTOR

RC - Rescue Concentration

REV - Reveille

Rheb - RAS Homologue Enriched in Brain

RICTOR - Rapamycin-Insensitive Companion of mTOR

ROP2 - RHO OF PLANTS 2

ROS - Reactive Oxygen Species

RpaA - Regulator Phycobilisome Association A

SasA - Synechococcus Adaptive Sensor A

SCN - Suprachiasmatic Nucleus

SESNs - Sestrins

SGK1 - Serum and Glucocorticoid-Induced Protein Kinase 1

SGG - Shaggy

SIN1 - SAPK-Interacting 1

SLC38A9 - Sodium-Coupled Amino Acid Transporter 9

S6K - S6 Kinase

S6K1 - p70 S6 Kinase 1

SC - Synthetic Complete Medium

TCA Cycle - Citric Acid Cycle

TG - Triglyceride

TIM - Timing of CAB Expression 1

tim - Timeless

TOR - Target of Rapamycin

TORC1 - TOR Complex 1

TORC2 - TOR Complex 2

TSC 1/2 - Tuberous Sclerosis Complex 1/2

TTFL - Transcription-Translation Feedback Loops

ULK1 - Unc-51 Like Kinase 1

VTA - Vacuolar TOR-Associated Protein

vri - Vrille

WCC - White-Collar Complex

WT - Wild-Type

## CHAPTER 01: Introduction

Life on earth has evolved in a rhythmic environment where the daily solar cycle prompts cyclical changes in environmental light intensity, levels of UV radiation, temperature, and food availability (Lakin-Thomas & Brody, 2004; Pittendrigh, 1993). In parallel with the geological history of Earth and under selection pressures imposed by daily environmental factors, organisms have developed internally generated biological rhythms to optimize their physiology and behaviour, in advance of the varied demands of the astronomical day/night cycles (Bhadra et al., 2018; Dunlap, 1999). These innate timing mechanisms have been a feature of life since the first cyanobacteria (a photosynthetic eubacteria) and their oxygen production shaped the future of this planet 3.8 billion years ago, and have been retained and refined by evolutionary mechanisms (Cohen & Golden, 2015; Foster & Kreitzman, 2009; Lowrey & Takahashi, 2004). The emergent property of time-telling is a universal phenomenon that is embedded within the genome of almost all living organisms from extremophilic archaeans and unicellular marine algae to higher plants, invertebrates, and vertebrates (Harmer et al., 2001; Montenegro-Montero et al., 2015; Paranjpe & Sharma, 2005; Sartor et al., 2019). The mechanisms underpinning such biological rhythms involve periodic oscillations in gene expression, protein modifications, and ultimately behaviour, therefore, they are not only a property of individual cells but a fundamental aspect of the organism's biology as whole (Foster & Kreitzman, 2017a). They regulate sleep-wake cycles, migration patterns (birds and butterflies), hibernation, pollen and seed germination, metabolism, blood pressure, hormone production, neurotransmitter release and cell-cycle progression (Buhr & Takahashi, 2013). The ubiquity of these rhythms underscores their importance and consequently their disruption is linked to many disease and pathological conditions including mental and psychiatric disorders, metabolic abnormalities and cancer (Vetter, 2020).

### 1.1 Circadian Rhythms

#### 1.1.1 Fundamental Properties of Circadian Rhythms

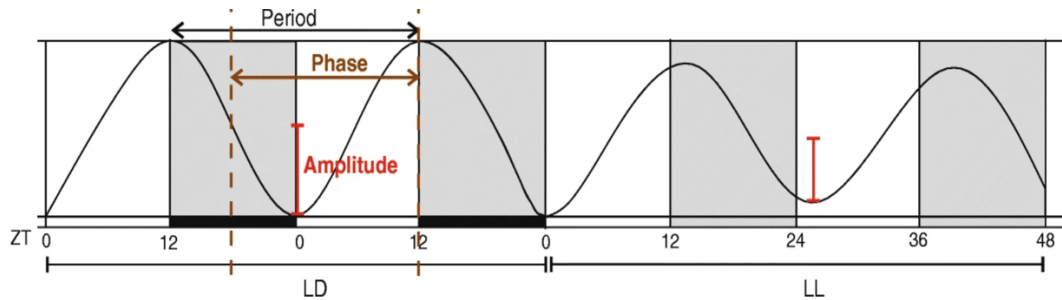
Circadian rhythms (from the Latin *circa*: approximately and *dies*: a day) are a subset of endogenously generated biological rhythms that oscillate with a 24-hour periodicity (Dunlap, 1999; Lakin-Thomas, 2000, 2006a) (Figure 1.1). These 24-hour phenomena are regulated by an

intrinsic oscillator, the core circadian clock. The imposition of an intrinsic circadian clock not only allows organisms to anticipate the periodic changes in the external environment but it also provides an internal temporal compartmentalization for all the essential biological processes, ensuring that they occur in the appropriate sequence (Bell-Pedersen et al., 2005; Lowrey & Takahashi, 2004). The internal timing mechanisms, to be biologically useful, must be entrained or synchronized with the rhythmic changes in the external environment. This entrainment is driven by recurring environmental Zeitgebers (German for 'time givers'), predominantly the light/dark cycle, humidity, temperature, rainfall, food, and predation (Harmer et al., 2001). However, these endogenous rhythms can still persist in the absence of external cues indefinitely (Dunlap, 1999; Lakin-Thomas, 2000). In constant conditions without exposure to entraining zeitgebers, rhythmic oscillations freerun. The period of the free-running oscillators no longer matches the 24 hours solar day but runs slightly faster or slower, and depending on the species, it can range from 22 to 25 hours, showing unequivocally that the rhythm is generated within an organism (Foster & Kreitzman, 2009).

Another fundamental feature of circadian rhythms that buffers them against inappropriate signals and allows them to persist under stable ambient conditions is temperature compensation, implying that the period of the oscillation does not change dramatically in response to the fluctuations in the ambient temperature (Diernfellner et al., 2007; Pittendrigh, 1954). Most biological reactions are temperature-dependent, meaning that the rate of these reactions doubles with every 10°C increase in the temperature (temperature coefficient  $Q_{10}=2$ ) until it reaches the maximum temperature at which the reaction stops. By contrast, circadian rhythms exhibit a  $Q_{10}=1$ , which prevents the clock from running faster at higher temperatures and slower at cooler conditions, emphasizing the stability and fidelity of endogenous rhythms (Barrett & Takahashi, 1995).

Overall, a circa 24-hour period length, the ability to use environmental cues as time signals and persistence in the absence of external factors, together with a compensation mechanism to maintain a steady period length under different conditions, are the cardinal features that make a biological rhythm a circadian rhythm and differentiates it from other types of rhythms with shorter period (ultradian) and longer period (infradian) (Baker et al., 2012; Wollnik, 1989).





**Figure 1.1. The schematic diagram representing the circadian rhythms in the form of sinusoidal waves when tested under entrainment conditions (LD: light-dark cycles) and free running conditions (LL: constant light).** Biological oscillations can be described by mathematical terms such as period, phase, and amplitude. Period is the time required for completion of one cycle, which is the distance from one peak to another. Phase is a relative event, referring to any time point on a rhythmic cycle relative to an external time point. Amplitude represents the degree of change in a physiological parameter over the course of the cycle and is typically measured as the height of the wave. ZT: Zeitgeber Time. (Adapted from Saini et al., 2019).

### 1.1.2 Historical Background

Most of the characteristics attributed to the circadian rhythms originated from a simple botanical observation made by the French astronomer *Jean-Jacques d'Ortus de Mairan* in the 18<sup>th</sup> century. *de Mairan* was aware that *Mimosa pudica* exhibits heliotropic behavior, meaning that different plant parts such as flowers and leaves follow a diurnal pattern in their folding and unfolding. They rise during the day in response to direction of the sun, while drooping at dusk (Golombek & Rosenstein, 2010; McClung, 2006). To test whether these movements were merely a response to light, he kept the plant in darkness and observed that even in the absence of external cues, the leaves retained their rhythmic behavior with a regular period for many days (Bass, 2012; Johansson & Köster, 2019). In 1832, the Swiss botanist *Alphonse de Candolle* repeated and expanded these observations and found that in constant darkness the rhythms of *M. pudica* leaf movement advanced to an earlier start each day, suggesting the free-running period of such rhythms. He further demonstrated that these rhythms could be inverted by reversing the light/dark alterations. The insightful observations made by early circadian pioneers have laid the groundwork for the modern-day chronobiology (study of biological rhythms) and provided a departure from the prevailing old age mentality that biological rhythms are simply passive reactions to external environmental fluctuations (Sollars & Pickard, 2015).

In 1954, Colin Pittendrigh showed that eclosion (hatching) in *Drosophila pseudoobscura* follows a circadian pattern (Golombek & Rosenstein, 2010). This observation suggested that there could be a genetic basis for rhythmic behaviours and paved the way for the subsequent molecular studies. The genetic dissection of circadian systems began with the identification of mutants with an altered circadian phenotype, particularly the period of free-running rhythms. The *per* (*period*) gene in the eclosion of *Drosophila*, *tau* mutant in the locomotor activity of golden hamster, and *frq* gene mutants in the sporulation rhythm of the bread mold *Neurospora* have become instrumental in molecular characterization of circadian machinery (Harmer et al., 2001; McClung et al., 1989; Ralph & Menaker, 1988).

### 1.1.3 The Molecular Basis of Circadian Rhythms

Circadian systems are conceptually composed of three components: entrainment input pathways that receive and relay environmental signals such as light, temperature, and pH to the central oscillator; a core pacemaker that functions as an endogenous, self-sustaining rhythm generator, which is capable of interpreting the external cues and sustaining rhythmicity in the absence of zeitgebers and output pathways that transiently regulate a diverse set of cellular processes through the rhythmic expression of clock-controlled genes (ccgs) in response to the fluctuations of the central oscillator (Bell-Pedersen et al., 2005; Harmer et al., 2001; Lakin-Thomas & Brody, 2004). This characterization might be an oversimplification, as emerging evidence suggests the dual functionality of clock components. For example, photoreceptors as part of photic input, can also modulate the output from the central oscillator while the output components can feedback on the central clock itself, affecting the pace of circadian machinery (Harmer et al., 2001; Saini et al., 2019). Therefore, instead of a linear model in which the information flows unidirectionally (input→central oscillator→output), a more comprehensive model would contain a complex network of multiple interconnected feedback loops at which there is no central oscillator per se, but the activity of several coupled loops contributes to the generation of a robust circadian rhythm (McClung et al., 2002; Saini et al., 2019). Nevertheless, the central oscillator paradigm, namely the transcription-translation feedback loops (TTFLs) of canonical clock genes, is a simplified model that can serve as a reference point for understanding the overall organization of feedback loops that comprise a part of all known circadian machinery (Dunlap, 1999; Lakin-Thomas, 2006b).

#### 1.1.3.1 Transcription-Translation Feedback Loops (TTFLs)

The molecular basis of circadian oscillatory systems is often attributed to transcription-translation feedback loops (TTFLs) (Hurley et al., 2016; Lakin-Thomas, 2019). According to this scheme, the positive elements, typically a heterodimeric complex activate the transcription of clock genes that yield clock proteins. These proteins then act as negative components by directly or indirectly suppressing the action of positive elements, therefore repressing their own transcription (Hurley et al., 2016; Lakin-Thomas, 2006b). The elements in the negative arm of the loop are then subjected to post-translational modifications such as step-wise phosphorylation followed by ubiquitin-proteasome mediated degradation. Upon the decay of these elements, the repression of the transcriptional activity of positive elements is lifted and the cycle is renewed (King & Takahashi, 2000; Lakin-Thomas et al., 2011). As a result, the delayed negative feedback loops at the transcription level of clock genes maintain the 24 hours periodicity of circadian oscillators and ultimately give rise to the rhythmicity of the organism as a whole. The organization of central pacemakers into TTFLs is a conserved blueprint observed in a broad phylogenetic spectrum of organisms from unicellular species to the organisms in the eukaryotic lineage and although the molecular players are different, the generic core mechanism involving positive and negative regulation of gene expression is relatively similar (Bell-Pedersen et al., 2005; Dunlap, 1999; Lakin-Thomas, 2006b). Therefore, investigating the molecular basis of the circadian timekeeping system in one organism can generate a more comprehensive understanding about the oscillator function in others (Bell-Pedersen et al., 2005; Young & Kay, 2001). Each one of the following circadian systems is a great achievement in understanding the interaction between genes and their protein product which can ultimately give rise to rhythmic physiology and behaviour.

#### 1.1.4 The Molecular Mechanism of Circadian Rhythms in Cyanobacteria

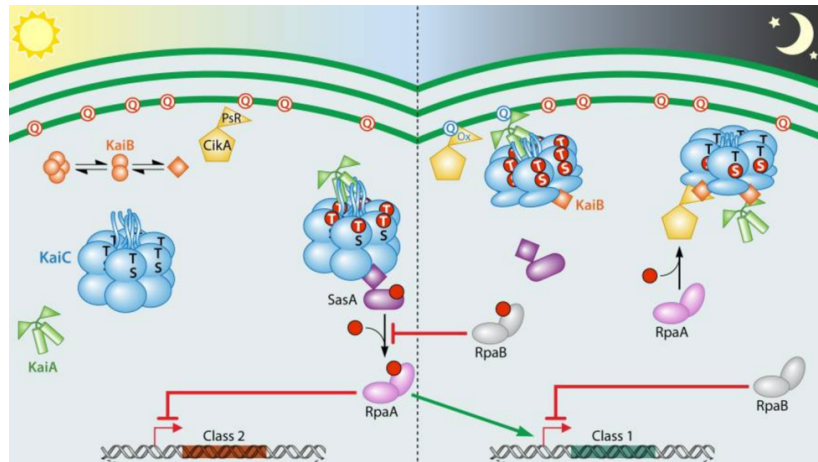
It was originally believed that circadian rhythms are solely a property of eukaryotic cells, as the regulated movement of proteins between the nucleus and cytoplasm is considered an essential aspect of the metazoan circadian clock (Cohen & Golden, 2015; Young & Kay, 2001). Based on this assumption, in rapidly dividing prokaryotes such as bacteria whose cell cycle is significantly

shorter than 24 hours and lack sufficient cellular complexity (no nuclei or membrane-enclosed organelles), the cellular functions would not be coupled to a circadian oscillator. However recent studies suggest that while only 10-20 percent of eukaryotic genes are rhythmically expressed, in cyanobacteria almost all essential genes are under circadian regulation (Foster & Kreitzman, 2017c; Vijayan et al., 2009). Indeed, the cyanobacterial clock synchronizes the global gene expression, cell division, metabolite partitioning and other essential metabolic processes such as photosynthesis, nitrogen fixation, and amino acid uptake from the environment (Taton et al., 2020).

In the cyanobacterium *Synechococcus elongatus*, the central oscillator is composed of three essential proteins KaiA, KaiB, and KaiC (kai is the Japanese word for “rotation”) and a set of mutually antagonistic input-output proteins, such as a redox-sensitive input sensor CikA (circadian input kinase) for the entrainment with the environment and two output components SasA (*Synechococcus* adaptive sensor A), and RpaA (regulator phycobilisome association A) for transcriptional regulation (Dong et al., 2010; Swan et al., 2018). The cyclical interactions between the three *kai* proteins and interplay between the phosphorylation-dephosphorylation state of KaiC constitutes a post-translational oscillator (PTO) which is believed to be the core of the molecular clockwork of cyanobacteria (Cohen & Golden, 2015). KaiC is a ring-shaped homohexamer that possesses multiple enzymatic activities. It has two homologous domains (CI and CII) that belong to AAA+ ATPase (adenosine triphosphate) family, additionally its CII domain also serves as an autokinase and a phosphotransferase (Tseng et al., 2017). During the subjective day, environmental information such as light is transduced through the phosphorylation of CikA; the activated CikA in turn stimulates kaiA through interaction with its receiver-like domain (Johnson et al., 2011). Next, KaiA associates with the C-terminal loop of unphosphorylated KaiC, promoting its autokinase activity. The sequential phosphorylation of two residues, serine 431 (S431) and threonine 432 (T432) stacks CI and CII domains of KaiC and results in conformational changes (Bell-Pedersen et al., 2005; Cohen & Golden, 2015). In the phosphorylated state, KaiC hexamer forms a complex with SasA. The histidine kinase SasA relay temporal information of the oscillator status to the rest of the cell to activate rhythmic gene expression through the phosphorylation of RpaA (Taton et al., 2020). The response regulator RpaA serves as a master transcription factor that can induce the expression of more than 134

genes, including *kaiBC* in *S. elongatus* (Markson et al., 2013). During the subjective night, KaiB binds to the N-terminus of hyperphosphorylated KaiC and initiates its dephosphorylation phase (Golden, 2020; Johnson et al., 2011). The Kai protein complex then dissociates into individual components and the cycle ends (Chang et al., 2015).

Perhaps one of the most remarkable features of the Kai-based PTO is its unique ability to be reconstituted in vitro when the three purified Kai proteins combined with ATP (Johnson et al., 2011). In vivo, the cyanobacterial PTO functions within a TTFL framework (Lakin-Thomas, 2006b; Qin et al., 2010; Swan et al., 2018). In this autoregulatory feedback loop, KaiA acts as positive element by enhancing the expression of *kaiBC* operon while KaiC serves as a negative element, inhibiting its own transcription (Young & Kay, 2001) (Figure 1.2).



**Figure 1.2. The schematic diagram representing the core post-translational oscillator (PTO) in the cyanobacterium *Synechococcus elongatus* circadian clock.** During the illuminated portion of the cycle (left panel), KaiA binds to the CII ring of KaiC stimulating its autophosphorylation activity. The interaction of SasA with hyperphosphorylated KaiC, triggers its phosphorylation, ultimately leading to activation of its binding partner RpaA. Based on the temporal information provided by CikA, RpaA-P represses the expression of dawn-peaking genes (class 2) while promoting the transcription of dusk-peaking genes (class 1). At night (right panel), the binding of KaiB to the Kai complex results in dissociation of KaiA and initiates the dephosphorylation of KaiC. (Adapted from Cohen & Golden, 2015).

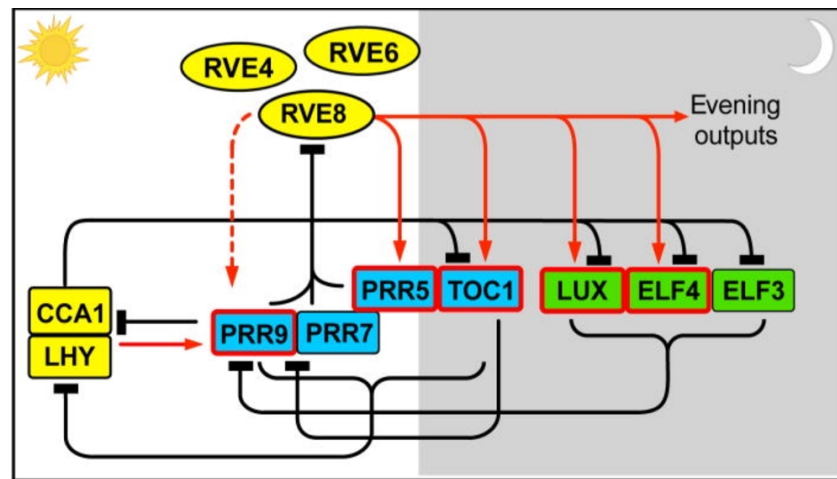
### 1.1.5 The Molecular Mechanism of Circadian Rhythms in Plants

As sessile organisms, plants cannot escape unfavorable environmental conditions. Additionally, due to their photosensitivity, they must possess a precise spatiotemporal programming to couple their metabolic activity and energy allocation to the diurnal changes in the environment. (Cervela-Cardona et al., 2021; Greenham & McClung, 2015; McClung et al., 2002). Plant circadian clocks work in resonance with the environment to confer optimal growth, propagation, and protection against biotic and abiotic stressors (Harmer, 2009). Indeed, the circadian system in plants regulates the majority of their essential physiological processes including photosynthesis, chlorophyll accumulation, CO<sub>2</sub> fixation, hormone signaling, floral transition, fragrance emission, and pathogen response (Creux & Harmer, 2019; Oakenfull & Davis, 2017). Plant's regulatory networks have evolved multiple layers of elaborate transcriptional and post-translational modifications to maintain circadian homeostasis and to generate waveforms of rhythmic gene expression and activity (Seo & Mas, 2014).

*Arabidopsis thaliana* is a small flowering plant whose circadian oscillator is based on multiple interconnected feedback loops which revolve around the negative regulation of differentially phased genes (Hsu & Harmer, 2014; Wang & Ma, 2013). In the core loop, two morning-expressed MYB- transcription factors, CIRCADIAN CLOCK ASSOCIATED1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY) directly bind to the promoter region of evening-expressed *TOC1* (*TIMING OF CAB EXPRESSION 1*) gene and repress its expression (Wang & Ma, 2013). At dusk, as the expression of the clock repressors CCA1 and LHY declines, and as TOC1 accumulates following its nuclear localization, TOC1 further downregulates the expression of morning-phased components (CCA1 and LHY) (Hsu & Harmer, 2014). At night, an Evening Complex (EC) including a MYB-like transcription factor LUX (LUX ARRHYTHMO), and two nuclear proteins ELF3 (EARLY FLOWERING 3), and ELF4 downregulate the transcription of *TOC1*, hence resuming *LHY* and *CCA1* expression for the following morning (Adams et al., 2015; Sanchez & Kay, 2016).

Additional feedback loops are mediated by the family of *Arabidopsis* Pseudoresponse Regulators (APRRs) which comprises of a series of sequentially expressed transcription repressors including PRR9, PRR7, and PRR5 (McClung, 2019). Collectively, PRR repressors together with their

homolog TOC1 restrict the expression of *CCA1* and *LHY* from late morning until the following dawn (Adams et al., 2015). REVEILLE (RVE 4, 6, and 8) is a set of rhythmically expressed transcriptional activators that upregulate the expression of afternoon and evening-phased genes including *PRR5*, *TOC1*, *ELF4*, *LUX*, and *PRR9*. RVE8 then becomes reciprocally regulated by PRR repressors (Adams et al., 2015; Sanchez & Kay, 2016) (Figure 1.3).



**Figure 1.3. The schematic diagram representing the multiple interlocked transcriptional-translational feedback loops (TTFLs) involved in the circadian network of *Arabidopsis thaliana*.** The clock components are depicted from left to right according to the time of their peak expression. The white and gray backgrounds represent the subjective day and night respectively. Solid red arrows indicate activation and the black perpendicular bars indicate repression. At dawn, the morning-phased elements *CCA1* and *LHY* repress the expression of the majority of clock genes including *TOC1*, *PRR* genes and the EC complex (*LUX*, *ELF3* and *ELF4*). The sequential expression of *PRR9*, *PRR7*, *PRR5*, and *TOC1* downregulates the transcription of *CCA1* and *LHY*. In the afternoon, RVE complex (*RVE4*, *RVE6*, and *RVE8*) promotes the transcription of *PRR9*, *PRR5*, *TOC1*, and the EC complex. In the evening, *TOC1* further represses the expression of the morning elements such as *CCA1* and *LHY*. (Adapted from Hsu & Harmer, 2014).

#### 1.1.6 The Molecular Mechanism of Circadian Rhythms in *Drosophila melanogaster*

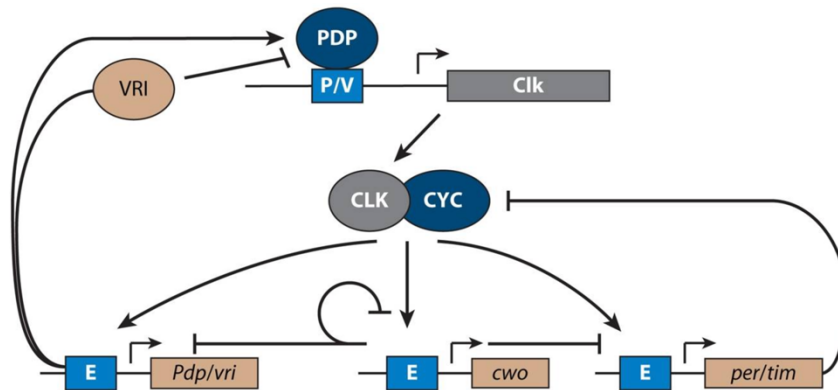
In the fruit fly *Drosophila melanogaster*, circadian clock gates various aspects of the organism's physiology and behaviour including eclosion, courtship, locomotor activity, learning, memory and temperature-preference rhythms (Kaneko et al., 2012; Tataroglu & Emery, 2014). It also contributes to the rhythmic coordination of chemosensory processes such as olfaction and gustation as well as regenerative responses in the intestine and other peripheral organs (Chatterjee et al., 2010; Karpowicz et al., 2013; Yildirim et al., 2022). Historically, *Drosophila* has been a pioneer in circadian research due to its advanced molecular and genetic toolkit. The identification of the first clock gene, *period* (*per*) and the isolation of mutant strains with altered

period length led to the foundational discovery of transcriptional-translational feedback loops (TTFLs) as the central mechanism driving the circadian rhythmicity which was honored with the 2017 Nobel Prize in Physiology and Medicine (Huang, 2018).

The dynamic force of the *Drosophila* molecular clockwork is the transcriptional drive provided by two bHLH-PAS (bHLH: basic-helix-loop-helix, PAS: Period-Arnt-Single-minded) transcription factors named CLOCK (CLK) and its heterodimeric partner CYCLE (CYC) which rhythmically bind to E-box (5' CACGTG 3') of *period* (*period*) and *Timeless* (*tim*) genes and promote their transcription (Dunlap, 1999; Hardin, 2011; Lakin-Thomas, 2006b; Tataroglu & Emery, 2014; Yildirim et al., 2022). As PER and TIM proteins accumulate, monomeric PER becomes subjected to extensive phosphorylation by DOUBLETIME (DBT) kinase which results in its proteasome-mediated degradation. The binding of TIM to PER-DBT complex, effectively protects PER from DBT-induced destabilization and leads to the cytoplasmic retention of PER-DBT-TIM complex (Allada & Chung, 2009; Huang, 2018). At night, the phosphorylation of PER by Casein kinase 2 (CK2) and TIM by Shaggy (SGG) promotes the nuclear localization of the PER-DBT-TIM complex. Once in the nucleus, the complex binds to CLK-CYC dimers and represses their transcriptional activity (Menet et al., 2010). *Drosophila* utilizes a cell-autonomous blue-light photoreceptor CYTOCHROME (CRY) to synchronize the endogenous clock with the light-dark cycles of the environment (Allada & Chung, 2009). Photon absorption at dawn triggers a conformational change in CRY structure and facilitates its binding to TIM; this leads to the dissociation of the PER-DBT-TIM complex, allowing CLK-CYC induced transcription to occur once again (Foster & Kreitzman, 2017b; Tataroglu & Emery, 2014).

In a second autoregulatory path, CLK-CYC dimer drives sequential expression of three additional genes: *vrille* (*vri*), *par domain protein 1* (*pdp1*) and *clockwork orange* (*cwo*) (Patke et al., 2020; Yildirim et al., 2022). As VRI levels rise, it binds to VRI/PDP1-box (V/P-box) in the *clk* promoter and inhibits its transcription (Hardin, 2011). Meanwhile PDP1 antagonistically counteracts VRI by providing positive input to fuel *clk* expression (Dubowy & Sehgal, 2017). CWO reinforces the inhibitory effects of PER-DBT-TIM, by downregulating the CLK-CYC transcription in a negative autoregulatory fashion (Patke et al., 2020) (Figure 1.4).





**Figure 1.4. The schematic diagram representing the molecular components of transcriptional-translational feedback loops (TTFLs) in the circadian clockwork of *Drosophila melanogaster*.** In the core TTFL, the heterodimeric transcription complex CLK-CYC binds to E-box element (E) in the promoter region of *per* and *tim* and drives their transcription. PER and TIM undergo various modifications, which lead to their nuclear localization where they repress CLK-CYC activity. The CLOCK-CYCLE complex also promotes the expression of *pdp*, *vri*, and *cwo*. In a secondary loop, PDP1 activates the transcription of *clk*, while VRI inhibits it. CWO feeds back to negatively regulate the activity of the positive elements CLK-CYC (Adapted from Allada & Chung, 2009).

### 1.1.7 The Molecular Mechanism of Circadian Rhythms in Mammals

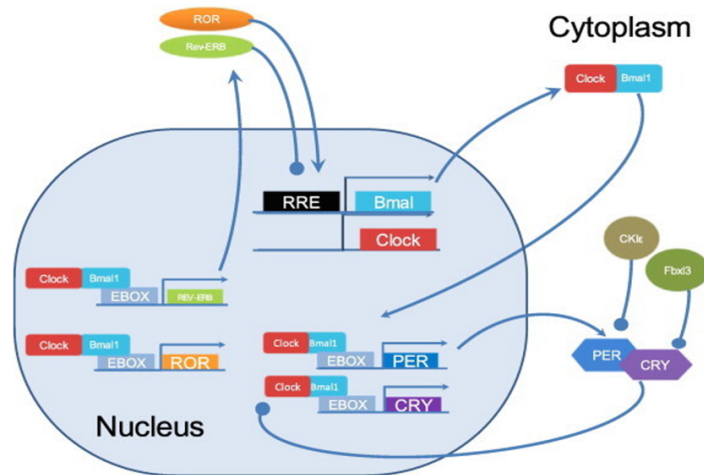
In mammalian systems, the circadian clock manifests as intrinsic and entrainable 24-hour oscillations at molecular, physiological, and behavioral levels (M. D. Li et al., 2021). These cyclical rhythms are evident in a vast array of cellular processes including thermoregulation, humoral and immune responses, gene expression, metabolism, synaptic plasticity, cell division, and DNA damage responses (Allada & Bass, 2021; Evans & Gorman, 2016; Eckel-Mahan & Storm, 2009; Ko & Takahashi, 2006). Mammalian clocks are at the pinnacle of cellular and molecular complexity; they represent a hierarchical network in which an ensemble of 20,000 coupled neurons comprises the principal circadian pacemaker in the suprachiasmatic nucleus (SCN) of the hypothalamus (Finger & Kramer, 2021; Partch et al., 2014; Buhr & Takahashi, 2013). The SCN orchestrates independent peripheral oscillators to generate a coherent rhythm at the organismal level which is tuned with environmental fluctuations (Li et al., 2021; Reppert & Weaver, 2002). In humans, the SCN is the only clock component that can be entrained by light via the activity of photoreceptors found exclusively in the eyes (Buhr & Takahashi, 2013).

Exposure to light activates the melanopsin-containing intrinsically photosensitive retinal ganglion cells (ipRGCs) in the retina, the photic input then travels as excitatory signals through

the retinohypothalamic tract (RHT) and impinges on the SCN neurons (Paul et al., 2020; Reinke & Asher, 2019). Once the pacemaker is light-entrained, it synchronizes the peripheral clocks via various endocrine and neuronal pathways, and thus restores circadian homeostasis throughout the body (Honma, 2018; Li et al., 2021; Reinke & Asher, 2019).

At the cellular level, the molecular clock machinery comprises a set of transcriptional-translational feedback loops (TTFLs) that work together to generate robust 24-hour rhythms in the expression of canonical clock genes (Walker et al., 2020). In the primary feedback loop, two bHLH-PAS transcription factors, CLOCK (CLK, Circadian Locomotor Output Cycles Kaput) and BMAL1 (Brain Muscle Arnt-Like1) heterodimerize and initiate the rhythmic transcription of the *period* (*per1-3*) and *cytochrome* (*cry1-2*) genes (Takahashi, 2017). As PER and CRY increasingly accumulate in the cytoplasm, the PER homodimers become phosphorylated by a group of auxiliary enzymes belonging to the CK1 family (Casein kinase1). The phosphorylation of PER followed by its ubiquitin-mediated degradation is a key regulatory node in setting the period of the clock (Eckel-Mahan & Storm, 2009; Partch et al., 2014). At night, the binding of CRY to PER-CK1 dimer stabilizes the complex and leads to the nuclear translocation of PER-CK1-CRY (Foster & Kreitzman, 2017b). In the nucleus, PER and CRY negatively regulate their own transcription by inhibiting the positive elements (CLK-BMAL1) (Ono, 2022). This negative feedback loop would repeat indefinitely, taking approximately 24 hours for each cycle to complete.

The core loop is interlocked with a secondary TTFL through CLOCK–BMAL1 activation of retinoic acid-related orphan nuclear receptors, REV-ERB $\alpha$  and ROR $\alpha$  (Allada & Bass, 2021). The REV-ERB $\alpha$  and ROR $\alpha$  proteins subsequently compete for binding to retinoic acid-related orphan response elements (ROREs) within the promoter region of *Bmal1*, where ROR $\alpha$  activates *Bmal1* transcription while REV-ERB $\alpha$  inhibits it (Li et al., 2021). This antagonistic act on the *Bmal1* promoter ensures that BMAL1 levels peak at dawn and fall during the day, cycling antiphase to the rhythms of PER and CRY (Finger & Kramer, 2021; Foster & Kreitzman, 2017b). Dec1 and Dec2 (mammalian homologs of *Drosophila cwo* gene) are additional elements that are positively regulated by CLK-BMAL1 heterodimers which in turn reinforce the inhibitory roles of PER and CRY by downregulating the CLK-BMAL1 transcription, and therefore contributing to the negative arm of the feedback loop (Honma, 2018) (Figure 1.5).



**Figure 1.5. The schematic diagram representing the molecular circuitry involved in the regulation of the mammalian circadian clock.** In the core TTFL, CLOCK-BMAL1 heterodimers act as positive elements and drive the transcription of negative components *period* (*per*) and *cryptochrome* (*cry*). As PER and CRY accumulate in the cytoplasm, they form a dimer and translocate to the nucleus to repress the activity of positive regulators (CLOCK-BMAL1). PER and CRY are subsequently degraded, through the activity of auxiliary enzymes such as CK1 and Fbx13, respectively; then, the next cycle starts. In a secondary loop, the transcriptional activator ROR $\alpha$  and the inhibitor REV-ERB $\alpha$  antagonistically modulate *Bmal1* transcription (Adapted from Robinson & Reddy, 2014).

#### 1.1.7.1 Synchronization of Peripheral Clocks by the SCN

The master pacemaker, located in the suprachiasmatic nucleus (SCN), maintains phase-alignment with subsidiary oscillators in the peripheral organs such as liver, heart and skeletal muscles via autonomic innervation, body temperature, humoral and local signals such as metabolites, as well as feeding-related cues (M. D. Li et al., 2021; Mohawk et al., 2012). The neuronal control of peripheral clocks occurs through both sympathetic and parasympathetic routes (Vujović et al., 2008). This is exemplified in the SCN projections via paraventricular nucleus (PVN) to the liver which influences the rhythms of glucose and glycogen metabolism and gives rise to pronounced daily rhythms in plasma glucose concentrations, glucose tolerance and insulin sensitivity (Greco & Sassone-Corsi, 2019; M. D. Li et al., 2021). Interestingly, SCN-lesioned mice display no rhythmicity in glucose and insulin levels (Greco & Sassone-Corsi, 2019).

In addition to the autonomic nervous system, endocrine signals are also mediators of circadian physiology. Glucocorticoid hormones such as cortisol (steroidal hormone released from the adrenal glands) are secreted in a circadian manner to phase-entrain the peripheral systems and

align them with the periods of energy intake and expenditure (Crespo et al., 2021; Tsang et al., 2013; Walker et al., 2020). Glucocorticoid input acts through GREs (Glucocorticoid-response elements) present in the regulatory region of core clock genes (*Bmal1*, *Cry1*, and *Per1-2*) and leads to the rhythmic expression of oscillatory components of the peripheral clocks (Greco & Sassone-Corsi, 2019). Glucocorticoids can also contribute to the rhythmic synchronization of peripheral metabolism through temperature entrainment. Peripheral oscillators including liver, kidney, and lungs are extremely sensitive to temperature changes (Mohawk et al., 2012). Glucocorticoids are thought to induce rhythmic thermogenesis in Brown adipose tissue (BAT) and contribute to the heat entrainment of extra-SCN oscillators via transcriptional regulation of clock genes through the activity of transcription factor HSF1 (Heat Shock Factor 1) (Greco & Sassone-Corsi, 2019; Mohawk et al., 2012).

Feeding rhythms can also act as a potent synchronizer for most peripheral clocks (Dibner & Schibler, 2015). In nocturnal rodents, time-restricted feeding regimen during the subjective day (period of rest) can shift the phase of circadian gene expression within the peripheral tissues and misalign the phase of peripheral clocks from the master pacemaker which is resilient to feeding rhythms (Asher & Sassone-Corsi, 2015; Dibner & Schibler, 2015). Unlike the SCN, the hepatic clock is particularly sensitive to entrainment by temporal changes in dietary intake, as the repertoire, phase, and amplitude of hepatic transcriptome change rapidly in anticipation of the timing of scheduled meal (Mohawk et al., 2012; Serin & Acar Tek, 2019). Interestingly, imposing a feeding rhythm to oscillator-deficient (*Cry1/Cry2*-deficient) mouse liver is sufficient to rescue the hepatic transcriptional rhythms (Vollmers et al., 2009). This food-driven circadian rewiring is likely to be a synergistic effect of both clock-independent metabolic regulators and the molecular clock which allow the organism to adapt to nutritional challenges (Greco & Sassone-Corsi, 2019).

### 1.1.8 The Circadian Clock mechanisms in *Neurospora crassa*

#### 1.1.8.1 *Neurospora crassa* life cycle

The filamentous ascomycete fungus *Neurospora crassa* is a eukaryotic organism widely distributed in nature (Baker et al., 2012; Sun et al., 2012). As a saprophytic organism (decomposing organic matter), *N. crassa* can also grow on carbohydrate-rich substrates,

particularly in moist tropical habitats (Kuo et al., 2014). Owing to its rapid growth and heat requirement for activation of its ascospore (sexual spores), it is an early colonizer of burnt vegetation (Davis & Perkins, 2002; Dunlap et al., 2007). *Neurospora* commonly exists in a vegetative state as an incomplete septate syncytium, meaning that the cellular compartments in the tubular hyphae (individual filaments) are delineated by incomplete cross walls, allowing for cytoplasmic streaming to carry organelles along the hyphae in the direction of growth (Davis & de Serres, 1970; Dunlap et al., 2007; Galagan et al., 2003). Frequent fusion among multinucleated hyphal filaments yields an interconnected haploid mycelial network with different genotypes, comprising a heterokaryon organism (Baker et al., 2012; Hickey et al., 2002).

*N. crassa* alternates between sexual and asexual reproductive cycles based on environmental factors such as nutrient availability, light, and temperature (Dunlap et al., 2007). In the haploid asexual phase, mycelia undergo a developmental switch and form specialized aerial hyphae which in turn produce chains of asexual spores known as macroconidia (Borkovich et al., 2004). Macroconidia (also referred to as conidia) are characteristically orange (due to presence of carotenoid pigment), multinucleated, and hydrophobic spores that are easily dispersed by light air movement (Baker et al., 2012). Microconidia, another type of asexual spore, are formed later during the growth by processes distinct from macroconidia formation; however, since they are uninucleate, they are less viable (Davis & de Serres, 1970; Davis & Perkins, 2002). A colony can constitute millions of macroconidia and microconidia and continue to grow and repeat the asexual cycles until suitable nutrients are no longer present.

In conditions inimical to vegetative growth, *N. crassa* undergoes sexual reproduction by producing nascent fruiting bodies known as protoperithecia (female structure) (Aramayo & Selker, 2013). Mating is achieved by chemotropic growth of specialized female hyphae projecting from protoperithecia towards the male cell (typically a conidium) in a process involving pheromones (Borkovich et al., 2004). Following fertilization, meiotic segregation, and mitotic growth, haploid ascospores (sexual spores) are formed and upon their germination the sexual life cycle returns to its starting point (Davis & Perkins, 2002).

*Neurospora crassa* is a tractable model system that has been instrumental in unveiling the molecular and genetic basis of circadian rhythms (Koritala & Lee, 2017). It can express a multitude of discernible rhythms in growth, morphology, and even gene expression, which together comprise a rich biology adaptive to the organism's success (Dunlap et al., 2007). These rhythmic events are a consequence of a well-orchestrated array of various subcellular processes, including light perception, transcriptional and post-translational modifications, signal transduction pathways, as well as chromatin remodelling events, which collectively generate a robust pacemaker capable of governing various aspects of fungal biology (Montenegro-Montero et al., 2015). Perhaps the clearest manifestation of the endogenous clock is embodied in the daily appearance of asexual spores (conidiation) that can be easily observed as an alternating zone between conidia (bands) and mycelia (interbands) when grown on a solid agar in growth tubes, also known as race tubes. (Baker et al., 2012; Lakin-Thomas et al., 2011). This particular characteristic, as well as other attributes such as fast growth, short life cycle, ease of culturing, haploid genetics and manifold similarities to animal systems, has positioned *Neurospora* at the forefront of circadian research (Hurley et al., 2016; Lakin-Thomas et al., 2011; Roche et al., 2014).

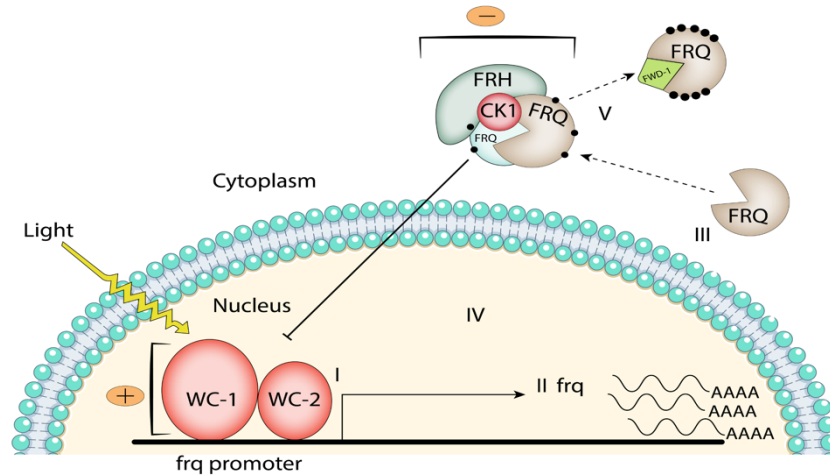
#### 1.1.8.2 The Molecular Mechanism of Circadian Rhythms in *Neurospora crassa*

The *Neurospora* circadian oscillator consists of an interlocked autoregulatory transcriptional-translational feedback loop run by a core clock complex that is temporally regulated by a series of ancillary proteins (Hurley et al., 2015). The core complex incorporates positive feedback from a heterodimeric transcriptional complex formed by the White Collar 1 (WC-1) and White Collar 2 (WC-2) to drive the expression of key protein in the negative arm of the loop, FREQUENCY (FRQ) (Hurley et al., 2016; Lakin-Thomas, 2006b; Lakin-Thomas et al., 2011). Subsequently, the negative element downregulates its own expression on a time delay, reflecting the ~24-hour period of the oscillator (Baker et al., 2012; Ratnayake et al., 2018).

In the late subjective night, the two GATA-type zinc finger transcription factors WC-1 and WC-2 interact with each other through their PAS (Per-Arnt-Sim) domains and form the white-collar complex (WCC) (Liu & Bell-Pedersen, 2006; Young & Kay, 2001). WCC rhythmically binds to the C box (Clock Box) on the *frequency (frq)* gene promoter and induces its expression. (He et

al., 2003). As FRQ protein accumulates, it forms a homodimer and associates with its partner protein FRH (Frequency Interacting RNA Helicase) (Liu & Bell-Pedersen, 2006). The interaction with FRH stabilizes FRQ as an Intrinsically Disordered Protein (IDP) and maintains its proper structure (Dunlap & Loros, 2017). The FRQ-FRH complex (FFC) then translocates into the nucleus and rapidly represses *frq* transcription by phosphorylating and inhibiting the transcriptional activity of WCC through the recruitment of CK I (casein kinase I) (Cha et al., 2015). As the negative feedback reaches its completion, the extensive phosphorylation of FRQ by CKI and CKII triggers its precipitous turnover through FWD-1 (F-box/WD-40 repeat-containing protein)-mediated ubiquitination and proteasomal degradation (Baker et al., 2012; Lakin-Thomas, 2019). Thus, by late subjective night, and in the absence of FRQ, the WCC restarts the cycle by activating *frq* expression (Montenegro-Montero et al., 2015).

In addition to the negative feedback loop, there is also a nested positive loop in which FRQ facilitates the stabilization of WC-1 and independently promotes the transcriptional activation of WC-2 (Baker et al., 2012; Cha et al., 2015). WC-1 is a flavin-containing blue light photoreceptor that possesses a specialized PAS domain known as LOV (Light, Oxygen, Voltage sensing) at its N-terminus, which allows the fungus to sense the environment and entrain the endogenous oscillator by modulating the expression of many clock-controlled genes, including *frq* (Lakin-Thomas et al., 2011) (Figure 1.6).



**Figure 1.6. The schematic diagram representing the molecular component of FWO (FRQ-WCC-Oscillator) as the core transcriptional-translational feedback loop (TTFL) involved in the circadian system of *Neurospora crassa*.** The FWO incorporates positive feedback from the heterodimerization of WC-1 and WC-2 (the WCC), which binds to the *frq* gene promoter and activates its transcription (I). The *frq* transcripts are then subjected to post-transcriptional modifications such as splicing that yields various mRNAs differing in their 5' UTR (untranslated region) (II). Following translation, as FRQ proteins accumulate in the cytoplasm, they form a heterodimer with FRH and CKI (III). This negative regulatory complex then translocates into the nucleus where it promotes the inhibition of WCC and thus downregulates the *frq* expression (IV). Ultimately, the continuous phosphorylation of FRQ leads to an open conformation, which promotes its degradation via interaction with ubiquitin ligase FWD-1(V). The degradation of FRQ releases the inhibition on WCC and reinitiates the cycle. (Adapted from Montenegro-Montero & Larrondo, 2013).

### 1.1.8.3 The FRQ-less oscillator in *Neurospora crassa*

While the importance of the FRQ-WCC oscillator (FWO) in the generation and maintenance of circadian rhythmicity in *N. crassa* is a well-accepted paradigm, this TTFL model remains inadequate in explaining the existence of residual rhythmicity in the absence of canonical clock genes (*frq* or *wc* genes) (Lakin-Thomas, 2006a; Li et al., 2011). The putative rhythms observed in the absence of a functional FWO are collectively coined as FLOs (FRQ-less oscillators) and although some of them lack one or more features of a true circadian rhythm, they point to the existence of autonomous oscillators that function independent of the canonical TTFL and may contribute to the robustness of the core pacemaker in *Neurospora* cells (Lakin-Thomas, 2006a; Nsa et al., 2015).

The conditional rhythmicity in *frq* and *wcc* null mutants can be induced by altering the genetic background, adding certain chemicals to the growth medium, or changing the geometry of the



culture vessel (Lakin-Thomas et al., 2011). For example, rhythmic conidiation in *frq* null mutant strains (*frq*<sup>9</sup> and *frq*<sup>10</sup>) can be observed when grown on extra-long race tubes. Additionally, supplementing certain chemicals such as farnesol or geraniol in the growth medium can produce reliable rhythms in the strains that are believed to be arrhythmic under standard conditions (*frq*, *wc-1*, and *wc-2* null mutants) (Lakin-Thomas et al., 2011). The FRQ-less conidiation rhythms can also be seen in cultures exposed to discrete temperature cycles and short heat pulses (Lakin-Thomas, 2006a).

Lipid deficiencies can also restore FLO-based rhythms under appropriate conditions (Lakin-Thomas & Brody, 2000). Introducing lipid-deficient mutations such as the fatty acid-requiring *cel* (*chain-elongation*) or the choline-requiring *chol-1* (*choline-requirer*) mutations into *frq*, *wc-1*, or *wc-2* null genetic backgrounds can produce robust conidiation rhythms with uncompensated long periods (Lakin-Thomas & Brody, 2000). These developmental rhythms can be observed under particular conditions: unsaturated fatty acid supplementation in the case of the *cel* mutant and choline starvation for the *chol-1* mutant (Lakin-Thomas et al., 2011).

In addition to the FRQ-less conidiation rhythms, there are also several molecular species that exhibit rhythmicity in the absence of canonical FWO (Lakin-Thomas et al., 2011). The levels of neutral lipid diacylglycerol (DAG) oscillate with a period that correlates with the conidiation rhythms in both wild-type and *frq* null strains (Ramsdale & Lakin-Thomas, 2000). The rhythmic activity of the nitrate reductase enzyme can be observed in ammonia-free medium in *frq*<sup>9</sup> and *wc-1* null strains. Similarly, WC-1 protein levels also oscillate in FRQ-less strains, albeit with a lower amplitude compared to the *frq*<sup>+</sup> strains (Lakin-Thomas et al., 2011). Oxidation-reduction cycles of peroxiredoxin proteins also follow a circadian oscillation in *frq*<sup>10</sup> mutants which may be indicative of FLO-based rhythmic generation of reactive oxygen species (ROS) (Edgar et al., 2012).

Despite the identification of various FRQ-less rhythms, their true identity, exact components, and possible hierarchical relationship with the FRQ-WCC oscillator remain enigmatic. To address these questions, our laboratory has implemented two assay systems: a heat-entrained conidiation rhythm in FRQ-less strains that can be induced using cyclical heat pulses repeated at circadian intervals and a free-running conidiation rhythm in the *chol-1* mutant that is depleted of

choline (Lakin-Thomas, 2019). Based on these strategies several novel mutations affecting *Neurospora* FRQ-less rhythms have been identified (Eskandari et al., 2021; Li et al., 2011; Ratnayake et al., 2018). Interestingly, these FLO-affecting mutations (*prd-1*, *prd-2*, *uv90* (*vta<sup>ko</sup>*), and *gtr2*) also appear to influence rhythmicity in the presence of functional FWO which may point to the dual role of these proteins in coordinating the circadian network by connecting and synchronizing the individual oscillators (Adhvaryu et al., 2016; Eskandari et al., 2021; Ratnayake et al., 2018).

The *prd* mutations (*period*, *prd-1*, and *prd-2*) have been identified as clock-affecting mutations in both *frq-null* and *frq*-sufficient backgrounds. These mutations abolish the free-running rhythms in the *chol-1 frq<sup>l0</sup>* background grown on choline-deprived cultures. They also significantly impair the heat-entrained oscillator in *frq<sup>l0</sup>* strains while lengthening the period in *frq<sup>+</sup>* samples (Li & Lakin-Thomas, 2010). The subsequent mapping and characterization of *prd-1* gene (NCU07839) reveal that it encodes for a DEAD-box RNA helicase *dbp-2* that localizes to the nucleus and may play a role in RNA processing (Adhvaryu et al., 2016).

The UV90 (also known as *vta<sup>ko</sup>*) mutation severely disrupts the FRQ-less conidiation rhythms in the *chol-1 frq<sup>l0</sup>* background when grown on choline-deficient cultures. It also affects the heat-entrainable conidiation rhythm in choline-rich cultures (Li et al., 2011; Li & Lakin-Thomas, 2010). Additionally, UV90 dampens the amplitude of both the conidiation rhythm and the FRQ protein rhythms in the *frq<sup>+</sup>* backgrounds. Furthermore, this mutation increases the susceptibility of the *frq<sup>+</sup>* strains to phase entrainment by light or high temperature (Lakin-Thomas et al., 2011). Based on this evidence, it can be concluded that the UV90 gene product is a crucial component of the FRQ-less oscillator that interacts with FWO and maintains the amplitude of its rhythms, and thus facilitates the crosstalk between FLO-based rhythms and the canonical TTFL. Mapping of the *uv90* gene (NCU05950) led to the identification of VTA protein (vacuolar TOR-associated protein) which is homologous to a TOR pathway protein in yeast (EGO1 complex) and mammalian LAMTOR1 (Ratnayake et al., 2018). The VTA protein is a component of a key complex that anchors TOR complex to the vacuolar membrane and contributes to the transmission of nutritional signals by promoting anabolic processes (Lakin-Thomas, 2019; Ratnayake et al., 2018).

The VTA binding partner, GTR2, is another component of the TOR pathway. It is homologous to yeast's GTR2 and mammalian RAG C/D. Similar to *vta<sup>ko</sup>*, *Δgtr2* compromises the FRQ-less conidiation rhythms (Eskandari et al., 2021). Additionally, the expression of GTR2 was found to be rhythmic across two circadian cycles and functional VTA protein was essential for this rhythmicity. Interestingly, in the *Δgtr2* strain, the rhythmic levels of FRQ protein are dampened, indicating that GTR2 may contribute to the cyclical fluctuations of FRQ (Eskandari et al., 2021). Overall, these results point to the extensive interplay between metabolism and circadian rhythmicity, by suggesting the potential role of the TOR pathway in generating the FRQ-less oscillations in *Neurospora* (Lakin-Thomas, 2023).

### 1.1.9 Circadian Rhythmicity and Metabolic Processes

#### 1.1.9.1 Rhythmicity of Metabolic Processes

The circadian integration of metabolic systems has evolved as an anticipatory mechanism that optimizes energy equilibrium by temporally separating incompatible biological processes such as anabolism and catabolism whilst coordinating these events with the external environment (Loizides-Mangold et al., 2017; O'Neill et al., 2013). The mammalian circadian clock governs rhythmicity within organisms far beyond the sleep-wake and feed-fast cycles; it influences homeostasis in myriad aspects of cellular processes including carbohydrate and lipid metabolism, stress adaptation, cell survival and autophagy (Reinke & Asher, 2019). Perhaps a direct piece of evidence that emphasizes the close association between the clock and metabolism is the emergence of pathological metabolic states such as metabolic syndromes and obesity upon the targeted mutations of clock genes (Asher & Sassone-Corsi, 2015; Lakin-Thomas, 2019).

Genome-wide transcriptome profiling has revealed that approximately 15 percent of mammalian genes exhibit daily oscillations in their expression. Importantly, a large fraction of these rhythmic genes are involved in regulating intermediary metabolism (Albrecht, 2012). The liver, as a prototypical metabolic organ, is tightly regulated by the circadian clock and displays diurnal rhythms in all transcriptional and organelle-level mechanisms (M. D. Li et al., 2021). For example, the daily oscillation in plasma glucose concentration is the result of circadian regulation of glucose input through rhythmic expression of enzymes involved in gluconeogenesis such as phosphoenolpyruvate carboxy kinase 1 (PEPCK1) and glucose-6-phosphatase (G6PC),

as well as rhythmic glucose utilization via diurnal expression of GYS2 (glycogen synthase 2) (Kumar Jha et al., 2015). Additionally, molecular clock components, specifically REV-ERB $\alpha/\beta$ , drive rhythmic glutathione metabolism and therefore contribute to detoxification of the hepatic microenvironment against reactive oxygen species (ROS) (M. D. Li et al., 2021). The pivotal role of molecular clock machinery in regulation of glucose homeostasis is best exemplified in *Bmal1*-deficient mice, where liver-specific inactivation of *Bmal1* results in reduction in the expression of glucose transporter GLUT2 and leads to severe hypoglycaemia during the rest-phase (Crespo et al., 2021; Gnocchi et al., 2015). Timely degradation and recycling of clock proteins such as CRY1 also contribute to sustaining glucose homeostasis (Rijo-Ferreira & Takahashi, 2019). Clock repressor CRY1 suppresses gluconeogenesis by rhythmic inhibition of glucocorticoid receptors and interference with glucagon signaling (Bass & Takahashi, 2010; Toledo et al., 2018). Degradation of CRY1 through autophagic pathways (intracellular degradative processes that recycle cytosolic content in the lysosome) allows glucose synthesis to resume (Rijo-Ferreira & Takahashi, 2019; Wang et al., 2020). Notably, in metabolic syndromes such as obesity, autophagic degradation of CRY1 is elevated which subsequently leads to higher blood-sugar levels. By contrast, loss of autophagy and accumulation of CRY1 disrupts the hepatic clock and impedes the homeostatic balance of glucose metabolism (Rijo-Ferreira & Takahashi, 2019).

In addition to glucose, lipid homeostasis also appears to be under circadian control (Asher & Sassone-Corsi, 2015). Both lipid transport, via scaffolding apolipoproteins (APO), and their absorption in the intestinal lumen exhibit diurnal rhythmicity (Gnocchi et al., 2015; Serin & Acar Tek, 2019). Moreover, lipid biosynthesis including triglyceride (TG) and cholesterol also vary in a time-of day dependent manner (Kumar Jha et al., 2015). This rhythmic regulation is mainly achieved through the circadian expression of enzymes such as ACC (acetyl co-A carboxylase) and FAS (fatty acid synthase) involved in TG biosynthesis and HMG-CoA reductase ( $\beta$ -hydroxy-3-methylglutaryl-CoA reductase) in cholesterol formation. (Gooley, 2016). The clock protein BMAL1 is thought to regulate *de novo* lipogenesis in the liver through insulin-mTORC2-AKT signaling (Crespo et al., 2021). mTOR (mammalian target of rapamycin) is a major intracellular sensor that integrates nutritional and energy status to promote synthesis of primary building blocks such as proteins, lipids, and nucleotides (Shimobayashi & Hall, 2014). mTOR

exhibits robust circadian oscillations in various tissues including the SCN and liver (Lakin-Thomas, 2019; Ramanathan et al., 2018). mTOR inhibition in liver disrupts the intrinsic clock properties by lengthening the period and decreasing the amplitude of rhythms (Ramanathan et al., 2018). In *Bmal1*-deficient hepatocytes, mTOR-driven phospho-cycles of downstream targets are lost; this in turn, leads to the suppression of *de novo* lipogenesis and fatty acid oxidation (Crespo et al., 2021; Robles et al., 2017). Interestingly, constitutive activation of insulin-AKT (protein kinase B) signaling which leads to stabilization of mTORC2 components can normalize *de novo* lipogenesis in *Bmal1*<sup>-/-</sup> hepatocytes (Zhang et al., 2014). Thus mTOR signaling appears to have multifaceted role in the regulation of circadian clock, it can transduce rhythmic outputs from the clock to regulate physiological and biochemical processes. Furthermore, it can modify local clock function in response to metabolic inputs (Ramanathan et al., 2018).

#### 1.1.9.2 Crosstalk between Circadian Clock and Metabolic Networks

The circadian machinery exerts control over metabolic processes through the strict temporal regulation of various key steps and players involved in these pathways (Sahar & Sassone-Corsi, 2012). A paradigmatic example is the rhythmic expression of enzymes involved in the rate-limiting steps of metabolic pathways, as seen in the NAMPT-dependent NAD<sup>+</sup> salvage pathway (Basse et al., 2023). The molecular clock, CLK-BMAL1 heterodimer drives the expression of *NAMPT* (nicotinamide phosphoribosyl transferase) which is a key rate-limiting enzyme in recycling of redox cofactor NAD<sup>+</sup> (nicotinamide adenine dinucleotide) (Sahar & Sassone-Corsi, 2012). Therefore, through the oscillatory expression of NAMPT, the clock directs the circadian synthesis of NAD<sup>+</sup> and subsequently leads to the cyclical activity of NAD<sup>+</sup>-consuming enzymes such as chromatin remodelers, SIRT1 and SIRT6 (Asher & Sassone-Corsi, 2015). The NAD<sup>+</sup>-deacetylase sirtuins 1 and 6 (SIRT1 and SIRT6) partition the circadian epigenome by controlling the transcriptional specificity of distinct groups of genes involved in peptide and lipid metabolism, respectively and thereby contribute to the temporal segregation of metabolic events (Asher & Sassone-Corsi, 2015; Masri et al., 2014; Nakahata & Bessho, 2016; Sun et al., 2019). Notably, the disruption of NAD<sup>+</sup> oscillation alters the circadian periodicity of locomotor activities in mice which may point to the reciprocal regulation of circadian timing and metabolism (Lakin-Thomas, 2019; Sahar & Sassone-Corsi, 2012) (Figure 1.7).

Another mode of circadian regulation of metabolism is through interaction with nuclear receptors involved in metabolite sensing. The core clock circuitry directly regulates the expression of several nuclear receptors such as ROR $\alpha$ , REV-ERB $\alpha$ , as well as several isoforms of peroxisome proliferator-activated receptor (PPAR) (Grimaldi et al., 2010; Sahar & Sassone-Corsi, 2012). PERIOD proteins (PER1-3) physically associate with a plethora of nuclear receptors and fine-tune several metabolic pathways. For example, PER2's interaction with PPAR $\gamma$ , a nuclear receptor critical in adipogenesis and insulin sensitivity, alters lipid metabolism (Jing et al., 2021). PER2-deficient mice display lower levels of hepatic triglyceride levels while its silencing in mammary epithelial cells completely inhibits lipogenesis and desaturation (Adamovich et al., 2014; Jing et al., 2021). In addition to its role in regulating lipid metabolism, PER2 is also implicated in glycogen metabolism through its interaction with REV-ERB $\alpha$ . Ablation of both PER2 and REV-ERB $\alpha$  in *Rev-Erb<sup>-/-</sup> / Per2<sup>Brdm1</sup>* mutant mice blunts the activity of glucose-6-phosphate enzyme involved in glycogenolysis which in turn leads to lower and arrhythmic levels of glycogen in the hepatocytes (Sahar & Sassone-Corsi, 2012; Schmutz et al., 2010). The interaction of PER proteins with the nuclear receptors not only impacts various metabolic pathways but it also influences the molecular clockwork through the antagonistic effects of REV-ERB $\alpha$  and ROR $\alpha$  on *Bmal1* promoter, which is necessary for generating and maintaining rhythmicity (Crespo et al., 2021).

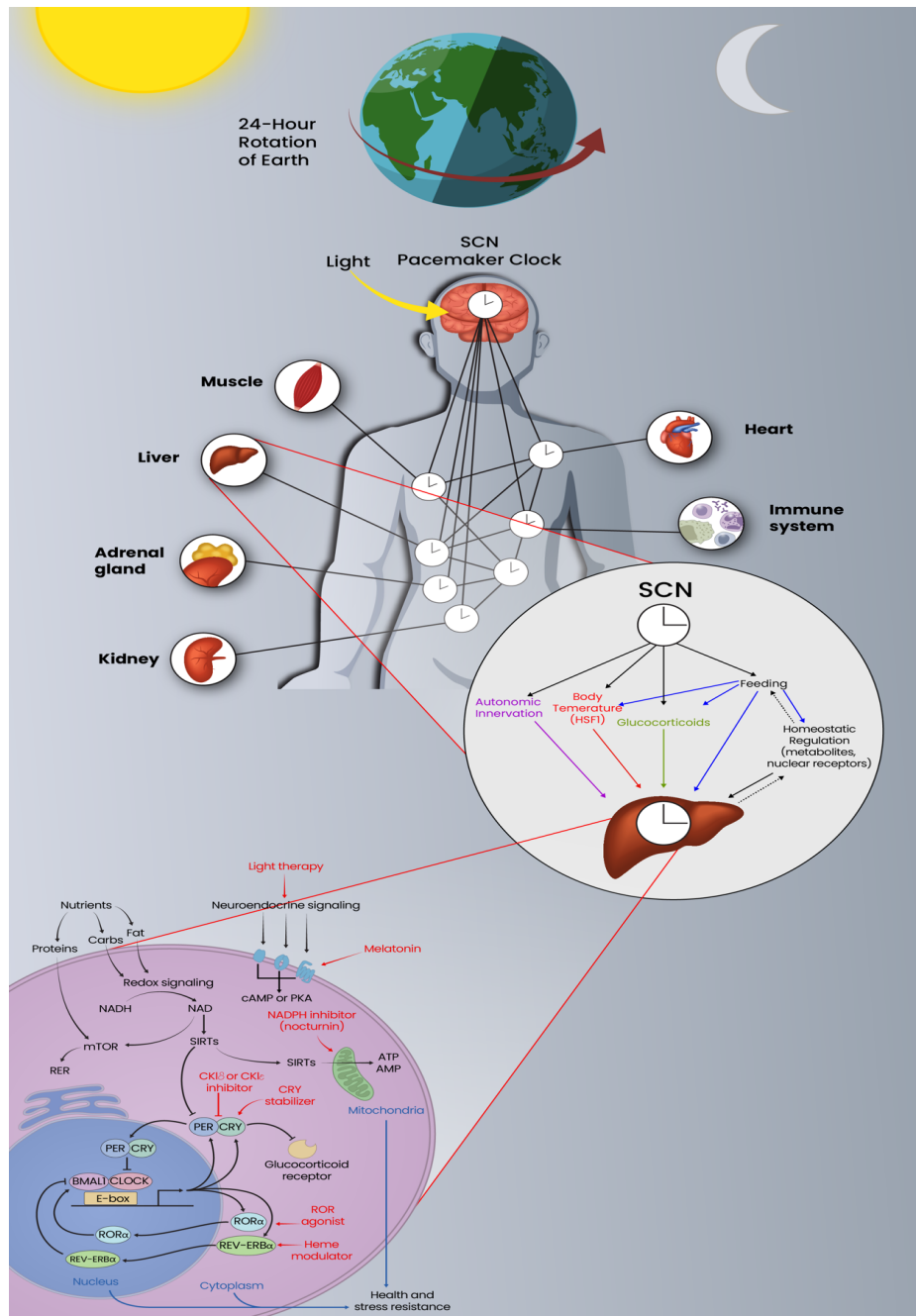
Although the central circadian machinery imposes rhythmicity on metabolic processes via a variety of output signals, metabolic signals can, in turn feedback and provide important input to the timekeeping systems and therefore fortify and enhance the rhythms of the SCN (Reinke & Asher, 2019). Satiety hormone leptin relays signals about the peripheral energy status to the central clock and influences energy homeostasis and food intake rhythms (Albrecht, 2012; Wang & Cheng, 2018). The circadian control of *leptin* expression in white adipose cells is driven by CLOCK-BMAL1-mediated transcriptional activation and consequently leads to daily oscillations in leptin levels in the blood (Dibner & Gachon, 2015). Leptin has a broad impact on metabolism; it suppresses food intake and stimulates metabolic events that lead to the dissipation of excess energy storage (Albrecht, 2012). This is achieved by regulating the activity of metabolic sensor AMPK which appears to couple metabolic processes to circadian clock. AMPK (adenosine monophosphate-activated protein kinase) is a heterodimeric protein kinase that responds to

cellular energy stress by sensing AMP/ATP ratio and transmits energy-dependent signals to the clock (Jeon, 2016; Jordan & Lamia, 2013). AMPK activation has pleiotropic effects in various tissues; it promotes a switch from ATP-consuming anabolic processes such as fatty acid and glycogen synthesis to energy-producing catabolic pathways such as fatty acid oxidation and glucose uptake (Barnea et al., 2012; Kola, 2008). AMPK's sub-cellular localization and activity appear to be under circadian control in mouse liver, fibroblasts, and the hypothalamus (Jordan & Lamia, 2013; Sahar & Sassone-Corsi, 2012). Leptin has a paradoxical tissue-specific effects on AMPK activity, it inhibits hypothalamic AMPK to suppress appetite while stimulating the AMPK activity in peripheral organs such as skeletal muscle both of which contribute to its role in establishing metabolic homeostasis (Jeon, 2016; Kola, 2008). One of the mechanisms by which leptin decreases the hypothalamic AMPK activity is through PI3K-AKT-mTOR pathway (Wang & Cheng, 2018). The action of anorexigenic hormones such as Leptin increases mTORC1 activity in hypothalamic neurons, leading to AMPK inhibition (Watterson et al., 2013). Activation of AMPK in peripheral tissues resets the clocks by two mechanisms: it can directly phosphorylate the core clock protein CRY1, leading to its destabilization and degradation, or it can indirectly affect the PER2 protein by activating its negative regulator CK1 $\epsilon$  (casein kinase1 $\epsilon$ ) (Jordan & Lamia, 2013). On the other hand, orexigenic hormone ghrelin (secreted from oxyntic cells of stomach) increases AMPK activity leading to inhibition of mTORC1 signaling (Watterson et al., 2013). In the fasted state, ghrelin can affect the clock gene expressions in the SCN and induces a phase shift in the internal clock; additionally, it can attenuate the light entrainment of the SCN (Kumar Jha et al., 2015).

There is ample evidence suggesting that metabolic regulation is more than a mere output function of the clock. Indeed, metabolic parameters are potent regulators that contribute to the plasticity of circadian system and allow it to adjust according to the metabolic requirements of the organism (Reinke & Asher, 2019). The extensive complexity of circadian transcriptional network has many advantages, given that each component of this system can serve as a node for transmitting circadian timing information to non-clock protein and thus, integrating cellular physiology with circadian function (Musiek & Holtzman, 2016). Indeed, optimal cellular function is the result of interflow among the intrinsic molecular clock, cellular metabolism, and

external factors; and any deviation from this alignment can be detrimental to the organism's health (Crespo et al., 2021; Robinson & Reddy, 2014).





**Figure 1.7. The schematic diagram representing the hierarchy of mammalian circadian clock and its molecular circuitry.** The suprachiasmatic nucleus (SCN) of the hypothalamus serves as the master pacemaker, entraining the endogenous peripheral clocks with the environment. The temporal information is relayed to the peripheral oscillators such as the liver through autonomic innervation, body temperature, glucocorticoid hormones, and feeding-related cues. Based on this information, the liver clock can regulate multiple metabolic pathways by regulating the rate-limiting steps, modulating of nuclear receptors, interacting with nutrient sensors, and controlling metabolites levels. At the cellular level, the molecular clock, together with tissue-specific factors drives the transcription machinery and generates robust oscillations in the expression pattern of these components according to the time-of-day dependent manner. Therapeutic agents affecting the circadian clock, such as light, and melatonin represent potential means for manipulating circadian disruption in specific tissues (Adapted from Allada & Bass, 2021; Mohawk et al., 2012).

### 1.1.9.3 Circadian Clock Disruption, Disease, and Adaptive Treatments

The circadian clock permeates all aspects of mammalian biology manifesting as rhythmic organization of behavioral, physiological, cellular, and neuronal processes (Reddy & O'Neill, 2010). This internal synchrony is orchestrated by hypothalamic suprachiasmatic nuclei that allow for anticipation and adaptation to the surrounding environment (Robinson & Reddy, 2014). While having this synchronicity is advantageous, the disturbance of clock machinery, as a result of genetic mutations or misalignment with exogenous cycles, can have adverse health consequences (Finger & Kramer, 2021).

#### Circadian Clock and Disease Manifestation

There are several ways in which diseases display circadian linkages: firstly, disease onset and its symptoms exhibit circadian preferences (Ruan et al., 2021). Among the best-known examples is the increased incidence of adverse cardiovascular events such as myocardial infarction, arrhythmias, and sudden cardiac death during the early morning hours (6 am-12 pm) possibly due to diurnal variation in blood pressure that undergoes a sharp surge upon awakening (Patel et al., 2008). Additionally, night-time exacerbation of inflammatory disease especially complications of lung pathophysiology such as asthma is another prominent example of the involvement of timekeeping processes in influencing the onset and severity of disease (Ruan et al., 2021; Sundar et al., 2015).

#### Influence of Age and Disease on the Clock Function

Under homeostatic conditions, the reciprocal interaction between the circadian systems and metabolic pathways drives cellular physiology. Therefore, it is possible that diseased states and natural aging events could lead to faulty clock function (Dibner & Schibler, 2015). Age-related changes in circadian organization are often the consequence of alterations in the SCN, peripheral oscillators, or the coupling mechanisms that link the systems together (Yamazaki et al., 2002). As the SCN function declines with age, the changes in the expression of molecular clock components especially those associated with temporal regulation of DNA damage responses, autophagy, and ROS (reactive oxygen species) clearance can compromise the timing of

physiological events and increase the susceptibility of certain tissues to damage, as seen in accelerated cataract formation and alterations in liver regeneration (Bass, 2012).

### Circadian Disruption in Sleep Disorders and Jet Lag

Certain diseases, such as sleep disorders and jet lag, are characterized by phase dissociation between the intrinsic circadian cycles and environmental zeitgebers (Allada & Bass, 2021). These disorders can arise due to artificial light exposure, shiftwork, and trans-meridian flights across multiple time zones as well as intrinsic clock gene defects. Some of the most severe cases of sleep disorder can be seen in individuals with ocular disease whose transmission of photic input from the retina to the SCN has been impaired (Skene & Arendt, 2007). The lack of light entrainment in the individuals experiencing the free-running sleep phase disorder leads to desynchronization of circadian rhythms and progressive sleep-wake phase delays, which results in altered patterns of alertness specifically severe insomnia at night and excessive daytime sleepiness (Atan et al., 2023). Remarkably, most patients with this condition reported that the free-running sleep phase disorder has caused more social and professional handicap than the visual impairment (Atan et al., 2023; Salva et al., 2017).

### Neurodegenerative Disease and Circadian Clock

In addition to the primary circadian dysfunctions caused by misalignment of the intrinsic clock with the surrounding environment, the progression of certain neuropsychiatric and neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease, can also disrupt circadian rhythmicity (Foster & Kreitzman, 2017a). These dysregulations are often accompanied by alterations in sleep-wake cycles. Given the considerable number of brain regions and neurotransmitters involved in regulation of sleep, it is becoming increasingly evident that abnormal sleep is a major comorbidity in the aforementioned diseases (Fishbein et al., 2021; Fisher et al., 2013). Patients with Alzheimer's disease initially exhibit out-of-sync and fragmented sleep patterns which as the disease progresses becomes severely debilitating to both patients and their caregivers and becomes the primary driver for institutionalization (Fishbein et al., 2021; Foster & Kreitzman, 2017a). The majority of Parkinson's disease patients (80-90 percent) experience sleep disorders as part of their symptoms (Kudo et al., 2011). Parkinson's disease is marked by neuronal cell loss accompanied by aggregation of abnormal protein

structures known as Lewy bodies and a reduction in dopamine signaling (Fishbein et al., 2021; Foster & Kreitzman, 2017a). In mouse models of Parkinson's disease, neuronal atrophy and loss of dopamine signaling contributes to low amplitude and misalignment of circadian rhythms especially in the brain stem nuclei implicated in sleep-wake cycles (Fishbein et al., 2021). The alterations in phase and amplitude of rest-activity as well as disruption in the rhythmicity of endocrine signals such as melatonin may occur a decade prior to the onset of clinical motor symptoms, suggesting a potential role of circadian dysregulation in disease pathogenesis (Kudo et al., 2011).

### Genetic and Environmental Factors in Clock Disruptions

Beside the circadian rhythm disruptions caused by technological advances associated with modern living such as atypical light exposure, certain genetic alterations can also increase the prevalence of rhythmic disorders (Walker et al., 2020). Familial advanced sleep phase disorder (FASPD) is a rare heritable chronotype (individual inclination in the preferred timing for sleep-wake cycles) with extremely advanced onset of sleep (Allada & Bass, 2021; Zou et al., 2022). This condition is characterized by mutations in casein kinase 1 (CK1 $\delta/\epsilon$ ) and its target, PER2 protein which result in reduction of the kinase activity in vitro leading to shorter circadian period (Rijo-Ferreira & Takahashi, 2019). Furthermore, polymorphism in circadian genes also correlates with metabolic disease. Mutations in melatonin (circadian sleep-promoting hormone secreted from pineal gland) receptor *MTNR1B* elevates fasting glucose levels and increases the risk of type-2 diabetes, implying that circadian disruption can induce ripple effects on metabolic processes leading to metabolic dysregulation (Ruan et al., 2021).

### Therapeutic Interventions

By addressing the causes of circadian disruption and harnessing the rich findings from clinical and translational research in circadian biology, novel therapeutic approaches have been invented to enhance individual and societal well-being (Fishbein et al., 2021). Light therapy is an effective and non-invasive option to improve sleep quality and cognitive function in individuals suffering from mood disorders such as seasonal affective disorder (SAD) characterized by severe seasonal variation in mood state (Blume et al., 2019). Anticancer therapy has also benefited from fluctuations in biological function in the form of chronotherapy. Some chemotherapy agents,

such as antimitotic drugs, and antimetabolites achieve the best efficacy and least toxicity when their administration is temporally synchronized with endogenous physiological rhythms especially with the expression window of their transporters and metabolizing enzymes (Allada & Bass, 2021). For example, time-of-day dependent delivery of oxaliplatin (cis-platinum derivative) based on the pharmacokinetics of the drug, reduces the associated negative side effects in patients with colorectal cancer (Allada & Bass, 2021).

### Future Directions

Advancements in the field of circadian biology began with establishing the genetic basis of behavior. Since then, our understanding of the molecular clock network and its implications in physiology and disease has expanded tremendously. Today, we can translate these insights into clinical practices to produce precise temporal diagnostic and therapeutic tools. The extensive crosstalk between the circadian clock and metabolic pathways has been leveraged in the development of small-molecule modulators that target the circadian system with the goal of reverting adverse metabolic conditions (Z. Chen et al., 2018; Reinke & Asher, 2019). Future studies are expected to further elucidate the interconnectedness of metabolic pathways and the circadian clock by identifying potential links between these two processes. The second part of this chapter will explore the TOR (target of rapamycin) pathway as a potential component of this complex interplay

## 1.2 TOR Signaling Pathway

### 1.2.1 TOR (Target of Rapamycin) pathway

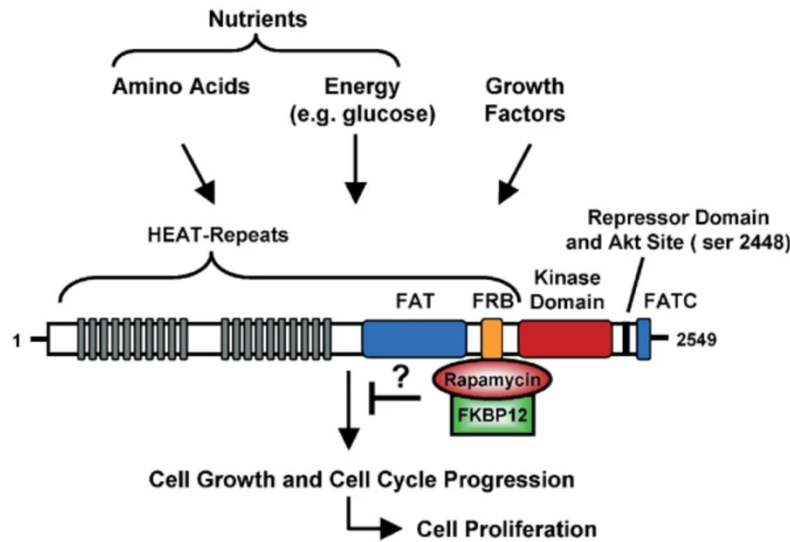
Growth and division of eukaryotic cells are intricate processes coupled to various stimuli, including nutritional availability, and a diverse set of intra- and extracellular cues, all of which are strictly regulated at many levels, especially when challenged by suboptimal conditions and stresses (Morozumi & Shiozaki, 2021; Roelants et al., 2017). The evolutionarily conserved TOR (Target of Rapamycin) signaling pathway has emerged as a universally important sensor, integrator, and controller of eukaryotic cell growth (Roelants et al., 2017). TOR (mTOR in mammals) generates a regulatory hub through which it facilitates growth by activating anabolic processes, such as protein, nucleotide, and lipid biosynthesis, along with mRNA translation, while inhibiting catabolic events such as autophagy, thereby maintaining metabolic homeostasis while promoting growth and development (Lutt & Brunkard, 2022). TOR was initially discovered in budding yeast *Saccharomyces cerevisiae* by a genetic screen for mutants resistant to the growth inhibitory effects of immunosuppressant rapamycin, and shortly thereafter, it was identified in mammalian cells (Loewith & Hall, 2011; Morozumi & Shiozaki, 2021). Given its broad control of essential cellular processes, any aberrancy in TOR signaling is often associated with severe pathologies; for example, loss of negative regulation of TOR pathway can result in unrestrained cell growth, as found in cancer, tumors, or epilepsy (Condon & Sabatini, 2019; Morozumi & Shiozaki, 2021). Conversely, decreased TOR activation can delay aging and extend lifespan in different organisms (Condon & Sabatini, 2019).

### 1.2.2 Conserved Domains and Structure of TOR kinases

The TOR proteins are atypical serine-threonine kinases belonging to the phosphoinositide 3-kinase (PI3K)-related kinase family (Saxton & Sabatini, 2017). TOR is assembled into two structurally and functionally distinct multiprotein complexes termed TOR complex 1 (TORC1) and TOR complex 2 (TORC2) (Loewith & Hall, 2011). The metazoan genome encodes a single TOR protein (human mTOR, 2549 residues) whereas *S. cerevisiae*, *S. pombe* and few other fungal species encode two paralogous TOR proteins (TOR1 2470 and TOR2 2474 residues; 70% identity in *S. cerevisiae*), possibly due to an ancient interspecies hybridization (Saxton & Sabatini, 2017; Thorner, 2022). TOR complex 1 is acutely sensitive to rapamycin while the

structural difference at the C-terminus of TOR complex 2 inhibits rapamycin binding and renders it insensitive to the inhibitory effects of this macrolide (Roelants et al., 2017). Interestingly, in budding yeast, *TOR2* is considered an essential gene while *TOR1* is not, since only TOR2 can serve as catalytic subunit for TORC2 whereas TORC1 can use either TOR1 or TOR2 as its catalytic subunit (Eltschinger & Loewith, 2016; Thorner, 2022).

All TOR protein orthologs possess similar motifs and domains structures. The domains found in TOR, in order from the N to the C terminus are:  $\alpha$ -helical HEAT (Huntington-EF3-PP2A-TOR1) repeats, the FAT (FRAP-ATM-TRAPP) domain, the FRB (FKBP-Rapamycin Binding) domain, the phosphoinositide-3 kinase domain, and the FATC (FAT C-terminus) domain (Loewith & Hall, 2011; Tatebe & Shiozaki, 2017). The tandem HEAT repeats occupy more than half of the TOR structure and can extend their superhelical structures to create a large interface for protein-protein interactions to accommodate the binding of other subunits of TOR complexes (Fingar & Blenis, 2004; Wullschleger et al., 2006). The FRB domain binds to the inhibitory FKBP1-rapamycin complex (FKBP12: FK506-binding protein which functions as the direct cellular receptor of rapamycin) and a single amino acid substitution in this domain confers complete resistance to rapamycin (Wullschleger et al., 2006). The catalytic kinase domain is flanked by FATC domain which is universally found in all PIKK (PI kinase-related protein kinase) family members and is essential for the kinase activity (Fingar & Blenis, 2004; Loewith & Hall, 2011) (Figure 1.8).



**Figure 1.8. The schematic diagram representing the conserved domain structures of TOR kinase in mammalian cells.** The N-terminal portion of TOR is composed of two blocks of ~20 HEAT repeats, which form pairs of interacting antiparallel  $\alpha$ -helices. The ~500-amino acid (aa) FAT domain includes modified HEAT repeats. The ~100-aa FRB domain is the FKBP12-rapamycin binding site that upon binding of this complex inhibits mTOR-dependent downstream signaling. The kinase domain phosphorylates Ser/Thr residues in protein substrates. The ~35 aa residue FATC domain is found in the C-terminal of PIKK family members and is essential for the kinase activity. mTOR integrates nutritional signals such as amino acids, glucose, and growth factors to modulate cell growth and cell cycle progression (Adapted from Fingar & Blenis, 2004).

### 1.2.3 TOR Complexes and the Maintenance of Cellular Homeostasis in Response to Various Inputs

#### 1.2.3.1 Composition of TOR complexes

The mTOR kinase nucleates two distinct protein complexes known as mTORC1 and mTORC2 (Laplane & Sabatini, 2012). Although the two mammalian TOR complexes share the same catalytic subunit, the biochemical and physiological distinction of the two is mainly determined by the complex-specific regulatory subunits (Tatebe & Shiozaki, 2017).

mTORC1 is composed of three defining subunits: mTOR, RAPTOR (regulatory-associated protein of mTOR), and mLST8 (mammalian lethal with SEC13 protein 8) (Tafur et al., 2020). RAPTOR facilitates the recruitment of the substrates to mTORC1 by binding to the TOR signaling (TOS) motif found on several mTORC1 substrates and is essential for the correct subcellular localization of mTORC1 (Condon & Sabatini, 2019; Saxton & Sabatini, 2017). mLST8 associates with the catalytic subunit of mTORC1 and is thought to stabilize the kinase activation loop (Saxton & Sabatini, 2017). In addition to the three core subunits, mTORC1 also

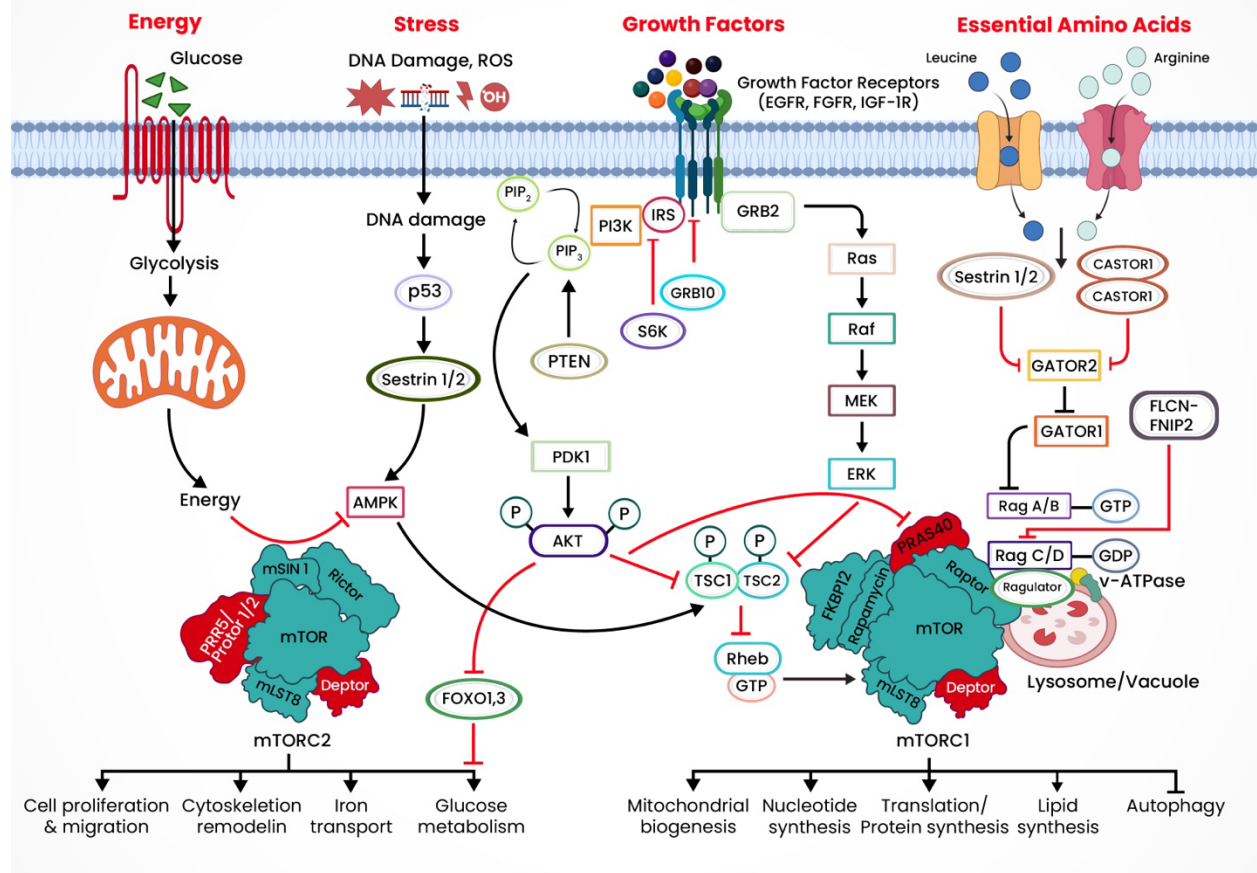


includes two inhibitory subunits: PRAS40 (proline-rich Akt substrate of 40 kDa) and DEPTOR (DEP domain containing mTOR interacting protein) (Laplante & Sabatini, 2012).

mTORC2 is composed of mTOR, RICTOR (rapamycin-insensitive companion of mTOR), SIN1 (SAPK-interacting1) and mLST8 (Shimobayashi & Hall, 2014). RICTOR plays indispensable roles for mTORC2 assembly and stabilization. The C-terminal region of SIN1 is composed of a PH (pleckstrin homology) domain, a lipid binding domain that selectively binds phosphoinositides in the phospholipid membrane while the conserved region in the middle (CRIM) is a ubiquitin-like domain characterized by acidic protrusion that functions as a substrate-recruiting domain of mTORC2 (Tatebe & Shiozaki, 2017). Furthermore, mTORC2 also contains the inhibitory subunit DEPTOR and Protor1/2 (protein observed with Rictor1 and 2) (Saxton & Sabatini, 2017) (Figure 1.9).

The TORC1 signaling in fungi shares a common architecture with its mammalian counterparts with a few minor exceptions (Tafur et al., 2020). In the budding yeast *S. cerevisiae*, TORC1 is composed of TOR1, Kog1 (the RAPTOR counterpart), Lst8 and the yeast specific subunit Tco89 (Tafur et al., 2020). TORC2 is exclusively constituted of TOR2, Lst8, and Avo3 (the ortholog of RICTOR) which is pivotal for TORC2 assembly, localization, and stability (Thorner, 2022). There are also Avo1 (mammalian ortholog mSIN1), the yeast specific Avo2, and Bit6 1/2 (counterparts of mammalian Protor1/2) (Thorner, 2022).

In addition to the differential sensitivity to rapamycin, the mTOR multimers have different upstream inputs and downstream outputs (Laplante & Sabatini, 2012). mTORC1 responds to cellular signals such as growth factors, amino acid availability, energy status, and stress signals to produce a variety of pro-growth outputs while mTORC2 primarily modulates cell survival, proliferation and cytoskeleton reorganization by activating several members of the AGC (PKA/PKG/PKC) family of protein kinases (Saxton & Sabatini, 2017; Tafur et al., 2020).



**Figure 1.9. The schematic diagram representing the major regulators and downstream effectors of mTORC1 and mTORC2.** mTORC1 is primarily composed of mTOR, Raptor, and mLST8, while the main subunits of mTORC2 are mTOR, Rictor, mLST8, and mSin1. Multiple stimuli such as growth factors, amino acids like arginine and leucine, energy sources such as glucose, and cellular stresses including DNA damage, and ROS stimulate mTORC1 activity through the GATOR2-GATOR1-Rag GTPases axis and the TSC2-Rheb GTPase route. mTORC1 activates anabolic processes such as mitochondrial biogenesis, nucleotide, protein, and lipid synthesis while blocking catabolic events such as autophagy. mTORC2 mainly responds to growth factors or insulin and regulates cell proliferation, migration, cytoskeleton remodeling, ion transport and glucose metabolism. The negative regulatory elements of mTORC1 (PRAS40 and Deptor) and mTORC2 ( PRR5/Protor1/2 and Deptor) are depicted in red (Adapted from Amemiya et al., 2023).

### 1.2.3.2 mTORC1 Activation

The mTORC1 pathway integrates inputs from at least five major intracellular and extracellular factors: growth factors (such as insulin), nutrients (amino acids), cellular energy (ATP/AMP ratio), oxygen levels, and stress signals such as DNA damage (Laplante & Sabatini, 2012). The extensive crosstalk between mTORC1 and lysosome, as the major depot for recycled biomolecules, grants the cell an exquisite sensitivity to the metabolic changes in their environment (Condon & Sabatini, 2019). The mTORC1 activation occurs under nutrient replete conditions and requires two independent events: the translocation of the complex to the lysosomal membrane, and activation by its membrane-bound activator, small GTPase Rheb (Rabanal-Ruiz & Korolchuk, 2018). Therefore, the mTORC1 activation is often thought of as a coincident detector or an AND gate where the availability of nutrients such as amino acids signals through the recruitment complex (Rag GTPases) to translocate the mTORC1 to the lysosome membrane while growth factor signals feed to the other side of the gate and culminates in the activation of Rheb which is essential for mTORC1 stimulation (Condon & Sabatini, 2019; Linde-Garelli & Rogala, 2023). The coordination of these signals via the coincidence detector ensures that mTORC1 is only active when all the requirements for cell growth are fulfilled (Condon & Sabatini, 2019).

#### Amino acid signaling promotes mTORC1 lysosomal translocation

Amino acid sufficiency regulates mTORC1 via the conserved Ras family of small GTPases (Rags), which direct the translocation of mTORC1 from an undefined cytosolic location to the lysosomal surface via a nutrient-sensitive interaction with Raptor (González & Hall, 2017; Sancak et al., 2010). Mammalian cells express four Rag proteins: RagA, RagB, RagC, and RagD (two in *S. cerevisiae* Gtr1 and Gtr2) which form obligate heterodimers with opposite nucleotide loading states (González & Hall, 2017; Laplante & Sabatini, 2012). Mammalian Rags localize to the lysosome irrespective of amino acid availability through their interaction with the pentameric lysosomal complex RAGULATOR (ternary EGO complex in *S. cerevisiae*, Ego1-Ego2-Ego3) (González & Hall, 2017). Amino acids especially leucine and arginine promote loading of RagA/B with GTP and RagC/D with GDP; the GTP-bound Rag A/B interacts with Raptor and results in localization of mTORC1 to the lysosomal membrane in close proximity to Rheb (Laplante & Sabatini, 2012).

In *S. cerevisiae* similar to mammalian cells, the Gtr1 and Gtr2 heterodimers containing, GTP-loaded Gtr1 and GDP-bound Gtr2 are tethered to the vacuolar membrane (analogous to mammalian lysosome) via their association with the EGO complex (Alfatah et al., 2021). In the presence of nitrogen and amino acid signals, the active conformation of Gtr1 and Gtr2 bind to Kog1 (equivalent of RAPTOR in mammals) and activate TORC1. However, unlike mTORC1, yeast TORC1 is constitutively localized on the vacuolar membrane regardless of nutritional availability (Sullivan et al., 2019).

#### Growth factor signaling-mediated activation of mTORC1

Growth factors stimulate mTORC1 activity through the PI3K- phosphoinositide-dependent kinase1 (PDK1)–AKT pathway. The AKT-dependent multisite phosphorylation of tuberous sclerosis complex (TSC1/2) which is a key negative regulator of mTORC1, inactivates this complex and promotes mTORC1 activation by relieving the inhibition of the small G-protein activator of mTOR, Rheb (RAS homologue enriched in the brain) (Shimobayashi & Hall, 2014). Independently of TSC, AKT directly phosphorylates PRAS40 the inhibitory component of mTORC1 and triggers its dissociation from the complex (Condon & Sabatini, 2019). In addition to PI3K/AKT, several other growth factor-signaling pathways such as mitogen-activated protein kinase MEK/ERK/RSK and MAPK/MK2 converge on TSC1/2 to drive Rheb-dependent activation of mTORC1 (Cam et al., 2010). At the lysosomal surface, the GTP-bound Rheb physically interacts with mTORC1 and stimulates its kinase activity (Condon & Sabatini, 2019) (Figure 1.9).

#### 1.2.4 Protein Synthesis downstream of mTORC1

Protein synthesis is one of the best characterized anabolic processes driven by mTORC1 (Laplante & Sabatini, 2012). The eukaryotic translation initiation factor 4E (eIF4E) binding protein 1 (4E-BP1) and p70S6 Kinase1 (S6K1) are the main regulators of protein synthesis downstream of mTORC1 (Albert & Hall, 2015; Saxton & Sabatini, 2017). The mTORC1-induced phosphorylation of 4E-BP1 relieves its inhibitory effects on the eIF4E, allowing the formation of eIF4F complex at the 5'-cap of mRNAs and thus, promoting the cap-dependent translational initiation (Ben-Sahra & Manning, 2017).

The ribosomal protein S6K1 belongs to the AGC family of protein kinases and requires phosphorylation at two sites for complete activation; mTORC1 phosphorylates Thr389 on the C-terminal hydrophobic motif of S6K1 while PDK1 (phosphatidylinositol-dependent kinase-1) involved in PI3K-dependent signaling is responsible for the second phosphorylation event (Finger & Kramer, 2021; Wullschleger et al., 2006). The activated S6K1 in turn phosphorylates the 40S ribosomal protein S6 at five serine residues, leading to increased translation of 5' TOP mRNAs (5' tract of oligopyrimidine) encoding for components of translational machinery including ribosomal proteins and elongation factors and thus, elevating the general translational capacity of the cell (Shimobayashi & Hall, 2014; Wullschleger et al., 2006). Interestingly, about 78% of mRNAs involved in ribosomal biogenesis are downregulated in S6K knockout murine cells, likewise, in mutated S6 protein where the five S6K target sites were changed to alanine, the ribosomal biogenesis was severely compromised, indicating the importance of mTORC1-S6K-S6 axis in promoting protein synthesis by increasing ribosomal biogenesis (Shimobayashi & Hall, 2014). Additionally, S6K also phosphorylates a variety of other proteins that are directly or indirectly involved in translational regulation or mRNA processing, for example it activates eIF4A RNA helicase which is essential for unwinding mRNAs at the beginning of translation by phosphorylating and recruiting its binding partner, eIF4B, while inhibiting eIF4A inhibitory protein PDCD4 (protein programmed cell death 4) (Albert & Hall, 2015; Ben-Sahra & Manning, 2017).

Similar to mammalian cells, yeast AGC kinase Sch9 (ortholog of mammalian S6K) is a direct substrate for TORC1 (Yan et al., 2006). TORC1-mediated phosphorylation of six target sites at the C-terminus of Sch9 is necessary for its activity (Loewith & Hall, 2011). Monitoring the Sch9 phosphorylation state by Western blotting serves as a convenient proxy for TORC1 activity, as it is rapidly dephosphorylated in cells exposed to rapamycin or nutrient starvation and other stress conditions (Loewith & Hall, 2011). The activation of Sch9 regulates many aspects of *S. cerevisiae* growth including ribosomal biogenesis, translational initiation, entry into quiescent G0, and stress responses (Novarina et al., 2021). TORC1 can also regulate yeast ribosomal biogenesis directly by regulating the expression of ribosomal protein (*RP*) genes. The TORC1-mediated phosphorylation of split zinc (Zn)-finger transcription factor Sfp1 results in the activation of many growth-promoting genes including ribosomal proteins (Guerra et al., 2022;

Loewith & Hall, 2011). Interestingly, unlike Sch9, the TORC1-induced activation of Spfl appears to be insensitive to the osmotic or nutritional stresses, suggesting that TORC1 regulates these two substrates via distinct mechanisms (Loewith & Hall, 2011). Despite TORC1's central role in controlling protein synthesis, it also appears to be regulated by this very process, as translational inhibition by cycloheximide or reducing the transcription of ribosomal proteins seems to hyperactivate TORC1 presumably by increasing in the concentration of cytosolic amino acid pools (Albert & Hall, 2015; Eltschinger & Loewith, 2016). Although the mechanistic details of this feedback regulation are unclear, they point to the pivotal role of TORC1 in maintaining protein homeostasis in the cell.

In addition to Sch9 and Spfl, serine/threonine protein phosphatases such as Sit4 (PP6-like) and Pph21/22 (PP2A-like) are also regulated by TORC1 in a nutrient-dependent manner (Di Como & Jiang, 2006; Finger & Kramer, 2021). When TORC1 is active, it phosphorylates the phosphatase-interacting protein, TAP42, which enhances the association of this protein with the catalytic subunits of the phosphatases, hence inhibiting their function (Loewith & Hall, 2011).

Presumably, the association of phosphatases with TORC1 via TAP42, restricts them from accessing the TORC1 substrates (Yan et al., 2012). TORC1 inactivation as a result of nutrient deprivation or rapamycin treatment leads to dephosphorylation of Tap42 and weakened association with the phosphatases. Dissociation of phosphatases from Tap42 is accompanied by activation of many downstream effectors of TORC1 including Npr1, Ure2 and Gln3 which are involved in nitrogen metabolism, TCA cycle, and stress response (Yan et al., 2006). Although the direct coupling mechanism of TORC1-Tap42-phosphatase complexes is unclear, Tap42 remains a prominent effector of TORC1 since mutation in several alleles of this protein can confer a rapamycin-resistance to the yeast cells (Finger & Kramer, 2021; Loewith & Hall, 2011).

### 1.2.5 Regulation of mTORC1: Integration of Metabolic and Autophagic Signals

#### 1.2.5.1 Amino acid sensing in the lysosome

mTORC1 senses both intra-lysosomal and cytosolic amino acid content through distinct mechanisms. Recent findings suggest that mTORC1 detects variations in amino acid levels in the lysosomal lumen via an inside-out mechanism that requires the lysosomal proton pump,

v-ATPase (vacuolar H<sup>+</sup>-adenoside triphosphate) (Saxton & Sabatini, 2017). Functionally, v-ATPase sets up a pH gradient that acidifies the lysosome and facilitates its degradative capacity (Condon & Sabatini, 2019). According to this model, v-ATPase directly interacts with the scaffolding RAGULATOR complex and triggers its GEF (guanine nucleotide exchange factors) activity toward RagA/B on the surface of the lysosome (Bar-Peled et al., 2012). Depletion of v-ATPase subunits blocks the amino acid-induced recruitment of mTORC1 and inhibits its downstream signaling (Laplante & Sabatini, 2012). Amino acid starvation appears to promote conformational change of v-ATPase and increase its interaction with RAGULATOR-Rag GTPases (Rabanal-Ruiz & Korolchuk, 2018). Interestingly, mTORC1 pathway regulates v-ATPase expression, suggesting a feedback loop between mTORC1 and amino acid availability in the lysosome (Laplante & Sabatini, 2012) (Figure 1.9).

In budding yeast, the TORC1 activity is dictated by the nucleotide-bound state of Gtr1, however, unlike mammalian cells, the EGO complex does not exhibit GEF activity toward Gtr1 (Binda et al., 2009). Instead, the GEF activity is performed by Vam6 which is a homotypic vacuolar fusion protein. Vam6 colocalizes with TORC1 and GTR1 on vacuolar membrane and is reported to activate TORC1 by stimulating GTP-Gtr1 (Valbuena et al., 2012).

Other lysosomal amino acid transporters are also involved in modulating mTORC1 activity. SLC38A9 (sodium-coupled amino acid transporter) is implicated in sensing specific amino acids such as glutamine and arginine within the lysosome (Rabanal-Ruiz & Korolchuk, 2018). This transporter regulates the efflux of amino acids from lysosome via direct interaction with the RAGULATOR-Rag GTPase complex. Overexpression of SLC38A9 results in the hyperactivation of mTORC1 even in the absence of amino acids, while its depletion inhibits mTORC1 activity in response to amino acid replenishment following starvation (Jung et al., 2015).

The proton-amino acid symporter PAT1/SLC36A1 is also required for amino acid-induced activation of mTORC1. This transporter is primarily involved in the export of alanine, glycine, and proline to the cytoplasm. PAT1/SLC36A1 interacts with Rag C/D through the FLCN (tumor suppress Folliculin)-promoted complex that functions as GAP (GTPase-activating proteins)

towards Rag C/D. (González & Hall, 2017; Rabanal-Ruiz & Korolchuk, 2018). This function is likely to be conserved between mammals and yeast species (Morozumi & Shiozaki, 2021).

#### 1.2.5.2 Amino acid sensing in the cytoplasm

Although the lysosomal lumen provides a complex readout of amino acid levels, the major contributors to mTORC1 activation are sensed in the cytoplasm. Indeed, many amino acids have their own specific cytoplasmic sensors (Rabanal-Ruiz & Korolchuk, 2018). mTORC1 signaling appears to be particularly responsive to the levels of essential amino acid leucine and conditionally essential amino acid arginine, as the starvation of these amino acids has potent inhibitory effects on mTORC1 activity (Condon & Sabatini, 2019; Lutt & Brunkard, 2022).

##### Leucine sensing mechanisms

Cytosolic leucine and arginine activate mTORC1 through distinct pathways comprised of the GAP activity towards the Rags (GATOR) complex (Takahara et al., 2020). GATOR1 (yeast ortholog SEACIT, composed of Npr12, Npr13, and DEPDC5) is tethered to the lysosomal surface via multiprotein complex KICSTOR where it interacts with RagA/B acting as a GAP and thus serves as negative regulator of mTORC1 signaling (Rabanal-Ruiz & Korolchuk, 2018; Saxton & Sabatini, 2017). GATOR2 (the yeast SEACAT, composed of MIOS, WDR24, WDR59, SEH1L, SEC13) inhibits GATOR1 activity and thereby relieves mTORC1 inhibition (Takahara et al., 2020).

Leucine-binding protein Sestrin2 serves as cytosolic leucine sensor that acts upstream of GATOR2 (Cangelosi et al., 2022). Sestrins (SESNs) are highly conserved stress-inducible proteins that mediate metabolic homeostasis in mammals by inhibiting mTORC1 signaling (González & Hall, 2017). Leucine binding to SESN2 triggers the dissociation of this protein from GATOR2, leading to GATOR2-mediated inhibition of GATOR1 and subsequent mTORC1 activation (Takahara et al., 2020). In contrast, upon leucine starvation, SESN2 binds and antagonizes GATOR2, leading to mTORC1 inhibition (Condon & Sabatini, 2019). In budding yeast, leucine activates TORC1 via Gtr1 (the ortholog of RagA/B), however, the lack of a SESTRIN ortholog implies the existence of other functional counterparts or possibly a different leucine-sensing mechanisms by TORC1 (González & Hall, 2017).



Leucyl-tRNA synthase (LARS, yeast ortholog LeuRS) which is involved in transferring leucine to its cognate tRNA, also acts as leucine sensor and modulates mTORC1 activity (González & Hall, 2017; Lutt & Brunkard, 2022). Leucine binding promotes lysosomal translocation of LARS and triggers its GAP activity towards RagD (González & Hall, 2017). Although the *S. cerevisiae* LeuRS is functionally different from LARS, it has been shown to interact with Gtr1 in a leucine-dependent manner and maintain the GTP-bound state of Gtr1, leading to the activation of TORC1 (Takahara et al., 2020).

#### Arginine sensing mechanisms

The cytosolic arginine sensor, CASTOR1 (cellular arginine sensor for mTORC1) forms a heterodimer with the highly related protein CASTOR2 (González & Hall, 2017). Much like Sestrin2, the binding of the CASTOR1-CASTOR2 heterodimer to GATOR2 inhibits its activity (Takahara et al., 2020). Arginine binding disrupts the dimer formation and inhibits their interaction with GATOR2, thus allowing free GATOR2 to inhibit GATOR1, which ultimately leads to mTORC1 activation (Condon & Sabatini, 2019; Saxton & Sabatini, 2017). Therefore, both leucine and arginine activate mTORC1 signaling partially by releasing the inhibitors for GATOR2, establishing this protein as the central node in mTORC1 stimulation in response to amino acids (Saxton & Sabatini, 2017).

In addition to CASTOR1, the lysosomal arginine transporter SLC38A9 also acts as an arginine sensor upstream of mTORC1 (Takahara et al., 2020). Arginine promotes the binding of SLC38A9 to RAGULATOR-Rags complex and induces its GEF activity towards Raga (Takahara et al., 2020; Wang et al., 2015). It has been suggested that both CASTOR1 and SLC38A9 function in parallel to regulate mTORC1 in response to arginine (Chantranupong et al., 2016). However, the residual arginine-induced activation of mTORC1 in *SLC38A9*-knockout *CASTOR1*-knockdown cells points to the existence additional, yet unidentified, arginine-sensing mechanisms by mTORC1 (González & Hall, 2017).

### Glutamine sensing mechanisms

Beyond leucine and arginine, other amino acids also activate mTORC1; the potency of this stimulation varies across biological contexts such as cell types, species, experimental conditions (Lutt & Brunkard, 2022). Unlike leucine and arginine, which signal to mTORC1 through the well-characterized Rag-GTPase pathway, glutamine activates mTORC1 via a different mechanism involving ADP-ribosylation factor 1 (Arf1) (Meng et al., 2020). Arf1 is Golgi-localized small GTPase involved in regulation of intracellular vesicle trafficking (Rabanal-Ruiz & Korolchuk, 2018; Takahara et al., 2020). Although the direct mechanism by which Arf1 promotes lysosomal translocation of mTORC1 remains unclear, new evidence points to the involvement of phosphatidic acids (PAs) generated by phospholipase D1 (PLD1) downstream of Arf1 (Takahara et al., 2020). Arf1 transduces glutamine signal to mTORC1 by activating PLD1, the PLD-derived phosphatidic acids are likely to stabilize mTORC1 on the lysosomal membrane and contribute to its activation (Bernfeld et al., 2018). Interestingly, Arf1-mediated mTORC1 activation by glutamine is relatively slow (~ 60 min) compared to potent activators of mTORC1 such as leucine and arginine that act through the Rag GTPases axis (~ 15 min) (Takahara et al., 2020). Therefore, it is tempting to speculate that mTORC1 is differentially regulated by various amino acids and the potency of the response is not unrelated to the route of action.

Similarly, in budding yeast, glutamine activates TORC1 independently from EGO-Gtr1/2 axis (Takahara et al., 2020). Pib2, a vacuolar membrane-associated phosphatidylinositol 3-phosphate (PI3P) binding protein localizes to the vacuole through its FYVE zinc finger domain and acts as a positive regulator of TORC1 in response to glutamine (Morozumi & Shiozaki, 2021).

#### 1.2.5.3 Amino acid sensing and regulation of autophagy

When adequate amino acids are present in the cellular environment, the transporters at the plasma membrane supply the cytosolic amino acids, which activate mTORC1. Conversely, in the absence of an amino acid source, mTORC1 signaling is suppressed, and autophagy is initiated to restore lysosomal amino acid pools through protein degradation (Condon & Sabatini, 2019). Autophagy is an evolutionarily conserved intracellular degradation pathway that contributes to cell survival by eliminating damaged intracellular proteins and organelles, and it sustains energy

homeostasis during the starvation periods by recycling cytoplasmic components to compensate for nutrient deprivation (Rabanal-Ruiz et al., 2017). In order to prevent a futile cycle between mTORC1-stimulated pro-growth events and autophagic degradation, mTORC1 tightly controls autophagic flux through the direct phosphorylation and inhibition of proteins involved in the initiation of autophagosome (double-membrane vesicles which carry autophagic cargo to the lysosome for degradation) formation, ULK1 (Unc-51-like kinase 1) and autophagy-related (ATG 13) protein (Condon & Sabatini, 2019; Mijaljica et al., 2012).

During amino acid starvation, lysosomal protein degradation supplies amino acids for both the replenishment of intermediates of the TCA cycle and also for the translation of specific proteins such as metabolic enzymes and transporters as part of adaptation to amino acid restriction, to alleviate the nutritional stress (Tan et al., 2017). Emerging evidence suggests that under prolonged starvation periods, ribosomes as a major cellular reservoir for amino acids and nucleotides can be selectively degraded through a process of ribophagy that is regulated by mTORC1 (Condon & Sabatini, 2019). Once amino acids produced during this process are released into the cytosol, they can reactivate mTORC1; this reactivation is autophagy-dependent and critical for cell survival (Condon & Sabatini, 2019).

#### 1.2.5.4 Glucose-Dependent Regulation of mTORC1

Glucose is a major requirement for life, and as a fundamental energetic substrate, its concentration is constantly sensed at the cellular level to ensure the maintenance of energetic homeostasis (Leprivier & Rotblat, 2020). Glucose transport across the plasma membrane is mediated by transporters such as GLUT1 (glucose transporter 1), whose inhibition has been shown to selectively inhibit the mTORC1 pathway (Condon & Sabatini, 2019). Although currently there is no consensus on how mTORC1 activity is controlled by glucose levels, it has been reported to involve AMPK (Leprivier & Rotblat, 2020). As described previously, AMPK is a conserved sensor of cellular energy status that becomes active in response to increased AMP/ATP ratios as a result of metabolic stress such as glucose deprivation (González & Hall, 2017). Upon glucose starvation, AMPK inhibits mTORC1 via two different mechanisms: by phosphorylating and activating the negative regulator of mTORC1, TSC2 or by inhibiting the regulatory subunit of mTORC1, RAPTOR (González & Hall, 2017). These regulatory events

together inhibit anabolic processes such as protein synthesis and cell cycle progression at the time of energy crisis (Leprivier & Rotblat, 2020). Importantly, in AMPK-knockout cells, glucose-starvation can still inhibit mTORC1, indicating the existence of other glucose sensors independent of AMPK (Efeyan et al., 2013). Indeed, some evidence suggests that Rag-GTPases especially RagA contribute to increased cell sensitivity to glucose starvation (Efeyan et al., 2013). In yeast, glucose levels are primarily sensed by the cyclic AMP (cAMP)-dependent protein kinase A (PKA) (Alfatah et al., 2021). In the inactive state, the catalytic subunit of PKA (Tpk1-3) is inhibited by the regulatory subunit Bcy1 (Broach, 2012). Upon glucose supplementation, the GTP-binding proteins Ras1/2 and Gpa2 activate adenyl cyclase enzyme, resulting in the accumulation of cAMP. cAMP binding dissociates Bcy1, alleviating its inhibitory effects on PKA (Alfatah et al., 2021). The active PKA phosphorylates several cellular proteins leading to enhanced protein synthesis and inhibition of stress responses (Alfatah et al., 2021). In contrast to PKA which becomes active in response to fermentable sugars, the TORC1-Sch9 (the mammalian S6 kinase, downstream effector of mTORC1) is only stimulated when a complete palette of nutrients such as amino acids, nitrogen, phosphate, and a fermentable carbon source is available (Botman et al., 2023). Although the two pathways can function independently, the large overlap of their targets allows them to rescue each other's effect. Interestingly, 90% of genes activated by Sch9 overlap with downstream targets of PKA, making TORC1-Sch9 axis as important as PKA signaling in mediating the glucose response (Botman et al., 2023).

Similar to mammalian cells, glucose starvation in *S. cerevisiae* is sensed by AMPK Snf1 (Sullivan et al., 2019). In glucose-starved cells, the Snf1-dependent phosphorylation of Kog1, results in its dissociation from TORC1 and leads to reduction in TORC1 activity (González & Hall, 2017). The Snf1 phosphorylation sites on Kog1 are located on a glutamine-rich prion-like motif, which is essential for Kog1 translocation to the perivacuolar sites upon glucose starvation (González & Hall, 2017; Sullivan et al., 2019). Interestingly, organisms such as *S. cerevisiae* and *C. elegans* that lack TSC orthologs, express this prion-like domain on their Kog1/RAPTOR proteins, while species such as *S. pombe* and mammals that lack such motifs, express TSC proteins, implying that mechanisms of AMPK-dependent inhibition of TORC1 may have diverged during evolution (González & Hall, 2017).

#### 1.2.5.5 mTORC1 Regulation in Response Stress Signals

Cells respond to intracellular and environmental stresses such as hypoxia (low oxygen levels), low ATP levels, and DNA damage by downregulating energy-demanding processes such as protein synthesis and arresting growth (Wullschleger et al., 2006). Hypoxic conditions inhibit mTORC1 in part through AMPK, but also through the induction of two homologous proteins REDD1 and REDD2 (Regulated in DNA damage and development 1) which promote the dissociation of the negative regulatory subunits of TSC, leading to its activation and subsequent suppression of mTORC1 signaling (Cam et al., 2010; Saxton & Sabatini, 2017). It appears that the stress sensor kinase ATM (Ataxia Telangiectasia Mutated) upregulates the expression of REDD through the activation of HIF1 (Hypoxia-inducible factor 1) transcription factor, as ATM-deficient cells fail to negatively regulate mTORC1 and exhibit continued phosphorylation of mTORC1 substrates S6 protein and 4E-BP when exposed to hypoxic stress (Cam et al., 2010). Other stress signals such as DNA damage downregulate mTORC1 signaling through the induction of p53 target genes including the regulatory subunits of AMPK and TSC2 which contribute to increased TSC activity (Saxton & Sabatini, 2017).

#### 1.2.5.6 mTORC1 regulation by lipids

mTORC1 as a sensor of nutrient sufficiency, is also regulated by the lipid second messenger phosphatidic acids (PA) (Popova & Jücker, 2021). PA is a central metabolite in the synthesis of membrane phospholipids and triglycerides, and as a consequence, its levels are tightly regulated to maintain lipid homeostasis (Foster, 2013). Three major metabolic pathways generate PA; perhaps the most significant one is the lysophosphatidic acid acyltransferase (LPAAT) pathway, where *de novo* synthesized or dietary fatty acids are acetylated onto G3P (glycerol-3-phosphate) to generate PA (Foster et al., 2014). Additionally, PA can be produced by the phosphorylation of diacylglycerol (DAG) via diacylglycerol kinase (DAGK), or through the action of phospholipase D (PLD) (Foster, 2013; Foster et al., 2014).

Recently, it has been reported that both amino acid-induced lysosomal translocation of mTORC1, as well as Rheb-dependent activation of this complex, require phosphatidic acids

(Frias et al., 2023). The PA-dependency of mTOR is presumably a means for sensing sufficient lipid precursors to meet the demands of membrane biosynthesis in growing cells (Foster et al., 2014). Interestingly, phosphatidic acid can serve as a broader indicator of nutrient sufficiency in the cell since its synthesis depends on several key metabolic pathways involving various nutrients. For example, fatty acids incorporated into PA exhibit glucose dependency since the conversion of glycolysis end product pyruvate to acetyl-CoA is necessary for fatty acid synthesis. Additionally, glutamine, which is a precursor to  $\alpha$ -ketoglutarate involved in the TCA cycle, also plays an important role in fatty acid synthesis. Furthermore, since PA synthesis is an ATP-dependent process, its availability can reflect sufficient energy levels necessary to invest in anabolic processes (Foster, 2013; Foster et al., 2014).

mTOR kinase activation by PA is induced by Rheb GTPase activation of the PA-generating enzyme PLD (Frias et al., 2023). The elevated expression of PLD1 and exogenous source of PA can both activate mTORC1 signaling in various types of cells (Laplane & Sabatini, 2012; Popova & Jücker, 2021). Importantly, PLD translocates to the lysosome in response to amino acids (Frias et al., 2020). PLD1, which is widely expressed in different tissues and cell types, hydrolyzes phosphatidylcholine, the most abundant membrane phospholipid, to generate choline and phosphatidic acid (Frias et al., 2020; Menon et al., 2017). Phosphatidic acid binds to a highly conserved sequence on the  $\alpha$ 4 helix of the FK506-binding protein–12-rapamycin-binding (FRB) domain of mTOR, leading to the stabilization and activation of both TOR complexes (Frias et al., 2023). Interestingly, PA binding to the FRB domain of mTORC1 appears to be sufficient for stimulating the substrate binding and the catalytic activity of this complex, even in the absence of amino acids and growth factors (Frias et al., 2020, 2023). It has been proposed that PA binding displaces PRAS40, the negative regulatory subunit of mTORC1, thus assisting in binding of substrates such as S6K to the complex (Frias et al., 2023).

### 1.2.6 mTORC2 Signaling Network

Although the mechanisms of mTORC1 activation by amino acids and growth factors are well-studied, the upstream regulatory mechanisms leading to mTORC2 activation remain largely elusive (Liu et al., 2015). mTORC2 appears to function as a primary effector of insulin-PI3K (phosphoinositide 3-kinase) signaling which is crucial in regulating cell growth, proliferation, and survival (Saxton & Sabatini, 2017). The mTORC2 subunit mSin1 contains a PH (pleckstrin homology) domain which is critical for the insulin-dependent regulation of mTORC2. In the absence of insulin, the mSin1 PH domain inhibits mTORC2 function while this autoinhibition is relieved by insulin-induced activation of PI3K and subsequent binding of PIP3 to this region. AKT can also phosphorylate mSin1; interestingly, partial activation of AKT promotes the activation of mTORC2 which in turn phosphorylates and fully activates AKT in a positive feedback looping system (Laplane & Sabatini, 2012; Saxton & Sabatini, 2017). The fully activated AKT can, in turn, activate or inhibit several substrates, including the transcription factor FoxO1/3, the metabolic regulator GSK3 $\beta$ , and the mTORC1 inhibitor TSC2 (Saxton & Sabatini, 2017). Besides AKT, mTORC2 also regulates the activity of SGK1 (serum and glucocorticoid-induced protein kinase 1) and PKC- $\alpha$  (protein kinase C- $\alpha$ ) which is involved in regulating the actin cytoskeleton (Fu & Hall, 2020).

In budding yeast, *S. cerevisiae*, TORC2 is primarily located at the plasma membrane and plays a major role in maintaining the proper distribution of lipid bilayer components such as sphingolipids, glycerophospholipids, sterols, and integral membrane proteins (Thorner, 2022). Therefore, conditions that can challenge plasma membrane integrity such as stressors affecting the membrane surface area by influencing lipid production and intracellular turgor are thought to be the main regulators of TORC2 (Eltschinger & Loewith, 2016). The best characterized and possibly the major TORC2 substrate is the AGC family protein kinase Ypk (Loewith & Hall, 2011). Ypk1 (mammalian ortholog SGK1) regulates the aminoglycerophospholipid asymmetry in the lipid bilayer by controlling the activity of flippase enzymes; additionally, it plays an important role in regulating the glycerol concentration especially under hyperosmotic stress (high salt concentration) and regulates the rate of endocytosis of integral membrane proteins (Roelants et al., 2017). Moreover, the TORC2-Ypk1 signaling may also contribute to

translational control as phosphorylation of ribosomal S6 protein has been partially attributed to YpK1 (Roelants et al., 2017; Yerlikaya et al., 2016).

### 1.2.7 Overview of Regulation of TOR Pathway in Plants

The knowledge of the TOR signaling pathway in plants lags behind mammals and yeast; while there are many notable similarities, plants lack homologs of some of the genes involved in mediating TOR signaling (Burkart & Brandizzi, 2021). The evolutionary conserved TOR protein in *Arabidopsis thaliana* and humans share 73% amino acid sequence identity in the kinase domain (Wu et al., 2019). The plant TOR Complex1 (TORC1) consists of two conserved regulatory subunits: LST8 (LETHAL-WITH-SEC13-PROTEIN8) and RAPTOR (REGULATORY-ASSOCIATED-PROTEIN-OF-MTOR1) (L. Li et al., 2021). In plants, TOR not only responds to conserved upstream signals such as glucose, amino acids, and energy but it also senses the plant-specific signals including light, phytohormones, and various nutrients such as inorganic nitrate, ammonium, phosphorous, and sulfate which might be due to the specific evolutionary adaptation of plants to their sessile and autotrophic lifestyle (Meng et al., 2022). It is believed that TOR in plants acts as a central hub in which the integration of both nutritional and hormonal signals leads to tight regulation of many downstream processes including the cell cycle progression, nutrient assimilation and transportation, primary and secondary metabolism as well as protein expression and ultimately growth and proliferation.

In *Arabidopsis thaliana*, both glucose metabolism and energy status play an important role in activating the TOR pathway via their synergistic effects with light, hormones, and other nutritional signals. Although glucose alone is sufficient in activating root meristem proliferation via TOR signaling, the TOR-mediated growth in leaf primordia requires both glucose and light input (Li et al., 2021). The light-induced synergistic effects are sensed by photoreceptors such as phytochrome (phy), and cryptochromes (CRYs) through the convergent CONSTITUTIVE PHOTOMORPHOGENIC1 (COP1)-auxin-RPS6 (ribosomal protein S6) axis (G. H. Chen et al., 2018). TOR activation in response to auxin (a type of plant hormone) signaling is mediated by Rho-like small GTPase, ROP2 (RHO-OF-PLANTS2), as plants expressing constitutively active ROP2 have high levels of TOR activation (L. Li et al., 2021).



Plants lack the metazoan and yeast RAG GTPases and Ragulator complex that are involved in amino acid sensing, implying that they utilize different, yet unknown, mechanisms for amino acid sensing. Recent observations suggest that specific amino acids such as proline and alanine when present at high levels can activate TOR pathway and lead to increased protein synthesis (Burkart & Brandizzi, 2021). Additionally, loss of function mutation in a specific gene (IPMS1: isopropylmalate synthase 1) involved in leucine biosynthesis leads to the accumulation of branched-chain amino acids (BCAA) specifically valine which ultimately results in TOR activation. Interestingly, the increased TOR activity due to the accumulation of BCAA can lead to a higher degree of actin bundling, since actin organization is dependent on TORC2 in both yeast and mammals; these findings suggest that although the canonical TORC2 components are absent in plants, TOR can still modulate actin cytoskeleton reorganization in a novel way (Burkart & Brandizzi, 2021; P. Cao et al., 2019).

#### 1.2.8 TOR Pathway in *Neurospora crassa*

The conservation of the TOR pathway in the fungal kingdom illustrates the evolutionary trajectory of a central nutrient-sensing cascade that enables these eukaryotic organisms to respond to their natural environment (Shertz et al., 2010). In this lineage, the TOR pathway has been best studied in the budding yeast *Saccharomyces cerevisiae*, the fission yeast *Schizosaccharomyces pombe*, and the human pathogen *Candida albicans*. However, little is known about TOR signaling in *Neurospora crassa*. Several rapamycin binding proteins, FKBP (FK506-binding proteins) have been identified in *N. crassa* (Park et al., 2011; Ratnayake et al., 2018). Additionally, a global Ser/Thr kinase gene deletion screening has led to the identification of yeast TOR1/TOR2 homologs via sequence similarity, although the unavailability of a homokaryon (all nuclei are genetically identical clones) from the Fungal Genetics Stock Centre may imply the lethality of the gene deletion (Money, 2017; Ratnayake et al., 2018). The same screening has identified the substrate of TOR kinase, STK10 as the homolog of the yeast Sch9 (mammalian S6K) whose deletion compromises the growth and leads to impaired osmoregulation (Park et al., 2011; Ratnayake et al., 2018).

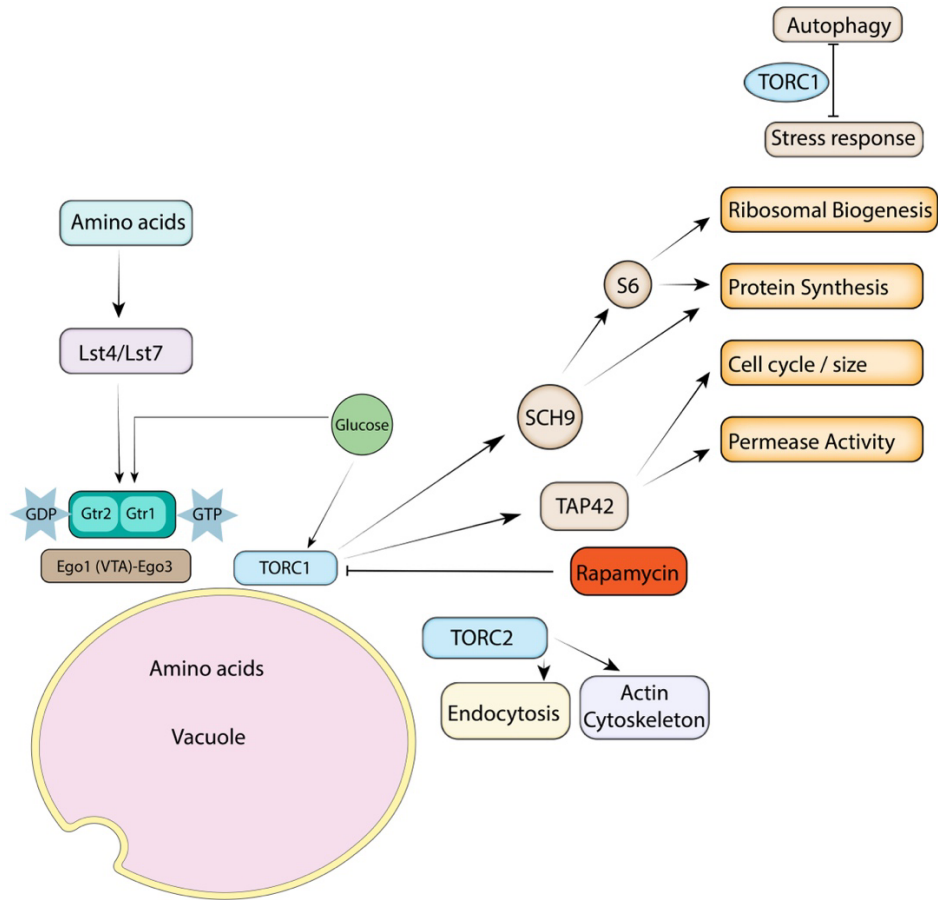
As mentioned previously (section 1.1.8.3), our lab's primary endeavour in the identification of the components of the FRQ-less oscillator as part of the circadian network of *Neurospora crassa*

has led to the discovery of components of TOR pathway, VTA and GTR2 (Eskandari et al., 2021; Li et al., 2011; Ratnayake et al., 2018). These components, in addition to their cognate roles in nutrient sensing and growth control, also appear to regulate circadian rhythmicity as the knockouts of *vta* and *gtr2* exhibit dampened conidiation rhythm and also compromised FRQ protein rhythms (Eskandari et al., 2021; Ratnayake et al., 2018). VTA (Vacuolar TOR associated protein), a homolog of yeast EGO1 and mammalian LAMTOR1, resides on the vacuolar membrane and is involved in transmitting the nutritional signals and regulation of the growth (Lakin-Thomas, 2019). The VTA binding partner, Gtr2, is a counterpart of yeast Rag-GTPase GTR2 and similar to VTA, aids in transmission of nutritional information to the TOR kinase (Eskandari et al., 2021) (Figure 1.10). Our laboratory has recently developed an assay for measuring the phosphorylation state of S6 ribosomal protein as a marker for TOR pathway activation in *Neurospora crassa* and based on this method we have discovered a robust circadian oscillation in the phosphorylation state of S6 protein when grown on solid agar cultures in constant darkness and temperature (Akhtari, 2023; Lakin-Thomas, 2023). These findings suggest that activity of TOR kinase in *Neurospora* is rhythmic. As mentioned earlier in section 1.2.4 and based on the sequence alignment from figure 1.11, S6 ribosomal protein is highly conserved among various eukaryotic species. The high degree of similarity especially in the core functional region of the protein reflects the essentiality of these regions in promoting the ribosomal structure and function.

Similar to the budding yeast ribosomal protein S6 (Rps6), which has two phosphorylation sites (Ser232 and Ser233) at the C-terminal region, the *Neurospora* S6 protein also appears to have two phosphorylation sites (González et al., 2015). Monitoring the phosphorylation state of *Neurospora* Rps6 by Western blotting reveals three distinct protein bands corresponding to the unphosphorylated (first band, lowest), monophosphorylated (second band, middle), and diphosphorylated (third band, top) forms of the protein. These bands are distinguished based on their molecular weights, approximately 48-63 kDa. Phosphatase treatment collapses the top two bands into the lowest band, confirming that the differences among the bands are due to phosphorylation levels (Akhtari, 2023).

Additionally, *prd-4* (period-4), the ortholog of checkpoint kinase 2 (CHK2) which is involved in DNA damage responses, has been found to be phosphorylated and activated by TOR kinase in

response to the translational stress (Diernfellner et al., 2019). Subsequently, Prd-4, phosphorylates the central TTFL protein FRQ and leads to the phase advancement of conidiation (Diernfellner et al., 2019). These findings, together with observations listed in Table 1.1, regarding the circadian regulation of TOR pathway in different organisms have redirected our focus towards exploring the potential links between the TOR pathway and circadian rhythmicity, by raising the possibility that TOR signaling might play a role in the generation of FRQ-less oscillation in *Neurospora crassa* (Lakin-Thomas, 2023). This thesis aims to investigate the metabolic regulation of the TOR pathway in *Neurospora crassa* to delineate the interplay between this pathway and circadian rhythmicity as the potential component of the FRQ-less oscillator, thereby contributing to a more comprehensive understanding of the molecular underpinning of the metabolic oscillators in the living organisms.



**Figure 1.10. Hypothetical diagram representing the TOR (Target of Rapamycin) pathway in *Neurospora crassa*.** VTA (Vacuolar TOR associated protein), a homolog of yeast EGO1, resides on the vacuolar membrane, tethering the small Rag-GTPase GTR2 to the vacuole and TORC1. VTA and Gtr2 are involved in transmitting the nutritional signals to TORC1 and the regulation of growth. Activation of TORC1 leads to phosphorylation of Sch9 (STK10), which subsequently results in the phosphorylation of S6 ribosomal protein and the activation of anabolic events. Since knocking out VTA and GTR2 proteins dampens the conidiation rhythms in *frq null* background and also compromises the FRQ protein rhythms in the *frq*-sufficient samples, they are thought to connect TOR pathway function with regulation of circadian rhythmicity. (Adapted from Eskandari et al., 2021).



Table1.1. Circadian clock-driven rhythmic TOR (Target of Rapamycin) pathway activity in various tissues and organisms measured by activation status of downstream effectors of the TOR signaling network.

| Organism/Cell Line                   | Observation                                                                                                                                                                                                                                 | Assay                                  | Reference            |
|--------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------|----------------------|
| Dorsoventral neurons of the SCN      | mTOR activity in the SCN follows a circadian pattern, with maximal activity during the subjective day and minimal activity at night. Importantly, p-S6 levels correlate with the expression pattern of clock gene period 1 ( <i>per1</i> ). | p-S6                                   | Cao et al., 2011     |
| Avian retinal cells                  | Activation of mTORC1 in chicken retina displays circadian regulation. Inhibition of mTORC1 dampens the L-type voltage-gated calcium channels (L-VGCCs) density at night.                                                                    | p-mTORC1, p-70S6                       | Huang et al., 2013   |
| Murine hepatocytes                   | Rhythmic expression and activation of components of mTORC1 correlate with the rhythmic expression of mRNAs encoding for translation initiation machinery.                                                                                   | p-S6, p-4E-BP                          | Jouffe et al., 2013  |
| Isolated murine hepatocytes          | mTORC1 activity measured by S6 phosphorylation levels oscillates daily. S6 phosphorylation is low during the light phase and high during the dark phase.                                                                                    | p-S6                                   | Cornu et al., 2014   |
| Isolated lung fibroblasts            | Circadian clock regulates the rhythmic activity of mTORC1 through the clock transcription factor BMAL1, and BMAL1 <sup>-/-</sup> cells exhibit increased mTORC1 activity and disrupted rhythmicity across different circadian cycles.       | p-S6                                   | Khapre et al., 2014  |
| Renal Carcinoma cell line            | 24-hour rhythms are observed in levels of p-mTOR, total mTOR, and p70S6K. Interestingly, mTOR protein oscillations are opposite to that of the LC3-II protein which is a marker of autophagy.                                               | p-mTORC1, p70S6K                       | Okazaki et al., 2014 |
| Mouse liver tissue                   | Rhythmic phosphorylation cycles of mTOR pathway effectors are lost in the livers from <i>Bmal1</i> <sup>-/-</sup> mice.                                                                                                                     | p-TSC2, ULK1                           | Robles et al., 2017  |
| <i>Drosophila melanogaster</i> brain | Expression of <i>Tor</i> gene follows a bimodal pattern in light/dark cycles. Silencing the <i>Tor</i> gene reduces the period of locomotor activity.                                                                                       | Expression patterns of <i>Tor</i> gene | Kijak and Pyza, 2017 |

| Organism/Cell Line                                                  | Observation                                                                                                                                                                                                                                       | Assay       | Reference            |
|---------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------|----------------------|
| <i>Arabidopsis thaliana</i>                                         | RPS6 phosphorylation peaks during the subjective night. In the absence of functional clock, the phosphorylation state of RPS6 lacks periodicity and does not follow a reproducible pattern.                                                       | p-RPS6      | Enganti et al., 2018 |
| Primary mouse hepatocytes, embryonic fibroblasts, and liver samples | mTORC1 activity oscillates diurnally, peaking during the subjective night. Upon fasting, the core clock protein Period 2 functions as a scaffolding protein that tethers TSC1, Raptor, and mTORC1 together and leads to the inhibition of mTORC1. | p-S6K, p-S6 | Wu et al., 2019      |

### 1.3 Hypothesis and Objectives

Our hypothesis is that the *Neurospora crassa* circadian network is likely comprised of multiple autonomous yet interconnected components in which the canonical FRQ-WCC oscillator (FWO) and other FRQ-Less oscillators (FLO) work together to generate robust rhythmic outputs. Previous work from our laboratory shows that when components of the TOR pathway are mutated, the FLO-based rhythms are compromised, suggesting that this pathway is likely involved in driving FRQ-less rhythmicity.

My long-term objective is to uncover the potential connections between the TOR pathway and the circadian clock, to delineate the role of the TOR pathway in *Neurospora* 's circadian network, and ultimately to elucidate the relationship between the metabolism and the circadian time-keeping systems.

My short-term objectives are, firstly, to identify the regulators of the *Neurospora* TOR pathway by using metabolites such as amino acids and lipids, which are known to be major regulators of this pathway in other well-characterized systems, including mammals and budding yeast (*S. cerevisiae*). Subsequently, I will analyze the effects of the factors that perturb TOR activity on the endogenous clock properties to determine whether altering TOR signaling impacts the clock-driven phenotypes.



## CHAPTER 02: Materials and Methods

### 2.1. *Neurospora crassa* strains:

All the *Neurospora crassa* strains used in this study were either obtained from the Fungal Genetics Stock Center (FGSC, Kansas State University, Manhattan, KS) or constructed in the laboratory by standard crossing techniques. All the strains carried the *ras-1<sup>bd</sup>*, *csp-1*, and *chol-1* mutations genotypes (Refer to Tables 2.1 and 2.2 for complete genotype and detailed description of each mutation and their associated phenotype). In order to measure the TOR pathway activity, S6 ribosomal protein was tagged with a FLAG epitope that facilitates the detection and isolation of the protein using commercially available anti-FLAG antibodies. The FLAG-tagged S6 gene (previously constructed in our lab) was crossed into all of the strains utilized in this study.

Table 2.1: The full genotype of *Neurospora crassa* strains used in this study. All strains carried the FLAG-tagged S6 gene.

| Strain              | Genotype                                         | Lab Stock Number |
|---------------------|--------------------------------------------------|------------------|
| Wildtype            | <i>csp-1; chol-1; ras-1<sup>bd</sup></i>         | 667              |
| $\Delta vta$        | <i>csp-1; chol-1; ras-1<sup>bd</sup>; vta</i>    | 694              |
| $\Delta gtr2$       | <i>csp-1; chol-1; ras-1<sup>bd</sup>; gtr2</i>   | 737              |
| Glutamine auxotroph | <i>csp-1; chol-1; ras-1<sup>bd</sup>; gln-1</i>  | 747              |
| Arginine auxotroph  | <i>csp-1; chol-1; ras-1<sup>bd</sup>; arg-13</i> | 744              |

Table 2.2. The full description of *Neurospora carassa* mutant strains used in this study and their associated phenotypes

| Name                                        | Abbreviation                              | Gene ID                       | Phenotype                                                                                                                                                                                                                                                                                                                                                                                                                                                                        |
|---------------------------------------------|-------------------------------------------|-------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Choline-1<br>(Choline-N-methyl transferase) | <i>chol-1</i>                             | NCU08045<br>Linkage group-IV  | The <i>chol-1</i> mutation is defective in the synthesis of the phospholipid phosphatidylcholine. On minimal medium, growth is slow. The membrane phospholipid composition is altered. The period of the conidiation rhythm is lengthened and temperature compensation is poor. Choline supplementation restores normal growth and rhythmicity. When <i>chol-1</i> strain is supplemented with choline the strain will be referred to as wild-type (Lakin-Thomas & Brody, 2000). |
| Band<br>(Ras-like protein)                  | <i>ras-1bd</i><br>(formerly bd)           | NCU08823<br>Linkage group-IV  | The bd mutation renders conidiation resistant to inhibition by excess CO <sub>2</sub> accumulated in closed culture vessels and enables the clear visualization of conidial banding (Lakin-Thomas & Brody, 2000)                                                                                                                                                                                                                                                                 |
| Conidial separation-1                       | <i>csp-1</i>                              | NCU02713<br>Linkage group-I   | The Csp-1 mutation is defective in the separation of macroconidia and reduces contamination (Lakin-Thomas & Brody, 2000).                                                                                                                                                                                                                                                                                                                                                        |
| Vacuolar TOR associated protein (UV90)      | <i>vta</i> knockout<br>( $\Delta vta$ )   | NCU05950<br>Linkage group-VI  | The <i>vta</i> knockout mutation results in the loss of FRQ-less conidiation rhythm in choline-starved <i>chol-1</i> background. It severely affects the heat entrainment of FRQ-less rhythm and dampens the amplitude of FRQ-WCC oscillator (Ratnayake et al., 2018).                                                                                                                                                                                                           |
| GTR2                                        | <i>gtr2</i> knockout<br>( $\Delta gtr2$ ) | NCU00376<br>Linkage group-III | The <i>gtr2</i> knockout mutation, similar to the <i>vta</i> ko, disrupts the FRQ-less conidiation rhythm in choline-deficient <i>chol-1</i> strain and compromises the heat-entrained FRQ-less rhythms. It also affects the amplitude of FWO (Eskandari et al., 2021).                                                                                                                                                                                                          |
| Glutamine auxotroph<br>(Gln synthase)       | <i>gln-1</i>                              | N/A                           | The <i>gln-1</i> mutation is defective in the conversion of L-glutamate to L-glutamine due to loss of function mutation in the gene encoding for Glutamine synthase (GS). It requires glutamine supplementation for normal growth. (Davila et al., 1983).                                                                                                                                                                                                                        |
| Arginine auxotroph                          | <i>arg-13</i>                             | N/A                           | The <i>arg-13</i> mutation is defective in the metabolism of L-arginine due to loss of function in <i>arg-13</i> gene. It exhibits slow growth rate in the absence of arginine supplementation (Davis, 1979).                                                                                                                                                                                                                                                                    |

## 2.2. *Neurospora crassa* culture systems:

### 2.2.1 Amino acid culture systems:

#### 1) Stationary liquid culture

Strains were maintained on slant cultures of minimal agar medium (agar-based growth medium solidified at an angle in a test tube, incubated at 30°C under constant light (LL), Appendix C). Conidia obtained from 6–8-day old minimal media slants were inoculated into 12-well plates. Each well contained 2 mL of liquid minimal media (MM), which included 2% glucose (as a carbon source), 1x Vogel's salt (providing both ammonium and nitrate salts for growth), and 100µM choline. Supplements were added as needed according to the experimental design. The cultures were grown for 48 hours at 22°C under LL until mycelial pads (densely packed hyphae) were formed. Pieces of mycelium were then transferred to 5ml petri plates containing fresh minimal media for a 4-hour recovery period to replenish the glucose and essential salts. Following this, the mycelial discs were placed in 5mL of fresh minimal media and harvested after 20 and 60 minutes using a suction filter. The samples were then frozen in liquid nitrogen and stored at -80°C.

The amino acid concentrations used in this study are based on the growth rate assays that were previously conducted in our laboratory to determine the “inhibitory” (the concentration of the amino acid that limits the growth of the auxotrophic mutant to 50% of the wild-type strain's growth) and the “rescue” concentration (which enables the auxotrophs to grow at a rate comparable to the wild-type), noted as IC (Inhibitory concentration) and RC (Rescue concentration) respectively.

#### 2) Solid agar culture

To assess the long-term effects of amino acid addition or depletion on the TOR pathway activity, cultures were grown on solid agar media supplemented with 0.5% maltose (preferred carbon source for solid culture experiments), 100µM choline, 2% agar, and 1x Vogel's salt with a trace of food coloring and supplements as needed. 150 mm diameter disposable petri plates were filled with 50 mL of sterile media and were left to cool down under UV light for 4 hours. To prevent the fungal hyphae from penetrating into the agar media and to facilitate sample collection, a layer

of cellophane that was cut to the size of the petri plate, then soaked and washed with distilled water and autoclaved for 15 minutes at 121°C, was laid on the solidified medium and left to further cool down under the UV light for a day. The next day, the plates were inoculated with fresh spore from the slant tubes at the edge of the plate and were incubated at 22°C in LL (constant light). Once the growth reached halfway across the plate (different length of time depending on the strain and the amino acid concentration), the final 1cm of mycelium growth front was scraped off the cellophane using a plastic spatula, the samples were then immediately frozen in liquid nitrogen and stored at -80°C.

#### Linear growth rate measurement on solid agar culture

To evaluate the effect of amino acid treatment on the linear growth rate, the mycelia growth front was marked daily. Once the growth period was completed (the cultures reached the edge of the plate), the bottom of each plate was scanned and analyzed using ImageJ software. Based on the position and timing of each mark, the average linear growth rate was calculated using two pre-coded Excel macros, FijiTau and MacTau (Lakin-Thomas, 1998).

#### Growth rate measurement by obtaining the biomass on solid agar culture

In order to determine whether the total growth rate was affected by amino acids, the mycelial biomass accumulation was measured. After the cultures reached the end of the petri plate, the entire mycelial growth front (~ 1cm) were harvested and dried for 48 hours in an 80°C oven in pre-weighed microfuge tubes to remove the excess moisture. The dried samples were weighed, and the net weights were calculated by subtracting the weight of the empty tubes.

#### 3) Shaking liquid culture with short-term (20 and 60 minutes) glucose-starvation period

To rule out the potential stimulatory effects of glucose on the TOR pathway activity of *N. crassa*, the effects of amino acid treatments were analyzed under short-term (20 and 60 minutes) glucose-starved conditions. Fungal spores from slant cultures were inoculated into 24-well plates, each well containing 1mL of liquid minimal media with 2% glucose, 1x Vogel's salt, 100 µM choline, and 0.2 % Tween 80 (added to prevent formation of spores). The cultures were grown for 48 hours at 22°C under LL. The mycelial discs (three per flask) were then transferred

to 150 mL flasks containing 50 mL of low-glucose minimal medium (0.08% glucose, 1x Vogel's salt, and 100 $\mu$ M choline). Amino acid supplements were added to the flasks and were shaken at 150 r.p.m on an orbital shaker at 22°C under LL (shaking ensures the even distribution of nutrients, pH, and oxygenation of the culture). The samples were harvested after 20 (if applicable) and 60 minutes, washed with water using a suction filter and were immediately frozen in liquid nitrogen and stored at -80°C.

#### 4). Shaking liquid culture with long-term (24-hour) glucose starvation period

To enable the fungus to adapt to the low-glucose culture environment described in the previous section, the cultures were subjected to a prolonged (24-hour) incubation period in non-growing, low-glucose media, after which the effects of glucose and amino acid treatments were tested on the TOR pathway activity of *N. crassa*. Fresh conidia from minimal medium slants were inoculated into 24-well plates containing 1 mL 2% glucose minimal media (2% glucose, 1x Vogel's salt, 100  $\mu$ M choline, and 0.2% Tween 80). The cultures were incubated for 48 hours at 22°C under LL. The mycelial mats (three per flask) were then transferred to 150 mL flasks containing 50 mL of low-glucose minimal medium (0.08% glucose, 1x Vogel's salt, and 100 $\mu$ M choline) and were shaken at 150 r.p.m for 24 hours at 22°C under LL. Following this, the glucose and amino acid treatments were added to the shaking cultures. The samples were then harvested after 60 minutes using a suction filter, frozen in liquid nitrogen, and stored at -80°C.

### 2.2.2 Lipid culture systems:

#### 1). Stationary liquid culture

In order to determine the effects of choline supplementation or depletion on the TOR pathway activity in *Neurospora crassa*, conidial inocula obtained from frozen stocks were incubated in 2 mL of liquid minimal media (MM) containing 2% glucose, 1x Vogel's salt, and 100 $\mu$ M choline (to confer normal growth to the choline-requiring *chol-1* strain). The cultures were grown for 48 hours at 22°C under constant light (LL). Following the formation of mycelial pads, they were transferred to 5mL fresh MM and were harvested after 1, 4, and 24 hours using a suction filter. For long-term storage, the samples were immediately frozen in liquid nitrogen and stored at -80°C.

To distinguish between the TOR pathway activity of *chol-1* mutant strains growing on choline-sufficient or choline-deprived conditions, the fungal spores were inoculated into 12-well plates, each well containing 2 mL of MM (+/-choline) and were incubated for 48 hours at 22°C under LL. Mycelial discs were then transferred to 5 mL fresh minimal media for a 4-hour recovery period to replenish the glucose and essential salts. Following this recovery period, the mycelial discs were placed in 5mL of fresh minimal media for the final incubation period and harvested after 5, 10, 20, and 60 minutes using a suction filter. The samples were then frozen in liquid N<sub>2</sub> and stored at -80°C.

The concentration of the diacylglycerol analog, DOG (1,2-dioctanoyl-*sn*-glycerol) used in this study was 250 µM. This concentration was chosen based on the previous observations that displayed the maximum effect on the period of conidiation (Ramsdale & Lakin-Thomas, 2000). DOG was diluted in dimethyl sulfoxide (Me<sub>2</sub>SO) to yield a final Me<sub>2</sub>SO concentration of 0.1% (v/v). DOG treatment was either added directly to the original growth media (48h initial growth medium) and harvested after 10, 20, 60 minutes and 4 hours, or added during the final incubation periods to the autoclaved growth medium. The control samples contained only the solvent (Me<sub>2</sub>SO) at the same concentration.

## 2) Solid agar Culture

To evaluate the long-term effects of choline-starvation on the TOR pathway activity of *Neurospora*, a solid agar culture system was employed. The cultures were grown on agar-based growth media containing 0.5% maltose, 2% agar, 1x Vogel's salt with or without choline supplementation, and a trace of food coloring for identification purposes. 50 mL of sterile growth media was filled into 150 mm diameter disposable petri plates, the cultures were left to solidify under UV light for 4 hours. Once the media cooled down, a layer of cellophane, which was previously triple-washed with distilled water and autoclaved for 15 minutes at 121°C, was laid on top of the growth medium to prevent the fungal hyphae from penetrating into the medium. The plates were left to dry under UV light for a day.

The following day, the cultures were initiated with conidial inoculations at the edge of the plates and were incubated at 22°C under LL. When the growth reached halfway across the media

(duration varied as choline-deprived *chol-1* mutant strain exhibits a slower growth rate compared to choline-sufficient cultures), the final 1cm of the mycelial growth front was scraped off the cellophane and immediately frozen in liquid N<sub>2</sub> and subsequently stored at -80°C.

### 3) Shaking liquid culture

To examine the effects of altered lipid composition on the TOR pathway activity of *Neurospora* in isolation from the potential stimulatory effects of glucose, the low-glucose shaking liquid culture system with short-term (20 and 60 minutes) and long-term (24 hours, if applicable) incubation periods were utilized. Fungal spores from slant cultures were inoculated into 24-well plates, each well containing 1mL of liquid minimal media with 2% glucose, 1x Vogel's salt, and 0.2 % Tween 80, with or without choline supplementation. The cultures were grown for 48 hours at 22°C under LL. The mycelial discs (five per flask) were then transferred to 150 mL flasks containing 50 mL of low-glucose minimal medium (0.08% glucose, 1x Vogel's salt, with or without choline). The flasks were shaken at 150 r.p.m on an orbital shaker at 22°C under LL. The samples were harvested after 20 and 60 minutes and 24 hours (if applicable), using a suction filter and were immediately frozen in liquid nitrogen and stored at -80°C.

The 250 µM DOG solution was added to low-glucose shaking flasks and the samples were harvested after 20 and 60 minutes. The Me<sub>2</sub>SO control samples contained the same concentration of the solvent only.

## 2.3. Protein Methods

### 2.3.1. Protein Extraction

Prior to the protein extraction, the harvested mycelial pads were stored at -80°C. Total protein was extracted by grinding the frozen samples with a liquid nitrogen pre-cooled mortar and pestle. The frozen mycelial powder was transferred to a 1.5 mL Eppendorf tube and suspended in ice-cold protein extraction buffer (PEB: 50 mM Tris pH 6.8, 2% SDS, 10% glycerol, and 1mM PMSF) at a volume of 1:1 (buffer: mycelial powder) ratio. The samples were then vortexed and heated at 80-100°C for 5 minutes, vortexed again, and placed on ice. The mycelial extracts were reheated at 100°C for 30 seconds, then centrifuged at 25°C and 12,000g for 10 minutes, and the supernatants were transferred to clean 1.5 mL Eppendorf tubes. To prepare dilutions for protein

assay, a 1:10 (5 µl of supernatant: 45 µl of PEB) and 1:3 (20 µl of supernatant: 40 µl of PEB) were made for each sample. From these dilutions, additional 1:100 (2.5 µl of 1/10 dilution: 22.5 µl of ddH<sub>2</sub>O) and 1:30 (2.5 µl of 1/3 dilution: 22.5 µl of ddH<sub>2</sub>O) was prepared.

### 2.3.2. Bio-Rad DC Protein Assay

To determine protein concentration, a standard curve of 0.5 mg/mL BSA (Bovine Serum Albumin) was used. The curve was generated using six concentrations of BSA: 0, 2.5, 5, 10, 15, 20 µl each added to 25, 22.5, 20, 15, 10, 5 µl of ddH<sub>2</sub>O, respectively. The Bio-Rad protein assay was performed according to the manufacturer's instructions (catalog number 500-0007). 3 x 300 µl aliquots of each sample from the 1:100 and 1:30 dilutions were loaded onto a 96-well plate and read using a plate reader spectrophotometer at 750 nm. The protein concentrations were calculated in mg/ml.

### 2.3.3. Protein Electrophoresis by SDS-PAGE

Discontinuous SDS-PAGE (Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis) was used to separate the different phosphorylated forms of the *Neurospora crassa* ribosomal S6 protein on a 12.5% acrylamide gel (Appendix C). 50 µl of (3.03 mg/mL stock concentration) functional molecule “Phos-tag” together with 50 µl of the divalent metal MnCl<sub>2</sub> (10 mM stock solution) were co-polymerized with the separating gel (Appendix C). Phos-tag selectively binds to phosphorylated amino acid residues such as serine, threonine, and tyrosine through its metal ion (usually Manganese, Mn) chelated with a dinuclear metal complex interacting with the negatively charged phosphate groups ( $-\text{PO}_3^{2-}$ ) on amino acids and facilitates their separation based on the phosphorylation levels (Fujifilm Wako Chemicals U.S.A. Corporation).

The protein samples (10 µg) were prepared using the concentrations determined by the standard curve and PEB for a total volume of 10 µl. Next, 0.1 µL of DDT (Dithiothreitol) and 0.5 µL of 0.4% bromophenol blue were added and the protein samples were vortexed and heated at 95-100°C for 2 minutes prior to gel loading. Tris-glycine-SDS buffer (Appendix C) was used as a running buffer and the protein separation was performed at 200 V for approximately 45-60 minutes. Following electrophoresis, the gels were washed with cathode buffer (Appendix C) and



EDTA (Ethylenediaminetetraacetic acid, pH 8), for three times, 15 minutes each time, to remove  $\text{MnCl}_2$ .

#### 2.3.4. Western Blotting

The proteins were blotted to a PVDF (polyvinylidene fluoride) microporous membrane (Millipore-Immobilon-P transfer membrane) using a semi-dry blotter and a three-buffer system (Anode buffer I, Anode buffer II, Cathode buffer, Appendix C) as instructed by Millipore. The area of each gel was calculated and the transfer was performed at a constant current of  $2 \text{ mA/cm}^2$  for 45 minutes.

#### 2.3.5. Immunodetection

Prior to antibody probing, the PVDF membrane was blocked with 1x blocking buffer (10x blocking buffer with 10% BSA diluted in 1x TBST (Appendix C) with continuous agitation for one hour at room temperature. Following this, the membrane was probed with primary antibody (monoclonal Anti-FLAG M2 from Sigma-Aldrich, diluted 1:10,000 in 1x blocking buffer) with continuous agitation for one hour. The membrane was then washed with TBST buffer three times, for 5 minutes each. Subsequently, the membrane was incubated with horseradish peroxidase-conjugated secondary antibody (Bio-Rad goat anti-mouse IgG-HRP, diluted 1:50,000 in 1x blocking buffer) for one hour at room temperature. The membrane was washed with TBST buffer four times, for 5 minutes each. Chemiluminescent detection reagents (Millipore Immobilon ECL) were applied and incubated for 5 minutes. The blot was exposed to a CCD camera (charge-coupled device) on the MicroChemi imager to capture the emitted light from the Chemiluminescent substrate.

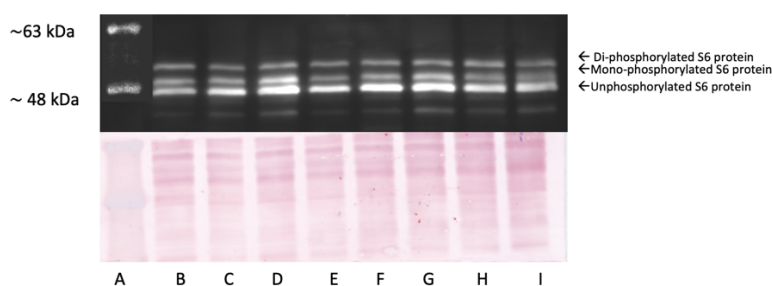
The *Neurospora crassa* S6 protein appears to have two phosphorylation sites, displaying three distinct protein bands on the western blot image which correspond to unphosphorylated (first band, lowest), monophosphorylated (second band, middle), and diphosphorylated (third band, top) forms of the protein. The bands are visible based their molecular weights, at approximately 48-63 kDa (Figure 2.1). Phosphatase treatment collapses the two top bands into the first (lowest) band, confirming that the difference in the bands is due to phosphorylation levels (Akhtari, 2023).

The ImageJ software was used to quantitate the signals of the S6 protein bands on the resulting image by measuring the mean grey value of the bands within a box of constant dimension. The net brightness of each band was determined by subtracting the background brightness. The chemiluminescent intensity of the three bands corresponding to the S6 protein was summed into one total value. To obtain the S6 phosphorylation levels, the value of the top band (highly phosphorylated) was divided by the total value (sum of the three bands).

#### 2.3.6. Staining of PVDF membrane with Colloidal Gold

For the total protein stain, the membrane was soaked in ddH<sub>2</sub>O and rinsed with 100% methanol for 1 minute. The membrane was then incubated with TTBS buffer (Appendix C) with continuous agitation, three times for 20 minutes each, and subsequently washed with ddH<sub>2</sub>O, three times for 2 minutes each. The membrane was then stained with Colloidal Gold (Colloidal Gold Total Protein Stain, Bio-Rad, catalog number 170-6527) with continuous agitation at room temperature until the bands were visible (duration of staining was varied depending on the strength of the stain and relative freshness) (Figure 2.1). The stained membrane was then rinsed with water and stored at 4°C.

For total protein quantification, the mean grey value of a representative section of each lane within a box of constant dimension was measured using ImageJ software to determine the total protein levels. To obtain the ratio of total S6 protein, the sum of the three bands in each lane was divided by the total protein. The background brightness was subtracted from each value.



**Figure 2.1. Sample western blot analysis of *Neurospora crassa* S6 ribosomal protein separated on 12.5% acrylamide gel.** The S6 protein has three bands corresponding to the different phosphorylation states: The bottom band (first) is the unphosphorylated form, the middle band (second) is the monophosphorylated version, and the top band (third) is the diphosphorylated form of the protein. The bands are located at around 48-63 kDa. The bottom panel is the immunoblot stained with Colloidal Gold total protein stain. The experiment analyzes the effects of leucine supplementation (with/without 0.5 mM Leu) on the S6 phosphorylation levels on low-glucose shaking culture without a 24-hour starvation period. (For the complete version of the blot, refer to the Appendix A).

| Lane | Sample                                       |
|------|----------------------------------------------|
| A    | BLUelf Protein Ladder                        |
| B    | $\Delta gtr2^-$ Leu, 20 min in shaking media |
| C    | $\Delta gtr2^-$ Leu, 60 min in shaking media |
| D    | $\Delta vta^+$ Leu, 48h initial Growth       |
| E    | $\Delta vta^+$ Leu, 20 min in shaking media  |
| F    | $\Delta vta^+$ Leu, 60 min in shaking media  |
| G    | $\Delta vta^-$ Leu, 48h initial Growth       |
| H    | $\Delta vta^-$ Leu, 20 min in shaking media  |
| I    | $\Delta vta^-$ Leu, 60 min in shaking media  |

## 2.4 Race Tube Assay

Since Torin compounds and DOG (1,2-dioctanoyl-*sn*-glycerol) are considered a biohazard, instead of regular glass tubes, sterile disposable plastic pipettes were used as race tubes. The 25 mL plastic pipettes (Falcon #357525) were filled with 10 mL of sterile minimal media (MM) containing 0.5% maltose, 2% agar, 1x Vogel's salts, and different choline concentrations (0, 10, 50, and 100  $\mu$ M). The growth medium was supplemented with various concentrations of Torin I (100, 200, 1000 nM) from 1 mM stock solution and Torin II (20, 40, 200, 500 nM) from 5 mM stock solution, these concentrations were adapted based on the previous growth rate assay done in our laboratory. To investigate the effects of elevated diacylglycerol (DAG) levels, 250 $\mu$ M of membrane-permeant DAG analog, DOG, 1,2-dioctanoyl-*sn*-glycerol (from 86 mg/ml stock) was used (this concentration was chosen based on the previous study that showed the maximum effect on the period of conidiation (Ramsdale & Lakin-Thomas, 2000)). All chemicals were diluted in Me<sub>2</sub>SO (DMSO, dimethyl sulfoxide) and added to autoclaved growth medium before

filling race tubes, to yield a final Me<sub>2</sub>SO concentration of 0.1% (v/v). The untreated control tubes contained only the minimal media without any treatments. Prior to the filling of the tubes, they were washed with the corresponding sterile MM to remove any potential impurities, then they were filled with the growth media and were left to solidify under the UV light for a few hours.

The tubes were inoculated with small mycelial plugs (to the size of the wide end of a sterile 1mL micropipette tip) obtained from the growing front on the starter or seed plates (50 mL petri plates) which contained the same MM (without the treatments) and were incubated at 30°C under constant light (LL) for 2 days. After the inoculation, the race tubes were incubated at 30°C in LL for 24 h to initiate the growth. Following this, the cultures were transferred to 22°C and were incubated in constant darkness (DD). The mycelial growth front was marked daily under the red safe light, noting the time and date. Once the growth period was completed, the race tubes were scanned using ImageJ software. The linear growth rate and the period of conidiation were calculated using two pre-coded macros, FijiTau and MacTau as described in Lakin-Thomas, 1998.

## 2.5. Statistical Analysis

Data analysis and graphing of all experiments were performed using GraphPad Prism v. 10.1.0 (264) software. ANOVA (Analysis of variance) was conducted for statistical significance; the significant results were followed with multiple comparison tests. The paired t-test was performed when comparing the means of two groups. The graphs were plotted as mean  $\pm$  S.E.M (Standard Error of the Mean), derived from n (the number of independent trials) experiments. The fold increase for the applicable experiments was calculated by  $\frac{\text{mean of the treatment}}{\text{mean of the control}}$ .

## CHAPTER 03: Results and Discussion

### 3.1 Amino Acid Regulation of TOR Pathway Activity in *Neurospora crassa*

#### 3.1.1 Introduction and Objectives

The target of Rapamycin (TOR) is an evolutionarily conserved serine/threonine protein kinase that orchestrates metabolic homeostasis by promoting the biosynthetic anabolic pathways while suppressing the catabolic events such as autophagy (Jewell et al., 2013). TOR complex 1 (TORC1), a homologue of the mTORC1 (mammalian target of rapamycin complex 1), is a key integrator of environmental cues that regulates cell growth in response to nutrient availability, energy status, and stress conditions (Avruch et al., 2009). Nutrient sufficiency, particularly glucose and amino acids, activates TORC1 and stimulates the phosphorylation of its downstream targets: the ribosomal protein S6 kinase (S6K, Sch9 in budding yeast) and eukaryotic translation initiation factor 4E binding protein (4E-BP1, Tap42 in budding yeast) (Avruch et al., 2009; Bröer & Bröer, 2017). The activated S6K1 phosphorylates the 40S ribosomal protein S6, leading to increased ribosomal biogenesis, while the phosphorylation of the translational repressors, 4E-BP1 ultimately results in cap-dependent translation initiation, which collectively increases the overall translational capacity of the cell (Melick & Jewell, 2020).

Amino acid availability modulates TORC1 activity via different mechanisms that largely involve the conserved RAG family of small GTPases, namely the Gtr1 (ortholog of mammalian Rag A/B) and Gtr2 (Rag C/D) proteins in *S. cerevisiae* (González & Hall, 2017). The Gtr1- Gtr2 heterodimer localizes to vacuolar membranes via an interaction with the EGO (Ego1-3) complex (Powis & De Virgilio, 2016). In the presence of amino acids, the active conformation of the Rag heterodimer (Gtr1GTP-Gtr2 GDP) directly binds to TORC1 via its regulatory subunit, Kog1 (Raptor in mammals) and stimulates its activity (Powis & De Virgilio, 2016).

Despite the significant progress in understanding the mechanistic regulation of the TOR pathway in response to amino acids in both mammals and *S. cerevisiae*, the detailed characterization of this pathway in the filamentous fungus *Neurospora crassa* is still unclear. In this section, I aimed to investigate the effects of various amino acids on the TOR pathway activity in *N. crassa*. I used a novel assay to examine the TOR pathway activity by measuring the phosphorylation state of endogenous S6 ribosomal protein in response to different amino acid treatments.

mTORC1 activity appears to be particularly sensitive to the levels of leucine and arginine, as the starvation of these amino acids has potent inhibitory effects on mTORC1 signaling (Lutt & Brunkard, 2022). However, yeast TORC1 responds best to glutamine as both an amino acid and nitrogen source (González & Hall, 2017). In *Neurospora crassa*, glutamine is also the preferred amino acid and nitrogen source for growth; arginine is also a prominent nitrogen source, as the fungi store large amounts of arginine in vacuoles and release it when glutamine is depleted (Davis & Perkins, 2002; Legerton & Weiss, 1984). Therefore, I chose to assay the effects of three amino acids: leucine (Leu), arginine (Arg), and glutamine (Gln) on the TOR pathway activity in *Neurospora*. Additionally, I sought to investigate whether, similar to the mammalian and yeast systems, the amino acid-mediated responses are transmitted to TORC1 through the Rag GTPase-EGO complex by using the *Neurospora* counterparts Gtr2 (equivalent of Gtr2 in budding yeast) and VTA (ortholog of Ego in yeast) knockout strains, which were previously identified and characterized in our laboratory based on their role in compromising the FRQ-less oscillator and dampening the FRQ protein rhythms (Eskandari et al., 2021; Ratnayake et al., 2018). Lastly, I analyzed the effects of amino acid starvation on the TOR pathway signaling in amino acid auxotrophs as nutritional stress in the form of nitrogen starvation has been shown to increase the cytosolic amino acid pool by mobilizing the metabolically inactive vacuolar pool and to accelerate protein turnover in the mycelia of *Neurospora crassa* (Legerton & Weiss, 1984).

### 3.1.2. Results & Discussion

#### 3.1.2.1 Characterization and optimization of the culture conditions

The metabolic regulation of the TOR pathway in *Neurospora crassa* has not been studied before; as a result, the kinetics of TOR activation and the degree of response remain largely unclear. Thus, due to the novelty of this project, the majority of the culture systems used during the course of this thesis were adopted from biochemical studies of circadian rhythms which were not primarily designed to assess the regulation of nutrient and stress-sensitive TOR pathway.

Assessing the TOR pathway regulation of *N. crassa* in different culture systems has provided an opportunity not only for the optimizing the culture conditions but also for providing a comprehensive analysis of *Neurospora* TOR pathway's response to various treatments including different amino acids that without a mixed approach would not be possible. For example, the stationary liquid culture system enables a variety of biochemical studies at short term intervals. However, liquid media can quickly become depleted of nutrients, which can impede the long-term observations (Hurley et al., 2015). To obviate such disparity, a solid culture system can be employed that simulates the fungus's natural habitat and allows for investigation of prolonged consequences of the tested treatments, but because the changes in local pH, waste accumulation, and nutrient availability are not rigorously controlled under such conditions, it can lead to slow diffusion of metabolic by-products and ultimately result in localized physiological changes (Cockrell et al., 2015). The shaking liquid media appear to be a more suitable approach for studying the metabolic states as continuous agitation allows for more uniform access to nutrients and provides ample oxygenation, ensuring a stable and consistent metabolic environment (Kelliher et al., 2020).

#### 3.1.2.2. Effects of Various Amino acids on the TOR Pathway Activity of *Neurospora crassa*

Main Result: Amino acid treatments increase TOR pathway activity, measured by S6 phosphorylation in wild-type *Neurospora crassa*, only after prolonged carbon-source starvation

TORC1 in both mammals and budding yeast senses amino acids to sustain normal growth, metabolism, and proliferation (D. Meng et al., 2020). Amino acids such as leucine and arginine are essential for attaining the maximal activation of mTORC1, while glutamine is a potent activator of yeast TORC1 (González & Hall, 2017; Sancak et al., 2008). If *Neurospora*'s TOR

pathway is regulated by amino acids similar to the aforementioned systems, then amino acid supplementation is expected to affect the phosphorylation levels of the downstream target of TORC1, the S6 ribosomal protein. To test this, I supplemented *Neurospora*'s minimal media (MM: containing 2% glucose as a carbon source, Vogel's salts, providing both ammonium and nitrate salts essential for the growth, and 100 $\mu$ M choline) with leucine (Leu, 0.5mM), arginine (Arg, 2.5mM), and glutamine (Gln, 5mM) and analyzed the effects of individual amino acids (which were supplemented as part of MM during all the incubation periods: 48h initial growth period, 4h recovery period, and the subsequent 20 and 60 min incubations) on the S6 protein phosphorylation levels after the initial 48-hour growth period and following 4-hour recovery period to replenish the carbon and nitrate sources. Amino acid concentrations used here are "rescue" concentrations determined previously based on the growth rate assays conducted in our laboratory. Because leucine and arginine activate mTORC1 rapidly (~15 min) while the glutamine response is relatively slow (~60 min), I chose to assay the amino acid responses after 20 and 60 minutes of the transfer to a fresh medium. Utilizing this method, I found that supplementing the *Neurospora*'s minimal medium with Leucine, arginine, and glutamine did not significantly alter the S6 phosphorylation levels compared to the non-amino acid treated controls at any of the tested time-points (Figure 3.1.1).

Since no short-term amino acid responses were found, I sought to analyze the effects of amino acids on cultures growing on media for long-term. To assess this, I incorporated the same amino acids: Leucine (0.5 mM), Arginine (2.5 mM), and Glutamine (5 mM) into the solid agar minimal media, containing maltose as the preferred carbon source for solid culture experiments, Vogel's salts, 100 $\mu$ M choline, and agar. The cultures were grown for 4 days until the mycelium growth front has reached the middle of the agar. As shown in Figure 3.1.2, the S6 phosphorylation levels appeared to be unaffected by any of the tested amino acids in comparison with the untreated control.

In a study by Alfatah et al., (2021), it was found that glucose is sufficient and necessary for the activation of TORC1 in *S. cerevisiae*. In the same study, wild-type yeast cells were initially grown in a Synthetic Complete medium (SC) with 2% glucose, then transferred to a SC without the glucose. After 60 minutes of the transfer, Sch9 (S6K in mammals, enzyme responsible for phosphorylating S6 protein) was completely dephosphorylated. TORC1 was reactivated upon



addition of the glucose (Alfatah et al., 2021). Based on these findings, I hypothesized that, if *Neurospora*'s TOR pathway regulation follows a pattern similar to the budding yeast, then analyzing the effects of amino acids in low-glucose cultures, where the stimulatory effects of glucose are depleted, would allow to study the isolated effects of amino acids on the TOR pathway activity. To examine this, the fungal samples after 48-hours initial growth in 2% glucose, were transferred to a low-glucose (0.08%) shaking cultures with the tested amino acids (Leu (0.5mM), Arg (2.5mM), and Gln (5 mM)) for 60 minutes. Utilizing this method, no statistically significant differences were found between the amino acid treated samples and the un-transferred and non-treated control (Figure 3.1.3).

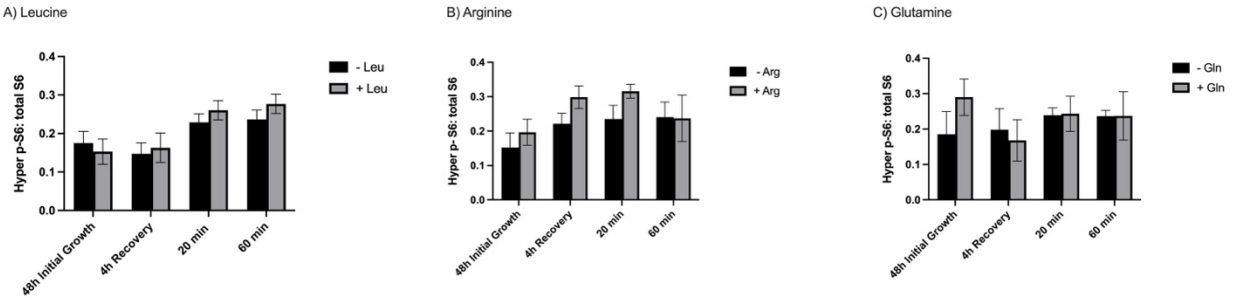
Following this, it was reasoned that a 60-minute incubation period in the low-glucose culture may not have been sufficient time to allow the fungus to equilibrate to this environment. Therefore, in the next series of experiments, after 48 hours of initial growth in 2% glucose, the fungal samples were transferred to low-glucose shaking cultures for 24 hours, then glucose (2%) and amino acid treatments (Leu (0.5mM), Arg (2.5mM), and Gln (5 mM)) were added directly into the shaking culture and harvested after 60 minutes. Interestingly, as shown in figure 3.1.4, after 24-hour incubation in the low-glucose environment, glucose supplementation resulted in a pronounced increase in S6 phosphorylation levels in comparison with the control sample (3.19-fold increase). Surprisingly, all three tested amino acids, leucine (2.07-fold), arginine (2.63-fold), and glutamine (2.30-fold) also significantly elevated the p-S6 levels compared to the control version, although to varying degrees. Perhaps another compelling finding here is the selective preference of *Neurospora*'s TOR pathway in using different metabolites. While glucose as the main preferred carbon source increased the phospho-S6 levels dramatically ( $p < 0.0001$ ), the amino acids that can serve as nitrogen sources such as arginine and glutamine elevated the TORC1 activity to a higher degree compared to leucine which acts as a poor nitrogen source for the fungus (Figure 3.1.4).

*Neurospora crassa* has modest nutritional requirements, the most important one being a carbon source such as glucose or sucrose, and a nitrogen source (Davis & Perkins, 2002). Wild-type *Neurospora* cells are able to synthesize all amino acids de novo but can also scavenge them from the environment. Although the fungus uses many amino acids as a nitrogen source, some amino acids can be used as alternative carbon sources as well (Davis, 2000). The results obtained from

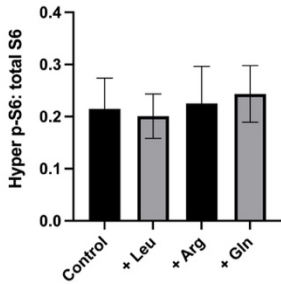
this section suggest that when the carbon sources such as glucose and maltose are readily supplied, the amino acid treatment does not affect the activity of TOR pathway (Figures 3.1.1 and 3.1.2). Even during the short-term glucose-starvation period, amino acids do not alter the phosphorylation of S6 protein (Figure 3.1.3). Only after prolonged glucose-starvation period, amino acids increase the phospho-S6 levels significantly (Figure 3.1.4). A possible explanation for these results is the presence of an essential regulatory mechanisms in many microorganisms including *Neurospora*, known as Carbon Catabolite Repression (CCR) pathway (Davis, 2000). CCR refers to a mechanism by which easily-used and favorable carbon sources such as glucose repress the enzymes required for the utilization of other less preferred compounds (Xiong et al., 2014). In *Neurospora*, the strength of CCR is tuned by glucose sensing and signaling. Although the crosstalk between CCR and TOR pathway has not been elucidated yet, interesting observations have been done in *S. cerevisiae* (Noël et al., 2021; Peter et al., 2006; Xiong et al., 2014). In budding yeast, glucose-mediated CCR was found to be a regulator of amino acid permeases, as the uptake of amino acids such as L-arginine and L-leucine was dramatically increased in the absence of glucose as the carbon source (Peter et al., 2006). Strikingly, the deletion of the yeast TORC1 abolished the CCR-induced amino acid uptake (Peter et al., 2006). An early study in *Neurospora* measured the activity of a catabolite-repressed enzyme, trehalase and found a clear inverse relationship between the activity of this enzyme and the mycelial dry weight when grown on different carbon sources (Hanks & Sussman, 1969). The dry weight was highest when glucose, sucrose, and maltose were used as carbon sources, while the activity of trehalase was lowest under these conditions, which may point to a similar CCR-mediated regulatory mechanisms in *N. crassa* (Hanks & Sussman, 1969).

Taken together, the results of this section provide novel and preliminary insights into the amino acid regulation of the TOR pathway of *Neurospora crassa*, emphasizing that only after prolonged glucose-starvation period, amino acid treatments can increase S6 phosphorylation levels. Further experiments that monitor the amino acid permease activities in the presence and absence of glucose can provide detailed description of whether glucose-induced CCR is responsible for the sensitivity or insensitivity of the TOR pathway to amino acids.

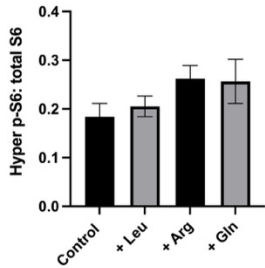
3.1.1) When glucose is available as a carbon source, amino acids (AAs) have no effect on the phosphorylation levels of S6



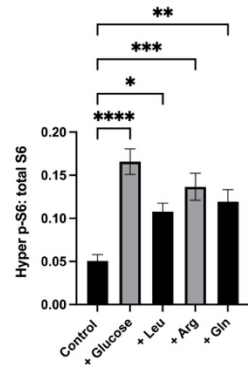
3.1.2) When maltose is available as a carbon source, AAs have no effect on the phosphorylation levels of S6



3.1.3) In low glucose-cultures with short-term glucose-starvation period, AAs have no effect on the phosphorylation levels of S6.



3.1.4) In low glucose-cultures with long-term glucose starvation period, AAs have significant effects on the phosphorylation levels of S6.



**Figure 3.1. Effects of various amino acids on the TOR pathway activity of *Neurospora crassa*.**

**3.1.1) When glucose is available as a carbon source, amino acids (AAs) have no effect on the phosphorylation levels of S6.** The wild-type *Neurospora crassa* (WT, strain #667) was grown on liquid minimal media (MM) culture with 2% glucose, 1x Vogel's salt, and 100µM choline for 48h at 22°C under LL. The mycelial pads were then transferred to fresh MM for 4 hours as a recovery period. The fungal discs were then transferred to a fresh MM and were harvested after 20 and 60 minutes. The amino acid treatment groups were supplemented with A: (+Leu, 0.5 mM leucine), B: (+Arg, 2.5 mM arginine), and C: (+Gln, 5 mM glutamine) as part of their MM. The S6 phosphorylation levels were measured by western blotting. The data are plotted as the mean± SEM of A (n=9), B (n=3), C (n=3) independent trials. A two-way ANOVA test was performed to determine the statistical significance (for detailed statistical analysis refer to Appendix B, table 3.1.1).

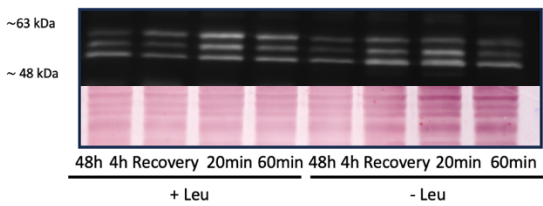
**3.1.2) When maltose is available as a carbon source, AAs have no effect on the phosphorylation levels of S6.** The wild-type (WT, strain #667) was grown on solid agar culture containing 0.5% maltose, 1x Vogel's salt, 100µM choline, 2% agar and without (control) or with amino acid treatments as follows: + Leu (0.5 mM leucine), +Arg (2.5 mM arginine), +Gln (5 mM glutamine) at 22°C under LL. The S6 phosphorylation levels were measured when the mycelial growth had reached the halfway across the agar medium using western blot analysis. A one-way ANOVA test was performed to determine the statistical significance. The data are plotted as the mean± SEM of n=3 independent trials (for detailed statistical analysis refer to Appendix B, table 3.1.2).

**3.1.3) In low glucose-cultures with short-term glucose-starvation period, AAs have no effect on the phosphorylation levels of S6.** The wild-type (WT, strain #667) was grown in liquid media with 2% glucose, 1x Vogel's salt, 100 µM choline, and 0.2 % Tween 80 for 48h at 22°C under LL. The fungal discs were then transferred to low-glucose media containing 0.08% glucose, 1x Vogel's salt, and 100µM choline with the amino acid treatments: + Leu (0.5 mM leucine), +Arg (2.5 mM arginine), +Gln (5mM glutamine). The flasks were shaken at 150 r.p.m on an orbital shaker at 22°C under LL. The samples were harvested after 60 minutes incubation period. The control sample was harvested after 48h initial growth. The S6 phosphorylation levels were monitored by western blotting. A one-way ANOVA test was performed to determine the statistical significance. The data are plotted as the mean± SEM of n=3 independent experiments (detailed statistical analysis are depicted at Appendix B, table 3.1.3).

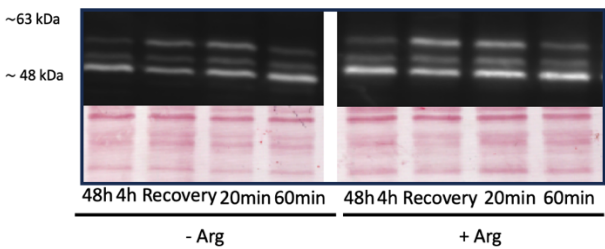
**3.1.4) In low glucose-cultures with long-term glucose starvation period, AAs have significant effects on the phosphorylation levels of S6.** The wild-type (WT, strain #667) *Neurospora* spores were grown on 2% glucose minimal media (2% glucose, 1x Vogel's salt, 100 µM choline, and 0.2% Tween 80) for 48h at 22°C under LL. The mycelial pads were then transferred to a low-glucose shaking flask (containing 0.08% glucose, 1x Vogel's salt, and 100µM choline) with continuous agitation at 150 r.p.m for 24h at 22°C under LL. The treatments: +Glucose (2% glucose), + Leu (0.5 mM leucine), +Arg (2.5 mM arginine), +Gln (5mMglutamine) were added directly to the shaking flasks and the samples were incubated for 60 minutes. The control sample was harvested after 24h incubation in low-glucose culture. The S6 phosphorylation levels were measure by western blotting. The data are plotted as the mean± SEM of n=6 independent trials. A one-way ANOVA followed by multiple comparison test was performed to determine the significance of the data (Appendix B, table 3.1.4). Asterisks (\*) denote the statistically significant differences between conditions as follows: \* p < 0.05, \*\* p < 0.01, \*\*\* p < 0.001, \*\*\*\* p < 0.0001. The immunoblots stained with Colloidal Gold total protein stain for each experiment are provided below.

3.1.1

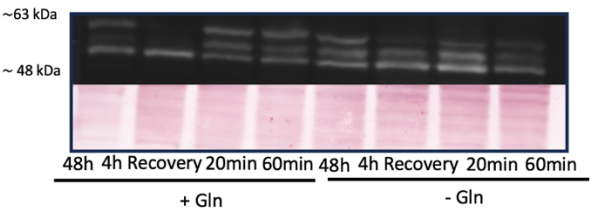
A) Leucine



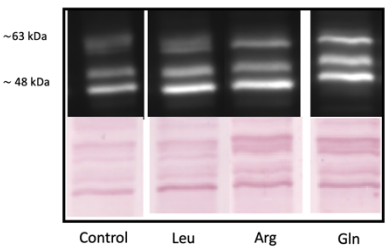
B) Arginine



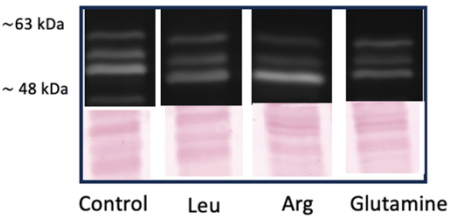
C) Glutamine



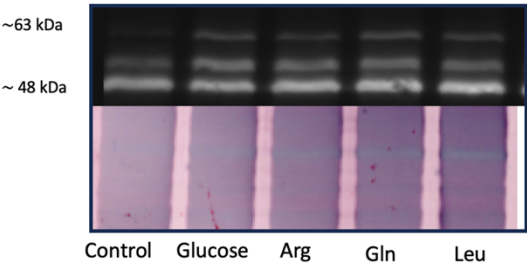
3.1.2



3.1.1



3.1.4



### 3.1.2.3 Amino Acid Regulation of *Neurospora crassa*'s TOR Pathway via GTR2-VTA Signaling

**Main Results:** The deletion mutations of *vta* and *gtr2* attenuate the amino acid-stimulation of TOR pathway in *Neurospora crassa* only after prolonged carbon-source starvation. The VTA protein plays a more pronounced role in metabolite-induced TOR activation compared to GTR2.

The amino acid-induced activation of TORC1 in both mammals and budding yeast is regulated by the conserved family of small Rag GTPases, namely the yeast's Gtr1 (homolog of mammalian Rag A/B) and Gtr2 (equivalent to mammalian Rag C/D) proteins (González & Hall, 2017). The Gtr1-Gtr2 heterodimer localizes to the vacuolar membrane via an interaction with EGO (Ego1-Ego2-Ego3) complex and transmit the amino acid signals to TORC1 via interaction with its regulatory subunit Kog1 (Alfatah et al., 2023; Powis & De Virgilio, 2016). If *Neurospora* follows the same consensus model of amino acid signaling for TOR activation via the Rag GTPase-EGO complex, then it is expected that the deletion of these components would compromise the amino acid-mediated S6 phosphorylation. In order to investigate this, I used the *Neurospora* knockout strains  $\Delta gtr2$  (GTR2<sup>KO</sup>, equivalent of Gtr2 in budding yeast) and  $\Delta vta$  (VTA<sup>KO</sup>, ortholog of Ego in yeast). Both of these strains were identified and characterized in our laboratory based on their role in disrupting the canonical (FWO) and non-canonical (FLO) circadian rhythmicity of *N. crassa* (Eskandari et al., 2021; Ratnayake et al., 2018).

Initially, I compared the ability of amino acids leucine (0.5 mM), arginine (2.5 mM), and glutamine (5 mM) to activate TORC1 in wild-type,  $\Delta vta$ , and  $\Delta gtr2$  strains grown on 2% glucose minimal media (MM) for 48 hours. Following this, the mycelial discs were incubated in fresh 2% glucose MM to replenish the glucose and Vogel's solution sources during the 4-hour recovery period. Additional mycelial samples were taken after 20- and 60-minute incubation in fresh MM with glucose. The amino acid treatments were added to the MM during all mentioned time points (48h initial growth period, 4h recovery period, and the subsequent 20 and 60 min incubations). In mammals and *S. cerevisiae*, both leucine and arginine activate TORC1 via the Rag GTPase-EGO (LAMTOR in mammals) complex; however, glutamine-induced activation of TORC1 utilizes a Rag-GTPase independent route (involving Pib2 in budding yeast and Arf1 in mammals) (Y. Meng et al., 2022; Takahara et al., 2020). Therefore, it was speculated that depending on the route of action of each amino acid, the deletion of VTA and GTR2 could affect

S6 phosphorylation differently. As shown in figure 3.2.1, the deletion of VTA and GTR2 proteins had no significant effects on the P-S6 levels compared to the wild-type strain at any of the tested intervals. Although, the S6 phosphorylation levels appeared to be unaffected by deletion mutations of *vta* and *gtr2* under these experimental conditions, the results obtained from this section accord with earlier observations in the previous section, implying that in the presence of glucose, neither the amino acids nor the deletion of proteins potentially involved in linking their signals have noticeable effects on the activity of *Neurospora*'s TOR pathway (Figures 3.1.1 and 3.2.1).

As mentioned previously, the TORC1 activity in *S. cerevisiae* is highly sensitive to glucose (Alfatah et al., 2021; Hughes Hallett et al., 2014). Recently, it has been reported that glucose can activate TORC1 through three distinct pathways involving both Rag-GTPase-dependent and -independent mechanisms; the addition of glucose to the yeast growth medium results in phosphorylation of Sch9 in the *Δgtr1* strain to the same extent as the wild-type only with 10 minutes delay in the response (Alfatah et al., 2021, 2023). Therefore, to account for this and to compare the effects of *vta* and *gtr2* deletions on the amino acid regulation of the TOR pathway without the interference of glucose, the next series of experiments were performed in low-glucose shaking cultures with short-term (20 and 60 minutes) and long-term (24 hours) glucose-starvation periods.

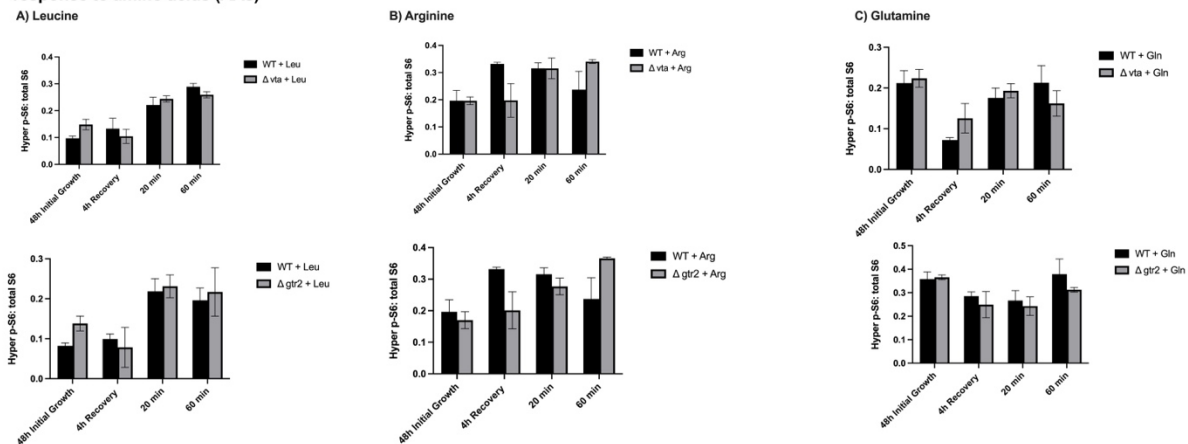
In the short-term glucose-starvation experiments, after the initial 48-hour growth in 2% glucose MM, the fungal discs were incubated in low-glucose (0.08%) shaking cultures and the aliquots were taken after 20 and 60 minutes. The amino acid treatments, leucine (0.5mM) and glutamine (5 mM) were incorporated as part of MM both during the initial growth and the short-interval incubation periods of all three tested strains (wild-type, *Δvta*, and *Δgtr2*) (arginine was not tested under these experimental conditions). Overall, based on the results of these experiments, in short-term glucose-starved cultures, knockouts of VTA and GTR2 did not significantly alter the S6 phosphorylation levels in response to leucine and glutamine stimulation compared to the wild-type strain (Figure 3.2.2).

Interestingly, when the amino acid supplements were added after the 24-hour glucose-starvation period, the deletion mutation of both VTA and Gtr2 resulted in significant differences in the

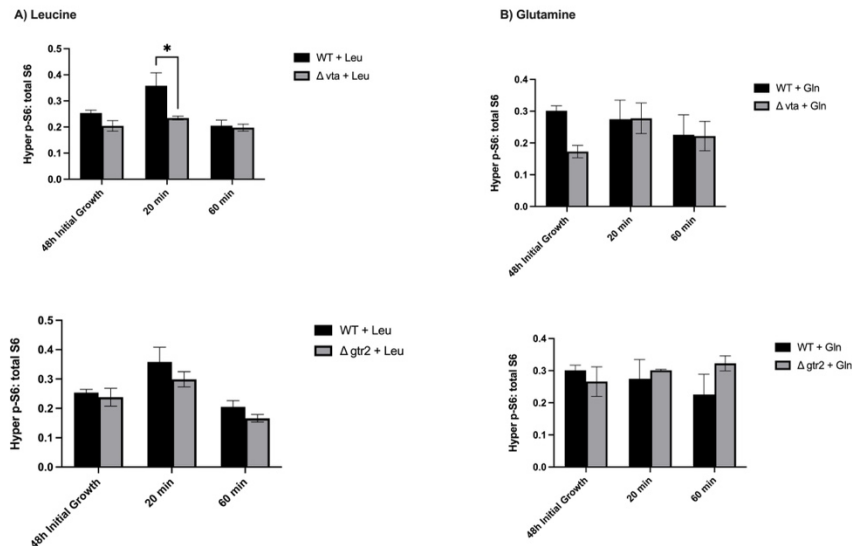
metabolite-induced phosphorylation of S6 protein in relation to the wild-type strain (Figure 3.2.3). As the results indicate, the *vta* gene product appears to play a more pronounced role in both glucose- and amino acid-induced activation of TORC1 which is evident from the marked diminished responses to glucose, leucine, and especially glutamine in  $\Delta vta$  strain compared to the wild-type counterpart. These results suggests that the VTA protein is an important mediator of nutrient-induced activation of the TOR pathway in *N. crassa*. The Gtr2 protein is likely to play a less essential role compared to the VTA. This might be due to the presence of the functional Gtr1 that may result in the functional overlap and potential redundancy between these two Rag GTPases. Indeed, the Vam6 (homotypic vacuolar fusion protein)-Gtr1-Ego axis is likely to be the main route to promote amino acid-dependent TORC1 activation in both *S. cerevisiae* and *S. pombe* (Powis & De Virgilio, 2016; Valbuena et al., 2012). In conclusion, the results of this section corroborate with the previous findings that only after prolonged carbon-source starvation, when amino acids responses are detected on the TOR pathway activity, the deletion mutation of *vta* and *gtr2* affect S6 phosphorylation levels (Figures 3.1.4 and 3.2.3).



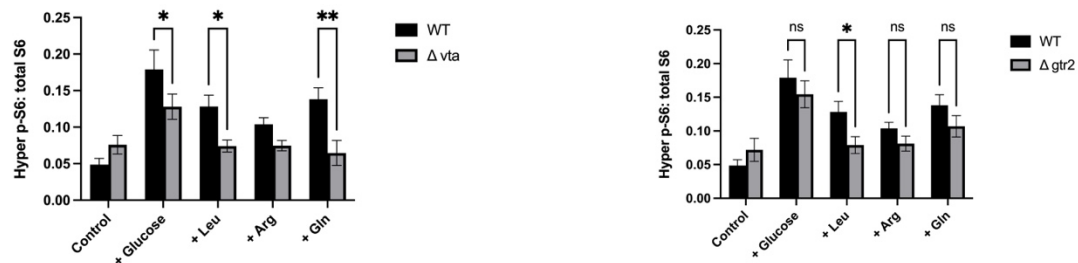
**3.2.1) When glucose is available as a carbon source, the deletion mutations of *vta* and *gtr2* have no effect on the phosphorylation levels of S6 in response to amino acids (AAs)**



**3.2.2) In low-glucose cultures with a short-term (20 and 60 minutes) glucose-starvation period, the deletion mutations of *vta* and *gtr2* have no overall effect on the phosphorylation levels of S6 in response to AAs**



**3.2.3) In low-glucose cultures with long-term (24 hours) glucose-starvation, the deletion mutation of the *vta* significantly diminishes S6 phosphorylation in response to metabolites while the, *gtr2* deletion exhibits less pronounced effects**



**Figure 3.2. Amino acid regulation of *Neurospora crassa*'s TOR pathway via GTR2-VTA signaling.**

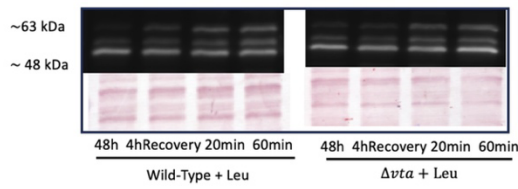
**3.2.1) When glucose is available as a carbon source, the deletion mutations of *vta* and *gtr2* have no effect on the phosphorylation levels of S6 in response to amino acids (AAs).** The wild-type (WT, strain#667), *VTA*<sup>KO</sup> (*Δvta*, strain#694), and *GTR2*<sup>KO</sup> (*Δgtr2*, strain#737) strains were grown on liquid minimal media (MM) with 2% glucose, 1x Vogel's salt, and 100μM choline for 48h at 22°C under LL. The mycelial discs were then incubated in a fresh 2% glucose MM for 4 hours as a recovery period. The fungal discs were then transferred to a fresh MM and were harvested after 20 and 60 minutes. The amino acid treatments leucine (+Leu, 0.5 mM), arginine (+Arg, 2.5 mM), and glutamine (+Gln, 5 mM) were supplemented in MM at all of the mentioned incubation periods of all three strains. The S6 phosphorylation levels were measured by western blotting. The data are plotted as the mean± SEM of leucine (*Δvta* n=3, *Δgtr2* n=3), Arginine (*Δvta* n=3, *Δgtr2* n=3), Glutamine (*Δvta* n=3, *Δgtr2* n=3) independent trials. A two-way ANOVA test was performed to determine the statistical significance (for detailed statistical analysis refer to Appendix B, table 3.2.1).

**3.2.2) In low-glucose cultures with a short-term (20 and 60 minutes) glucose-starvation period, the deletion mutations of *vta* and *gtr2* have no overall effect on the phosphorylation levels of S6 in response to AAs.** The wild-type (WT, strain#667), *Δvta*, (strain#694), and *Δgtr2* (strain#737) were grown in liquid media with 2% glucose, 1x Vogel's salt, 100 μM choline, and 0.2 % Tween 80 and the amino acid treatments +Leu (0.5 mM), +Gln (5mM) for 48h at 22°C under LL. Following this, the mycelial pads were incubated in low-glucose media containing 0.08% glucose, 1x Vogel's salt, and 100μM choline with the corresponding amino acid treatments as mentioned above. The flasks were shaken at 150 r.p.m on an orbital shaker and the samples were harvested after 20 and 60 minutes incubation period. The S6 phosphorylation levels were monitored by western blotting. A two-way ANOVA test was performed to determine the statistical significance. The data are plotted as the mean± SEM of leucine (*Δvta* n=3, *Δgtr2* n=3), glutamine (*Δvta* n=3, *Δgtr2* n=3) independent experiments (detailed statistical analysis are depicted at Appendix B, table 3.2.2).

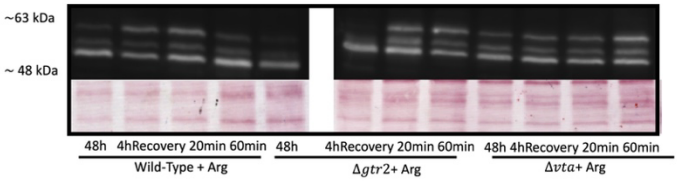
**3.2.3) In low-glucose cultures with long-term (24 hours) glucose-starvation, the deletion mutation of the *vta* significantly diminishes S6 phosphorylation in response to metabolites while the, *gtr2* deletion exhibits less pronounced effects** The wild-type (WT, strain#667) *Δvta*, (strain#694), and *Δgtr2* (strain#737) strains's spores were inoculated and grown on 2% glucose minimal media (2% glucose, 1x Vogel's salt, 100 μM choline, and 0.2% Tween 80) for 48h at 22°C under LL. The mycelial pads were then transferred to a low-glucose shaking flask (containing 0.08% glucose, 1x Vogel's salt, and 100μM choline) with continuous agitation at 150 r.p.m for 24h at 22°C under LL. The treatments: +Glucose (2% glucose), +Leu (0.5 mM leucine), +Arg (2.5 mM arginine), +Gln (5mMglutamine) were added directly to the shaking flasks and the samples were incubated for 60 minutes. The control sample was harvested after 24h incubation in low-glucose culture. The S6 phosphorylation levels were measured by western blotting. The data are plotted as the mean± SEM of n=3 independent trials. A two-way ANOVA followed by a multiple comparison test was performed to determine the significance of the data (Appendix B, table 3.2.3). Asterisks (\*) denote the statistically significant differences between conditions (\* p < 0.05, \*\* p < 0.01) and ns depicts non-significance. The immunoblots stained with Colloidal Gold total protein stain for each experiment are provided below.

3.2.1

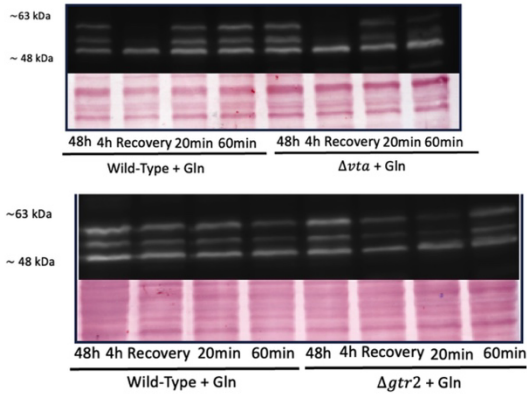
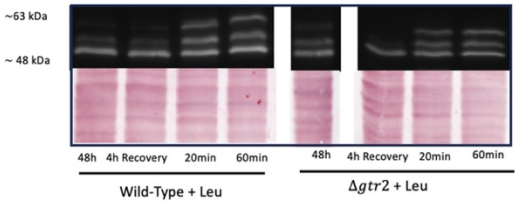
A) Leucine



B) Arginine

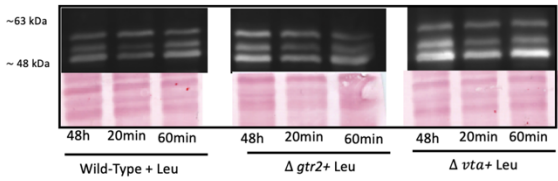


C) Glutamine

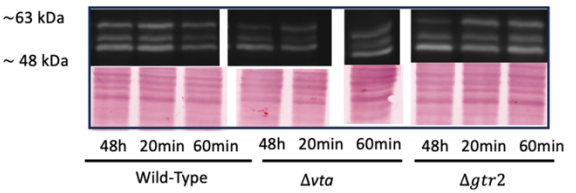


3.2.2

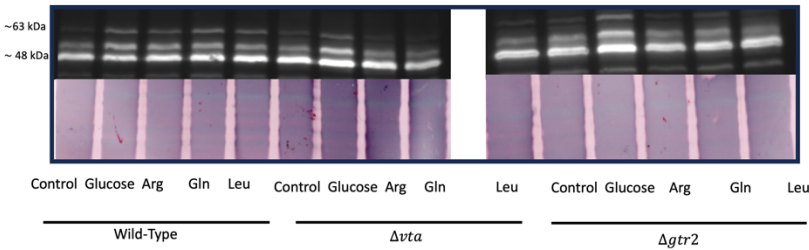
Leucine



Glutamine



3.2.3



#### 3.1.2.4. Effects of Amino Acid Starvation on *Neurospora crassa*'s TOR Pathway

Main Result: Starving amino acid auxotrophs for their required amino acid is likely to increase S6 phosphorylation levels. This elevation in phospho-S6 levels is not directed towards the growth of the organism, as measured by linear growth rate and biomass accumulation.

The TOR pathway serves as a pivot point between the anabolic and catabolic events; when adequate amino acid supply is available, mTORC1 is active, promoting anabolic processes such as protein synthesis to support the organisms' growth (Condon & Sabatini, 2019; Jewell et al., 2013). Conversely, under amino acid-starved conditions, mTOR signaling is repressed and autophagy is initiated to restore the lysosomal amino acid pool through protein degradation; this autophagy-dependent increase in cytosolic amino acid pool can subsequently feedback and re-activate mTORC1 to promote cell survival by increasing the expression of metabolic enzymes and amino acid transporters as part of adaptation to the amino acid insufficiency in the environment (Condon & Sabatini, 2019; Tan et al., 2017).

As mentioned previously, wild-type *Neurospora* cells are able to synthesize all amino acids de novo. Therefore, to determine whether similar to the mammalian systems, amino acid starvation can increase TOR pathway activity in *Neurospora*, I used amino acid auxotrophic mutants: the glutamine auxotroph (*gln-1*) and the arginine auxotroph (*arg-13*), which require glutamine and arginine supplementation for normal growth, respectively (for a detailed description of each mutation refer to Table 2.2). Based on the growth rate assay conducted previously in our laboratory, the “inhibitory” concentrations (i.e. the amino acid concentration that limits the growth of the auxotrophic mutant by 50% compared to the wild-type strain's growth) were determined to be 1 mM for the glutamine auxotroph and 0.025 mM for the arginine auxotroph. The “rescue” concentrations (the amino acid concentration that confers the wild-type growth to the mutant strain) were measured to be 5 mM glutamine for the Gln auxotroph and 2.5 mM arginine for the Arg auxotroph. I hypothesized that if nutritional stress in the form of amino acid-starvation can lead to an increase in protein degradation and subsequently increase in the amino acid reservoir, then incubation of the auxotrophic mutants with the inhibitory concentration of their required amino acid would lead to increased S6 phosphorylation while the supplementation with the rescue concentration might result in lower phospho-S6 levels. To test this, the wild-type and Arg auxotroph strains were grown on 2% glucose MM + the inhibitory arginine

concentration (0.025 mM) for 48h at 22°C under LL. The mycelia were then incubated in the same culture conditions for 4 hours. Following this, the mycelial discs were transferred to the fresh 2% glucose MM with the rescue arginine concentration (2.5 mM Arg) and harvested after 20 and 60 minutes. Similarly, the wild-type and glutamine auxotroph strains were initially grown on the inhibitory glutamine concentration (1mM Gln) for 48h and the subsequent 4h recovery period. The fungal discs were then transferred to the rescue concentration (5 mM Gln) for 20 and 60 minutes. As shown in figure 3.3.1, except for 48h initial growth period, no noticeable differences in the S6 phosphorylation levels were detected between the Arg auxotroph and wild-type growing on the inhibitory or rescue arginine concentrations. Similarly, the glutamine auxotroph had similar phospho-S6 levels as the wild-type strain with the exception of 20 minutes incubation in the rescue glutamine concentration (Figure 3.3.1). Overall, these results suggest that under these experimental conditions when the samples are constantly supplemented with glucose, the autophagy-induced elevation in the cytosolic amino acid pool and the subsequent increase in S6 phosphorylation is not seen. This observation aligns with the prior studies regarding the glucose-induced inhibition of catabolic processes and autophagic events in the budding yeast; when yeast cells are grown in a medium lacking glucose, the synthesis of gluconeogenic enzymes and the Snf1 (the *S. cerevisiae* homolog of AMPK) and the cyclin-dependent kinase Pho85 activate the autophagy-related proteins Atg1 and Atg13. In contrast, when glucose is added to the medium, glucose-induced PKA activation together with TOR kinase 1, inhibits the catabolic events and suppresses the autophagic signals (Moruno et al., 2012).

A study by Corral-Ramos et al. (2022), reported that leucine starvation is a potent activator of autophagy in *S. pombe* cells auxotrophic for leucine after a very long period of nitrogen starvation (20-25 days). Considering this, I sought to analyze the effects of amino acid starvation on *Neurospora*'s auxotrophic strains growing on solid agar for a long time (5-6 days, duration varied depending on the strain and the amino acid concentration). Although the results obtained in this section are not statistically significant which might merely be due to a small sample size (n=3), observing the trends in each individual trial, reflects the increased S6 phosphorylation levels for Arg auxotroph growing on the inhibitory arginine concentration. The phospho-S6 levels resembled the wild-type strain when the fungal samples were grown on the rescue arginine

concentration (Figure 3.3.2). However, the trends for the glutamine auxotroph are less pronounced and similar to the wild-type strain at both inhibitory and rescue glutamine concentrations (Figure 3.3.2). Nitrogen starvation has been reported to accelerate the protein turnover in mycelia of *Neurospora* and lead to an increase in the cytosolic arginine concentration; this is due to the mobilization of metabolically inactive arginine and purines in response to both arginine and glutamine scarcity, implying that glutamine starvation can also determine the metabolic fate of vacuolar arginine under unfavorable environmental conditions (Legerton & Weiss, 1984). These observations raise the possibility that arginine might play a more critical role in *Neurospora*'s metabolic adaptation than glutamine.

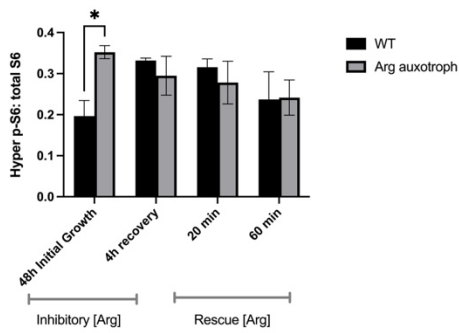
An increase in TOR activity is often associated with a robust rate of ribosomal biogenesis, an elevation in protein translation, and macromolecule biosynthesis, all of which ultimately lead to cell growth (increased size and mass) (Wullschleger et al., 2006). Thus, if amino acid starvation leads to an autophagy-induced elevation in the amino acid pool and subsequently an increase in S6 phosphorylation of auxotrophic mutants, then this potential elevation in ribosomal biogenesis would not be used to promote the organism's growth; instead, it would be used to increase the expression of metabolic enzymes and amino acid transporters essential to promote the survival of the organisms. To test this, I analyzed the relationship between S6 phosphorylation levels, linear growth rate, and biomass accumulation in auxotrophic mutants grown on both inhibitory and rescue concentrations in a solid agar medium. Interestingly, when the Arg mutant was starved for arginine (i.e. when grown on the inhibitory arginine concentration), the growth parameters such as biomass accumulation and linear growth rate were significantly lower compared to the wild-type strain, suggesting that increased S6 phosphorylation may indeed be a pro-survival signal to increase the ribosomal biogenesis and the translation of essential proteins (Figure 3.3.3.A). Similarly, when the glutamine auxotroph was starved for glutamine (when grown on the inhibitory glutamine concentration), the mass accumulation and the linear propagation were significantly diminished in relation to the wild-type strain (Figure 3.3.3.B). The two growth variables were comparable to the wild-type when supplemented with the rescue amino acid concentrations (Figures 3.3.3.A and B).

Taken together, it appears that under the inhibitory concentration of both amino acids, the increase in the phospho-S6 levels is not directed towards the growth of the organisms. The

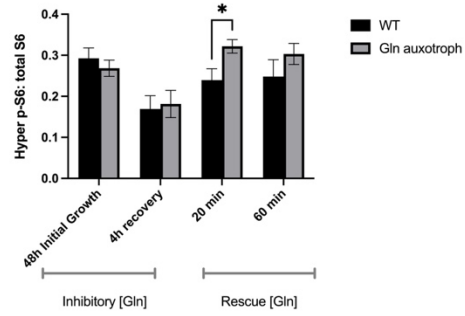
inhibitory conditions impose a stronger negative impact on the arginine auxotroph compared to the glutamine mutant. The results of these experiments can be further elucidated by increasing the sample size or by reproducing the experiments in low-glucose shaking cultures with a prolonged glucose and nitrogen starvation period.

3.3.1. When glucose is available, overall, no dramatic changes in P-S6 levels between the auxotrophic mutants and the wild-type strains growing on both the inhibitory and rescue AA concentrations were found

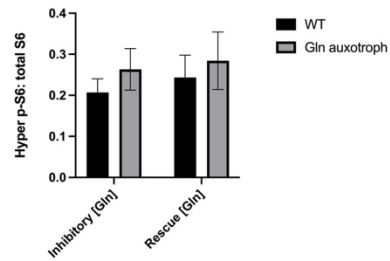
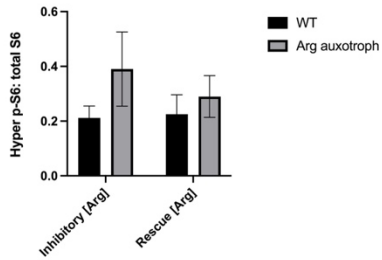
A) Arginine auxotroph



B) Glutamine auxotroph

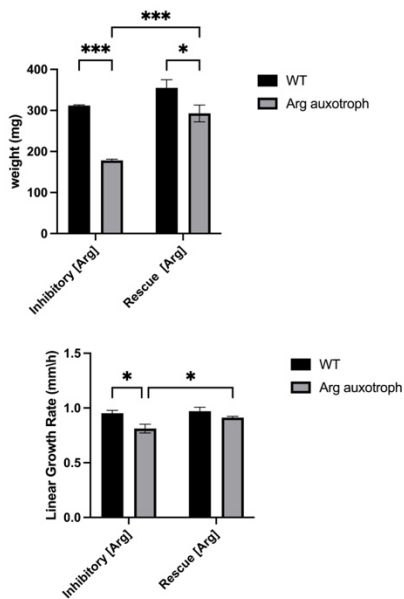


3.3.2. When auxotrophic mutants, especially arginine auxotrophs, are starved for their required amino acid for an extended period of time, S6 phosphorylation levels are likely to increase

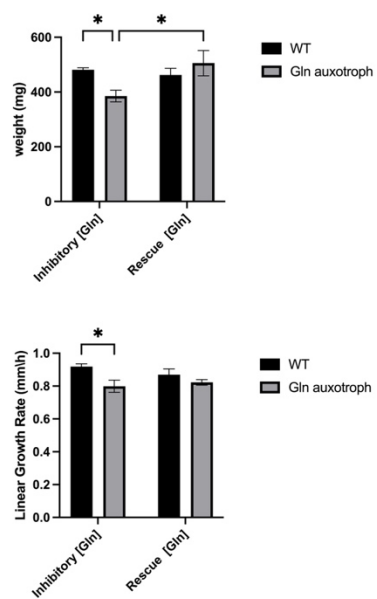


3.3.3. When the auxotrophic mutants are starved for their required amino acid, the linear growth rate and biomass accumulation are significantly lower than those of the wild-type.

A) Arginine auxotroph



B) Glutamine auxotroph



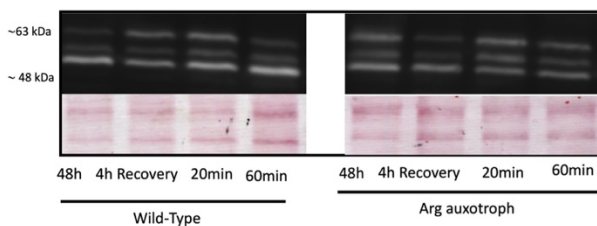


**Figure 3.3. Effects of amino acid starvation on *Neurospora crassa*'s TOR pathway.**

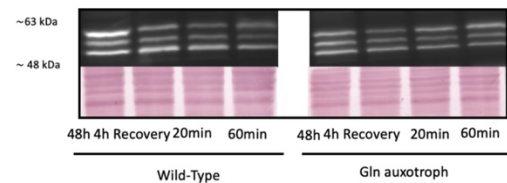
**3.3.1) When glucose is available, overall, no dramatic changes in P-S6 levels between the auxotrophic mutants and the wild-type strains growing on both the inhibitory and rescue AA concentrations were found.** The wild-type (WT, strain#667), arginine auxotroph (*arg-13*, strain#744), and glutamine auxotroph (*gln-1*, strain#747) strains were grown on liquid minimal media (MM) with 2% glucose, 1x Vogel's salt, and 100  $\mu$ M choline for 48h at 22°C under LL. The mycelial discs were then incubated in a fresh 2% glucose MM for 4 hours as a recovery period. During the 48h initial growth and the subsequent 4h recovery periods, the strains were incubated with the inhibitory amino acid concentrations (0.025 mM Arg for the arginine auxotroph and 1 mM Gln for the glutamine auxotroph). Following this, the mycelial discs were transferred to a fresh 2% glucose MM supplemented with the rescue amino acid concentration (2.5 mM Arg for the arginine auxotroph and 5 mM Gln for the glutamine auxotroph) and harvested after 20 and 60 minutes. The wild-type strain grown on both inhibitory and rescue arginine and glutamine concentrations serves as control. The S6 phosphorylation levels were measured by western blotting. The data are plotted as the mean  $\pm$  SEM of Arginine auxotroph (n=3) and glutamine auxotroph (n=9), independent trials. A two-way ANOVA test was performed to determine the statistical significance (\*  $p < 0.05$ ). (for detailed statistical analysis refer to Appendix B, table 3.3.1). **3.3.2) When auxotrophic mutants, especially arginine auxotrophs, are starved for their required amino acid for an extended period of time, S6 phosphorylation levels are likely to increase.** The wild-type (WT, strain #667), Arginine auxotroph (*arg-13*, strain#744), and Glutamine auxotroph (*gln-1*, strain#747) were grown on solid agar culture containing 0.5% maltose, 1x Vogel's salt, 100 $\mu$ M choline, 2% agar with inhibitory concentrations (0.025 mM Arg and 1mM Gln) and the rescue concentrations (2.5 mM Arg and 5mM Gln) at 22°C under LL. The samples were harvested and S6 phosphorylation levels were measured when the mycelial growth had reached the halfway across the agar medium using western blot analysis. A two-way ANOVA test was performed to determine the statistical significance. The data are plotted as the mean  $\pm$  SEM of Arginine auxotroph (n=3) and glutamine auxotroph (n=3), independent trials (for detailed statistical analysis refer to Appendix B, table 3.3.2). **3.3.3) When the auxotrophic mutants are starved for their required amino acid, the linear growth rate and biomass accumulation are significantly lower than those of the wild-type.** The wild-type, arginine auxotroph, and glutamine auxotroph were measured as described above. For linear growth rate measurements (mm/h), the samples were grown at 22°C at LL and the mycelia growth front was marked daily. Once the cultures reached the edge of the plate, the average linear growth rate was then calculated using two pre-coded macros, FijiTau and MacTau. The biomass accumulation measurement (mg) was conducted after the cultures reached the end of the petri plate; the mycelia were dried for 48 hours in an 80°C oven. The dried samples were weighed, and the net weights were calculated by subtracting the weight of the empty tubes. The data are plotted as the mean  $\pm$  SEM of Arginine auxotroph (n=3) and glutamine auxotroph (n=3), independent trials. A two-way ANOVA test was performed to determine the statistical significance (\*  $p < 0.05$ , \*\*\*  $p < 0.001$ ). (for detailed statistical analysis refer to Appendix B, table 3.3.3). The immunoblots stained with Colloidal Gold total protein stain for each experiment are provided below.

3.3.1)

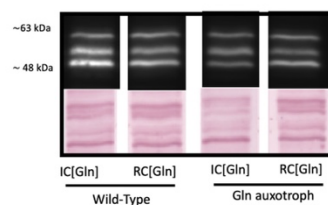
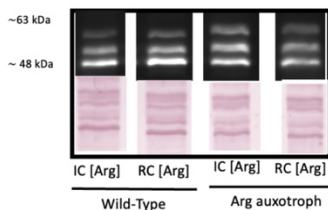
A) Arginine Auxotroph



B) Glutamine Auxotroph



3.3.2)



## 3.2: Lipid Regulation of TOR Pathway Activity in *Neurospora crassa*

### 3.2.1 Introduction and Objectives

The mammalian target of rapamycin (mTOR) is a vital sensor and integrator of various environmental cues that links external nutrient signals to cell growth and homeostasis (Laplante & Sabatini, 2012). Both mTORC1 and mTORC2 are tightly regulated by lipid second messenger phosphatidic acid (PA), which has been shown to directly bind to the rapamycin-binding domain (FK506-binding protein–12-rapamycin-binding, FRB) of mTOR complexes and promote their stabilization (Menon et al., 2017). PAs as the central metabolite of membrane phospholipid biosynthesis are mainly synthesized by the phospholipase D (PLD)-mediated hydrolysis of phosphatidylcholine or through diacylglycerol kinase (DAGK)-induced phosphorylation of diacylglycerol (DAG) (D. A. Foster, 2013; Popova & Jücker, 2021). The elevated expression of PLD enzymes, DAGK isoforms and the exogenous supply of fatty acids promoting *de novo* synthesis of PAs are all reported to increase mTORC1 activity in various tissues (Menon et al., 2017; Popova & Jücker, 2021; You et al., 2014).

Unlike mammalian systems, less is known about the ability of the TOR complexes to respond to the presence of essential nutrients such as lipids in other organisms. In fact, there is almost no published data on the lipid regulation of TOR pathway in *Neurospora crassa*. Therefore, this chapter aims to explore the effects of changes in the lipid composition on the TOR pathway activity of *Neurospora*.

Phosphatidylcholine (PtdCho) is one of the most abundant phospholipids in the membranes of *Neurospora crassa* which is synthesized by sequential methylation of phosphatidylethanolamine (PtdEtn) (Lakin-Thomas, 1996; Lakin-Thomas et al., 1997). The choline-requiring *chol-1* mutant strain of *Neurospora* is defective in the first methylation step of synthesis of PtdCho from PtdEtn due to the lack of S-adenosyl methionine phosphatidylethanolamine N- methyl transferase, thus requires choline supplementation for normal growth (Lakin-Thomas & Brody, 2004). Choline deficiency alters membrane phospholipid composition by decreasing PtdCho levels while increasing PtdEtn, phosphatidylinositol (PtlN), and neutral lipids (Lakin-Thomas & Brody, 2004). *Chol-1* mutant strain growing on choline-depleted cultures exhibits slow growth rate, lengthened period of the conidiation rhythm and poor temperature compensation (Lakin-Thomas,

1996; Lakin-Thomas et al., 1997). Choline supplementation restores normal growth and rhythmicity of this strain (Lakin-Thomas & Brody, 2004). Although the biochemical pathway linking the altered lipid composition of the *chol-1* strain to circadian rhythmicity has not yet been characterized, it appears that an increase in the diacylglycerol (DAG) may be responsible for these changes; interestingly, artificial elevation in DAG levels using membrane-permeant DAG analog, DOG (1,2-dioctanoyl-*sn*-glycerol), further lengthens the period of conidiation, emphasizing the potential role of diacylglycerol in regulating the circadian rhythmicity in *Neurospora* (Ramsdale & Lakin-Thomas, 2000).

Taking advantage of this system, I reasoned that if the elevated DAG levels in the choline depleted *Chol-1* strain are accompanied by increased activity of DAGK and subsequent elevation in PA concentrations, then choline-starvation of this strain would result in increased TOR pathway activity, if this system follows a similar pattern to mammalian TOR complexes. Additionally, I hypothesized that when the elevated DAG levels in choline-starved *chol-1* mutant strain are combined with an exogenous DAG analog, DOG, then the potential stimulatory effects on the TOR pathway should be greater than in choline-sufficient conditions due to increased PA synthesis.

### 3.2.3. Results & Discussion

#### 3.2.3.1 Effects of Choline Starvation on the TOR Pathway Activity of *Neurospora crassa*

Main Result: Choline starvation of *chol-1* mutant strain increases TOR pathway activity only in choline-depleted cultures growing without glucose supplementation over an extended period of time.

Phosphatidic acid (PA) is a lipid second messenger that participates in diverse sets of intracellular signaling events (Fang et al., 2001). One of the major cellular mechanisms for generating PA is through the phosphorylation of the neutral lipid diacylglycerol (DAG) (D. A. Foster, 2013). PAs have been shown to stabilize the mTORC1 on the lysosomal membrane and stimulate the activation of its substrates S6K1 and 4E-BP1 phosphorylation in human embryonic kidney (HEK) cells (Popova & Jücker, 2021). The choline-requiring *chol-1* mutant strain of *Neurospora* is deficient in the formation of monoethylethanolamine, which is the first methylation step in the conversion of PtdEtn (phosphatidylethanolamine) to PtdCho

(Phosphatidylcholine) (Lakin-Thomas, 1996, 1998). The *chol-1* strain has a lipid profile similar to the wild-type when supplemented with choline (Davis & Perkins, 2002). However, in the absence of choline supplementation, the altered lipid composition results in the accumulation of DAG in *Neurospora*'s membranes (Ramsdale & Lakin-Thomas, 2000). If the elevated DAG levels in choline-deprived *chol-1* strain is accompanied by increase in its phosphorylation and conversion to PAs, then choline-starvation of *chol-1* strain would increase TOR pathway activity, measured by phosphorylation levels of its downstream target, S6 ribosomal protein. To test this, the fungal spores were initially grown in high choline (100  $\mu$ M) MM cultures for 48 hours to ensure consistent growth rate among the samples. Following this, the mycelial discs were transferred to a fresh MM containing Glucose + choline, Glucose, Choline, and water to possibly mimic the starvation conditions (Note: all MM media contained 1x Vogel's salt). The samples were harvested after 1-, 4-, and 24-hours incubation periods; these time intervals were chosen to encompass the possible short-term (1 hour), mid-term (4 hours), and long-term (24 hours) effects. As expected, glucose supplementation, alone or in combination with choline significantly increased S6 phosphorylation levels compared to un-transferred controls especially at 4-hour intervals (Figure 3.4). This finding aligns with the previous observations in section 3.1, regarding the stimulatory effects of glucose on the TOR pathway activity of *N. crassa*. Interestingly, the lack of statistically significant differences between the "Glucose + Choline" and the "Glucose" groups suggest that choline supplementation failed to further stimulate TORC1 under these experimental conditions. This is also evident in the choline-treated group where in the absence of glucose, choline-supplementation did not alter S6 phosphorylation compared to the water-treated (starved) or un-transferred (control) variants. These results also point to the possibility that choline-depletion of *chol-1* strain does not affect TOR pathway activity when its potent stimulator, glucose is readily available (Figure 3.4).

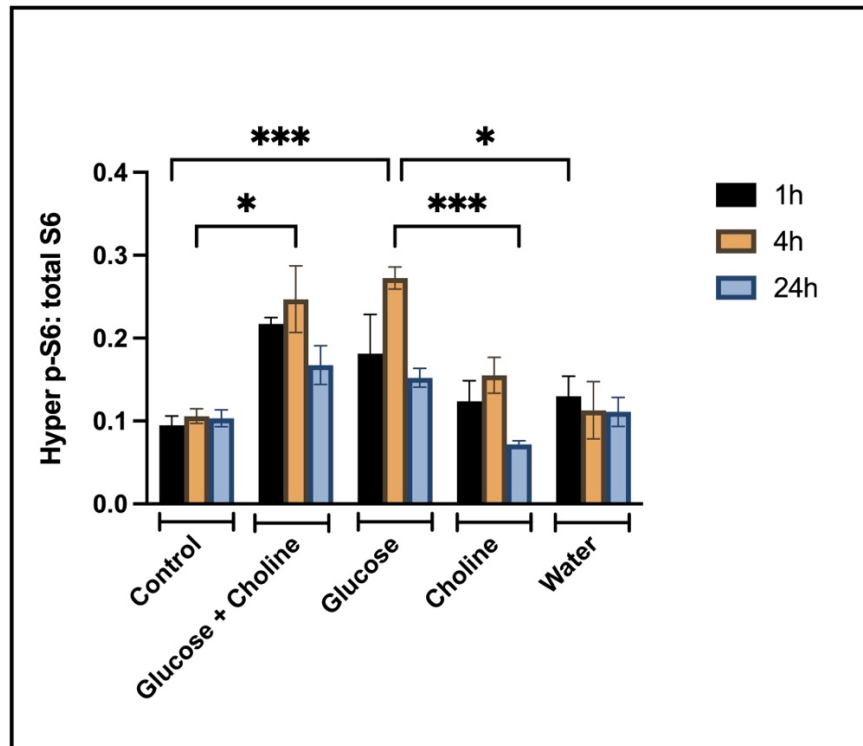
Since no notable differences between the choline-starved and choline-sufficient conditions were detected under the aforementioned experimental setup, I sought to investigate the effects of choline-depletion on cultures grown without choline supplementation even during the 48-hour initial growth period. I reasoned that in the previous condition, where *chol-1* strain was initially grown on choline-rich media, the cultures might not have been fully starved for choline following this period. To account for this, I compared the S6 phosphorylation levels between samples grown

with (+choline) or without choline (-choline) after 48h initial growth and 4h recovery period. I also analyzed the short-term effects following incubation periods of 5, 10, 20, and 60 minutes in 2% glucose fresh MM. As shown in Figure 3.5.1, choline-starvation of *chol-1* mutant strain did not significantly alter the S6 phosphorylation levels compared to the choline-treated samples at any of the tested time points, except the 4-hour recovery period.

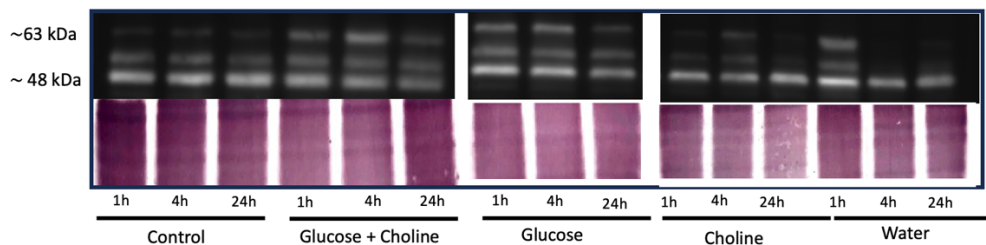
As mentioned previously, in *S. cerevisiae*, glucose, as the major and preferred carbon source is necessary and sufficient for activation of TORC1 (Alfatah et al., 2021; Kayikci & Nielsen, 2015). Similar trends have also been observed in *Neurospora*'s TOR pathway regulation (This thesis, section 3.1). Therefore, to circumvent this and to investigate the effects of choline starvation in the *chol-1* strain in isolation from the stimulatory effects of glucose, a low-glucose shaking culture system was adopted. In this approach, the fungal samples after 48-hours initial growth in 2% glucose (+/-choline treatment) were transferred to low-glucose (0.08%) shaking cultures (+/-choline) for 20 min, 60 min, and 24 hours to monitor both short- and long-term responses. In the short-term glucose-starvation periods (20 and 60 minutes), S6 phosphorylation appeared to be unaffected by choline starvation in relation to choline-rich conditions (Figure 3.5.2). A potential issue that arises here is that short-term incubation in a low-glucose culture might not have been enough duration to allow the fungus to adapt to low-glucose environment. Surprisingly, when the incubation period in low-glucose cultures were lengthened to 24 hours, choline-starvation (-choline) significantly increased P-S6 levels compared to the +choline conditions (Figure 3.5.3).

Based on this observation, I hypothesized that the choline starvation of choline-requiring mutant appears to only affect TOR pathway activity in cultures growing on choline-depleted cultures for a long period of time and this response can only be observed in the absence of glucose. Hence, to examine this, the cultures were grown on solid agar minimal media with or without choline supplementation. The cultures were grown for a few days until the mycelium growth front had reached the middle of the agar. As seen in Figure 3.5.4, choline-starvation of *chol-1* strain significantly increases S6 phosphorylation levels compared to choline-rich variants.

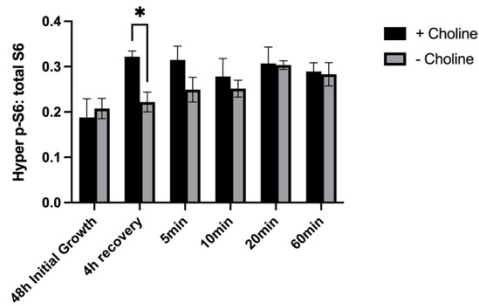
Overall, the results obtained in this section suggest that the altered lipid composition in choline-deprived *chol-1* mutant strain increases TOR pathway activity only in choline-depleted cultures growing without glucose supplementation over an extended period of time.



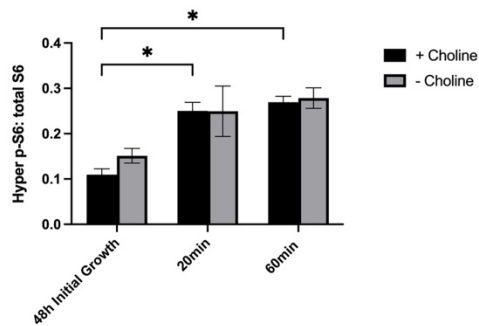
**Figure 3.4. Effects of choline starvation on the TOR pathway activity of *chol-1* mutant strain of *Neurospora crassa*.** The *chol-1* mutant strain (#667) was grown in 2% glucose liquid minimal media (MM), 1x Vogel's salt, and 100  $\mu$ M choline for 48 hours at 22°C under constant light (LL). The mycelial discs were then transferred to fresh MM containing "Glucose + Choline" (2% glucose MM + 100  $\mu$ M choline), "Glucose" (2% glucose MM, No choline), "Choline" (MM + 100  $\mu$ M choline, No glucose), or "Water" (MM, No glucose, No choline). The fungal discs were then harvested after 1, 4, and 24 hours of incubation at 22°C under LL. The control samples were not transferred to a fresh medium following the initial 48-hour growth period. The S6 phosphorylation levels were measured by western blotting. The data are plotted as the mean  $\pm$  SEM of n=3 independent trials. A two-way ANOVA followed by multiple comparison test was performed to determine the significance of the data (for detailed statistical analysis refer to Appendix B, table 3.4). Asterisks (\*) denote the statistically significant differences between conditions as follows: \* p < 0.05, \*\* p < 0.01, \*\*\* p < 0.001, \*\*\*\* p < 0.0001. The immunoblot stained with Colloidal Gold total protein stain is provided below.



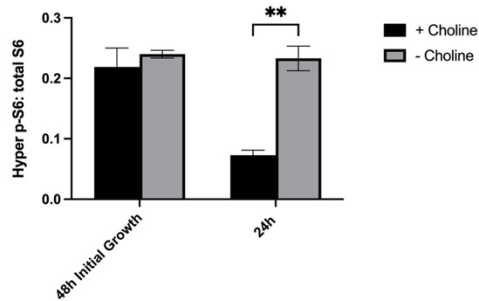
3.5.1. When glucose is available, the choline starvation of *chol-1* strain does not overall affect S6 phosphorylation levels



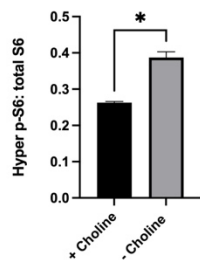
3.5.2. In low-glucose cultures with short-term (20 and 60 minutes) glucose-starvation periods, choline-depletion of *chol-1* does not affect S6 phosphorylation levels



3.5.3 In low-glucose cultures with long-term (24h) glucose-starvation period, choline-depletion of *chol-1* significantly increases S6 phosphorylation levels



3.5.4. Choline starvation of *chol-1* significantly increases S6 phosphorylation levels in cultures grown on solid agar culture long-term



**Figure 3.5. Effects of choline starvation on the TOR pathway activity of *chol-1* mutant strain of *Neurospora crassa*.**

**3.5.1) When glucose is available, the choline starvation of *chol-1* strain does not overall affect S6 phosphorylation levels.** The *chol-1* mutant strain (#667) was grown in 2% glucose liquid minimal media (MM), 1x Vogel's salts, and with (+ choline, 100  $\mu$ M choline) or without choline (-choline) for 48 hours at 22°C under constant light (LL). The mycelial discs were then transferred to fresh 2% MM with or without choline for a 4-hour recovery period. The fungal discs were subsequently transferred to fresh 2% glucose MM +/- choline supplementation and were harvested after 5, 10, 20, and 60 minutes. S6 phosphorylation levels were measured by western blotting. The data are plotted as the mean  $\pm$  SEM of n=3 independent trials. A two-way ANOVA followed by multiple comparison test was performed to determine the significance of the data (for detailed statistical analysis, refer to Appendix B, Table 3.5.1). Asterisks (\*) denote the statistically significant differences between conditions: \*  $p < 0.05$ .

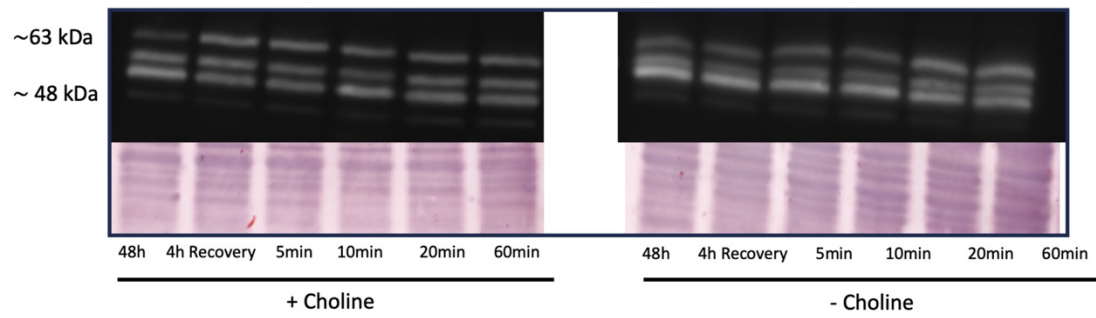
**3.5.2) In low-glucose cultures with short-term (20 and 60 minutes) glucose-starvation periods, choline-depletion of *chol-1* does not affect S6 phosphorylation levels.**

**3.5.3) In low-glucose cultures with long-term (24h) glucose-starvation period, choline-depletion of *chol-1* significantly increases S6 phosphorylation levels.** The *chol-1* strain (#667) was grown in liquid media with 2% glucose, 1x Vogel's salt, 0.2 % Tween 80, and with (+ choline, 100  $\mu$ M choline) or without choline (-choline) for 48h at 22°C under LL. The fungal discs were then transferred to 50 mL of low-glucose media containing 0.08% glucose, 1x Vogel's salt, and +/- choline for 20 and 60 minutes (Figure 3.5.2) or 24 hours (Figure 3.5.3). The flasks were shaken at 150 r.p.m on an orbital shaker at 22°C under LL. S6 phosphorylation levels were measured by western blotting. The data are plotted as the mean  $\pm$  SEM of (n=3 for figure 3.5.2) and (n=3 for figure 3.5.3) independent trials. A two-way ANOVA followed by a multiple comparison test was performed to determine the significance of the data (for detailed statistical analysis, refer to Appendix B, Tables 3.5.2 and 3.5.3). Asterisks (\*) denote the statistically significant differences between conditions: \*\*  $p < 0.01$ .

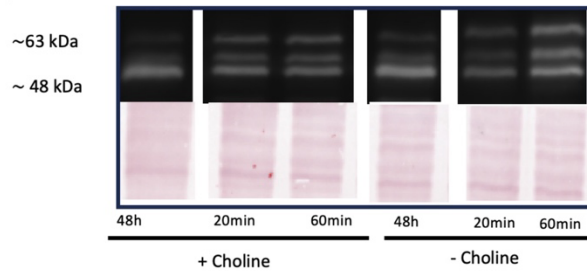
**3.5.4.) Choline starvation of *chol-1* significantly increases S6 phosphorylation levels in cultures grown on solid agar culture long-term.** The *chol-1* strain was grown on solid agar culture containing 0.5% maltose, 1x Vogel's salts, 2% agar and with (+ choline, 100 $\mu$ M choline) or without (-choline) at 22°C under LL. The S6 phosphorylation levels were measured when the mycelial growth had reached the halfway across the agar medium using western blot analysis. A paired T-test was performed to determine the statistical significance. The data are plotted as the mean  $\pm$  SEM of n=3 independent trials. Asterisks (\*) denote the statistically significant differences between conditions: \*  $p < 0.05$ . The immunoblots stained with Colloidal Gold total protein stain for all of the experiments are provided below.



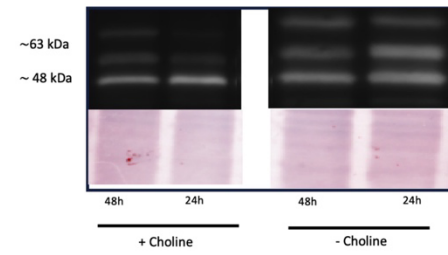
3.5.1



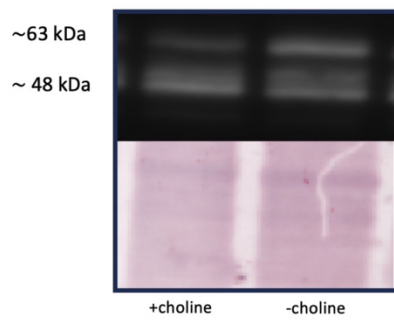
3.5.2)



3.5.3)



3.5.4)



### 3.2.3.2 Effects of Increased Diacylglycerol levels on the TOR Pathway Activity of *Neurospora crassa*

Main Result: When the already increased endogenous diacylglycerol levels in the choline-deprived *chol-1* strain are further artificially elevated, the excess diacylglycerol levels inhibit the TOR pathway activity of *Neurospora*

Diacylglycerol (DAG) is a cardinal signaling lipid that serves as an intermediate in the synthesis of membrane phospholipids and the storage lipid, triacylglycerol (TG) (Van Meer et al., 2008). Diacylglycerol kinase (DAGK)-mediated phosphorylation of DAG results in its conversion to phosphatidic acid, which has been shown to increase the activity of mTORC1 in various tissues (Frias et al., 2023). Under homeostatic conditions, cellular steady-state levels of DAG are usually very low (Rockenfeller et al., 2018). Phosphatidylcholine (PtdCho) accounts for more than 45% of phospholipids in *Neurospora*'s membranes (McKeon et al., 1997). Defects in the synthesis of PtdCho in the choline-requiring *chol-1* mutant strain of *Neurospora* result in accumulation of DAG and lengthen the period of conidiation (Ramsdale & Lakin-Thomas, 2000). When the increased DAG levels are further elevated with exogenous supplies of membrane-permeant DAG analog, DOG (1,2-dioctanoyl-*sn*-glycerol), the effects on the period of conidiation are doubled (Ramsdale & Lakin-Thomas, 2000). Taking advantage of this system, I reasoned that if *Neurospora*'s TOR pathway is positively regulated by PAs, as seen in mammalian settings, then increasing the already elevated DAG levels in the choline-starved *chol-1* strain, would further stimulate S6 phosphorylation levels.

To investigate the role of elevated DAG levels on the TOR pathway activity of *Neurospora*, I made use of a small, membrane-permeant DAG analog, DOG (1,2-dioctanoyl-*sn*-glycerol) at a 250  $\mu$ M concentration that was previously shown to have maximum effect on the period of conidiation (Ramsdale & Lakin-Thomas, 2000). The mycelial discs were initially grown on 2% glucose minimal medium (MM) with or without choline supplementation for 48h under LL. Following this, the DOG solution was added directly (without minimal media) to the growth media and the cultures were incubated for 10, 20, 60 minutes and 4 hours. The same concentration of the DOG solvent, Me<sub>2</sub>SO (Dimethyl sulfoxide, DMSO) was added to the control samples. I proposed that the potential effects of DOG on the TOR pathway would be more pronounced in the -choline

(without choline) samples that already have accumulated endogenous DAG compared to the +choline (with 100  $\mu$ M choline) variants. As seen in Figure 3.6.1 A, DOG administration did not alter S6 phosphorylation levels compared to the Me<sub>2</sub>SO-containing samples in a choline-sufficient background. Similarly, in choline-deprived cultures, the exogenous elevation of DAG levels (+DOG) did not significantly change phospho-S6 levels except at the 60-minute interval, where the DOG treatment lowered P-S6 compared to DMSO (Figure 3.6.1 B).

The limitation of the previous approach might be that when DOG was administered directly, because the cultures were stationary, there is a possibility that DOG was not well-mixed and equally incorporated into the medium so that not every part of the mycelium had equal access to the treatment. Thus, in an alternative method, following the initial 48h of initial growth and 4-hour recovery in 2% glucose MM in the absence of choline supplementation (to increase the endogenous DAG levels), the cultures were transferred to + choline (A) and -choline (B) MM with DOG treatment and harvested after 5, 10, 20, and 60 minutes. The time intervals in this experiment were shortened based on a previous observation that suggested that the signaling changes induced by DOG are relatively fast (up to 8 minutes) (Khatun & Lakin-Thomas, 2011). Utilizing this method, S6 phosphorylation levels appeared to be unaffected by DOG treatment in both + choline (Figure 3.6.2.A) and -choline (Figure 3.6.2. B) cultures.

All fungi including *Neurospora* depend on carbon sources, in fact, glucose and other easily-fermentable C-sources are both qualitatively and quantitatively the most important nutritional elements (Conlon et al., 2019). As seen in the previous experiments, when glucose is readily available in the growth media, other treatments such as different amino acids or choline starvation did not affect TOR pathway activity (Sections 3.1 and 3.2.3.1). Therefore, a low-glucose shaking culture system was adopted to study the effects of elevated DAG levels on the TOR pathway activity. In this approach, the fungal samples after 48 hours of initial growth in 2% glucose MM with (Figure 3.6.3 A) or without choline ( Figure 3.6.3 B) supplementation, were transferred to a low-glucose (0.08%) shaking cultures with the DOG or DMSO treatments and were incubated for 20 and 60 minutes. In choline-rich cultures, DOG did not significantly affect P-S6 levels in relation to the DMSO controls (Figure 3.6.3.A). Surprisingly, in choline-deprived cultures (-choline), where the already elevated endogenous DAG levels were further augmented

by exogenous DOG administration, the S6 phosphorylation levels were significantly diminished at both 20 and 60-minute intervals compared to the solvent-containing controls (3.6.3 B). This suggests that excess diacylglycerol levels can potentially inhibit the TOR pathway activity of *Neurospora*.

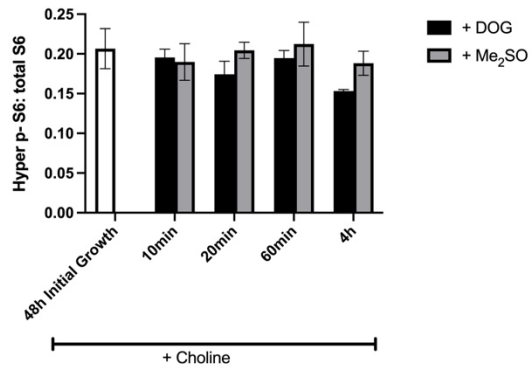
The maintenance of precise lipid symmetry is essential to ensure membrane integrity and proper signaling, as lipid molecules have distinct sets of characteristics regarding the bilayer formation, curvature determination, and membrane protein embedding (Rockenfeller et al., 2018; Van Meer et al., 2008). Lipid overload has been reported to induce cellular lipotoxicity (surplus of fatty acids causing cellular dysfunction) in higher eukaryotes; for example, high-fat diet in *Drosophila* or palmitic acid (16:0)-treated endothelial cells can induce severe lipotoxicity and increase ROS (reactive oxygen species) production (Asiimwe et al., 2023; Raja et al., 2022). A similar observation has been made in *S. cerevisiae*, where DOG treatment severely compromised cell survival and triggered necrotic cell death (Rockenfeller et al., 2018). Interestingly, the toxic effects of increased DAG levels were found to be linked to glucose metabolism (Rockenfeller et al., 2018). Although no link between lipid overload and TOR pathway regulation has been identified yet, it is tempting to speculate that if similar to the budding yeast, the *Neurospora*'s cells are negatively affected by the lipid perturbation induced by choline-starvation and DOG administration, then the TOR pathway as the main survival promoting signaling cascade would be downregulated.

Overall, the results obtained in this section suggest that if indeed the elevation in DAG levels is responsible for increased P-S6 levels in choline-starved *chol-1* strain, then that would imply that diacylglycerol-mediated regulation of *Neurospora*'s TOR pathway may operate in a spectrum, meaning that up to a certain threshold (as seen in choline-deprived *chol-1* strain) the elevation in DAG levels can positively stimulate TOR pathway activity (Figures 3.5.3 & 3.5.4). However, after this point when the accumulated endogenous DAG concentration is further amplified with the exogenous DAG analog, the excess diacylglycerol can inhibit TOR pathway activity (Figure 3.6.3. B). Interestingly, a similar dose-dependent response was seen in DOG-induced effects on the period of the conidiation rhythms; when the DOG concentration was increased from 250  $\mu$ M

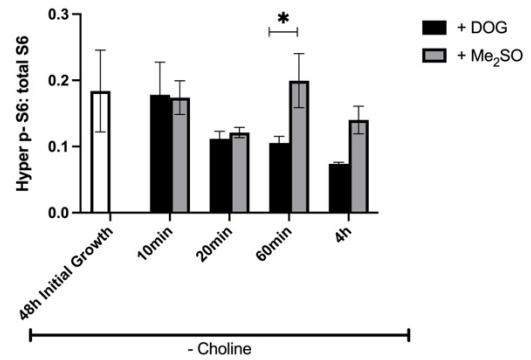
(the concentration used in this study) to 500  $\mu\text{M}$ , the period was not further lengthened instead it was reduced from ~135 to ~80 hours (Ramsdale & Lakin-Thomas, 2000).

### 3.6.1. When DOG is added directly to the growth media, it does not overall alter S6 phosphorylation levels in both choline sufficient or deprived cultures

A)

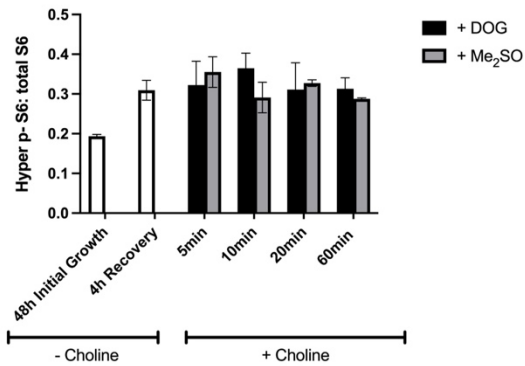


B)

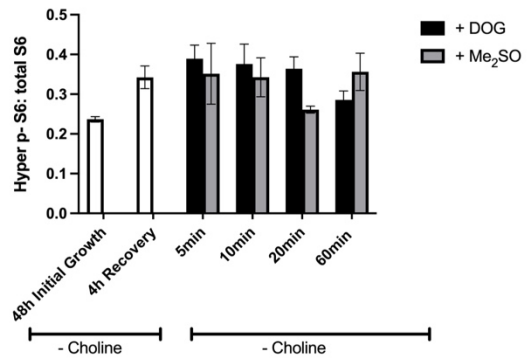


### 3.6.2. When cultures are grown on high glucose media, DOG supplementation does not change S6 phosphorylation levels

A)

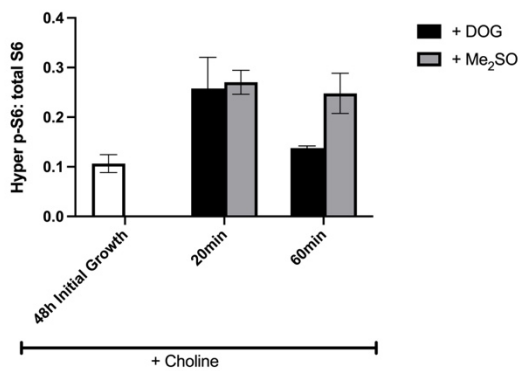


B)

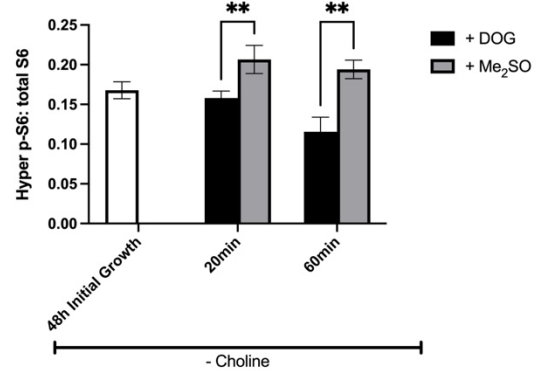


### 3.6.3. In low-glucose media, DOG treatment significantly lowers S6 phosphorylation levels only in choline-deprived cultures

A)



B)



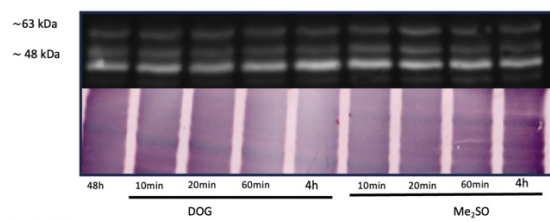
**Figure 3.6. Effects of increased diacylglycerol levels on the TOR pathway activity of *chol-1* mutant strain of *Neurospora crassa*.**

**3.6.1) When DOG is added directly to the growth media, it does not overall alter S6 phosphorylation levels in both choline sufficient or deprived cultures.** The *chol-1* mutant strain (#667) was grown in 2% glucose liquid minimal media (MM), 1x Vogel's salts, and with (A,+ choline, 100  $\mu$ M choline) or without choline (B, -choline) for 48 hours at 22°C under constant light (LL). Following this, a 250 $\mu$ M DOG solution was added directly (without MM) to the growth medium and the cultures were incubated for 10, 20, 60 minutes, and 4 hours. The same concentration of the DOG solvent, Me<sub>2</sub>SO (Dimethyl sulfoxide, DMSO) was added as the control samples. S6 phosphorylation levels were measured by western blotting. The data are plotted as the mean $\pm$  SEM of (A n=3) and (B n=3) independent trials. A two-way ANOVA followed by multiple comparison tests was performed to determine the significance of the data (for detailed statistical analysis, refer to Appendix B, Tables 3.6.1 A and B). Asterisks (\*) denote the statistically significant differences between conditions: \*  $p < 0.05$ .

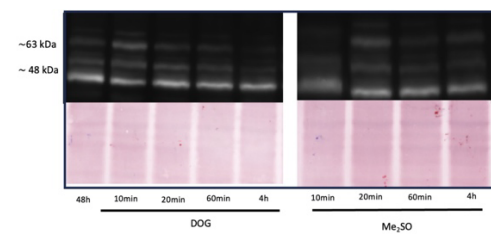
**3.6.2) When cultures are grown on high glucose media, DOG supplementation does not change S6 phosphorylation levels.** The *chol-1* mutant strain (#667) was grown in 2% glucose liquid minimal media (MM), 1x Vogel's salts, without choline (-choline) for 48 hours at 22°C under constant light (LL). Following this, the mycelial discs were transferred to a fresh 2% glucose MM without choline for a 4-hour recovery period. The fungal discs were subsequently transferred to fresh 2% glucose MM, +/- choline, with 250 $\mu$ M DOG and were harvested after 5, 10, 20, and 60 minutes. The control samples only contained the solvent Me<sub>2</sub>SO. S6 phosphorylation levels were measured by western blotting. The data are plotted as the mean $\pm$  SEM of (A n=3) and (B n=3) independent trials. A two-way ANOVA followed by multiple comparison test was performed to determine the significance of the data (for detailed statistical analysis, refer to Appendix B, Tables 3.6.2 A and B).

**3.6.3) In low-glucose media, DOG treatment significantly lowers S6 phosphorylation levels only in choline-deprived cultures.** The *chol-1* strain (#667) was grown in liquid media with 2% glucose, 1x Vogel's salts, 0.2% Tween 80, and with (A,+ choline, 100  $\mu$ M choline) or without choline (B,-choline) for 48h at 22°C under LL. The fungal discs were then transferred to 50 mL of low-glucose media containing 0.08% glucose, 1x Vogel's salt, and +/- choline with 250  $\mu$ M DOG for 20 and 60 minutes. The control samples contained the equivalent concentration of Me<sub>2</sub>SO. The flasks were shaken at 150 r.p.m on an orbital shaker at 22°C under LL. S6 phosphorylation levels were measured by western blotting. The data are plotted as the mean $\pm$  SEM of n=3 independent trials. A two-way ANOVA followed by a multiple comparison test was performed to determine the significance of the data (for detailed statistical analysis, refer to Appendix B, Tables 3.6.3 A and B). Asterisks (\*) denote the statistically significant differences between conditions: \*\*  $p < 0.01$ . The immunoblots stained with Colloidal Gold total protein stain for all of the experiments are provided below.

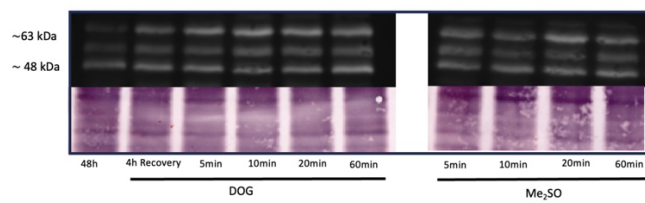
3.6.1.A)



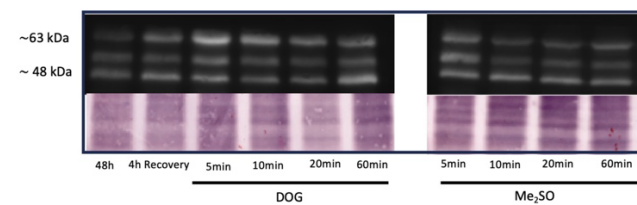
3.6.1.B)



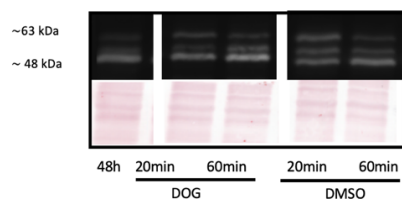
3.6.2.A)



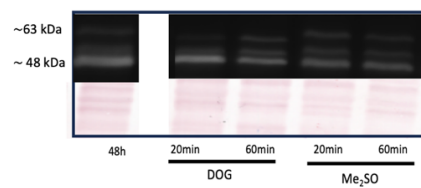
3.6.2.B)



3.6.3.A)



3.6.3.B)





### 3.3. TOR Pathway Perturbation and Its Effects on the Circadian Rhythmicity of *Neurospora crassa*

#### 3.3.1 Introduction and objectives

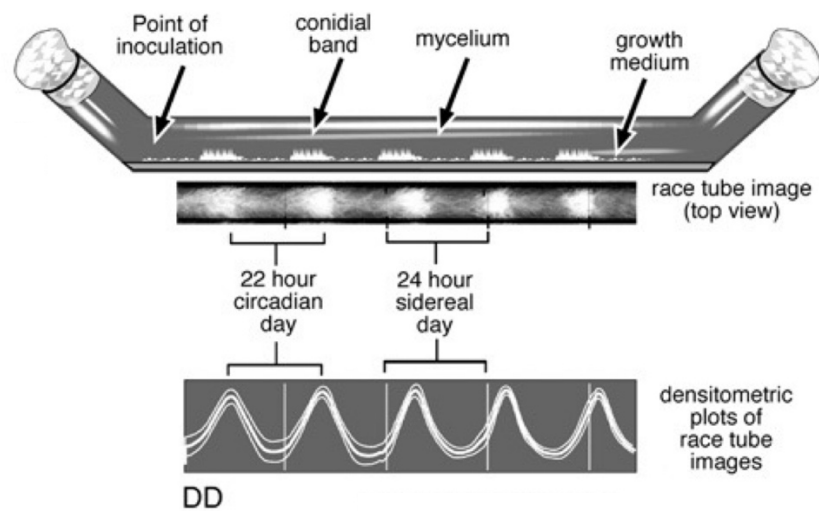
The regulation of metabolism by the circadian clock is thought to be a major reason behind the extensive representation of circadian rhythms within the phylogenetic tree (Hurley et al., 2015). Undeniably, much of cellular metabolism in the form of partitioning between the daytime activities involving energy production and nighttime tasks that favor the biosynthesis of cellular components, is clock-controlled (Ramanathan et al., 2018). High-throughput RNA-sequencing analysis reveals that approximately 10-50% of the *Neurospora crassa* transcriptome is reproducibly rhythmic at the mRNA level and out of this fraction, the genes that fall in metabolic pathways are highly enriched (Hurley et al., 2015; Hurley et al., 2016). The TOR pathway, as the major intracellular sensor that integrates nutritional signals and energy production to promote the growth and survival of the organism, exhibits robust circadian oscillation in its activity in various organisms and cells including *Neurospora* (Table.1.1). Our laboratory has become interested in discovering the links between the TOR pathway and the clock in *Neurospora*, due to the observations that when components of TOR pathway, namely the VTA (Vacuolar TOR Associated protein homologous to yeast EGO and mammalian LAMTOR1) and its binding partner Gtr2 (homologous to yeast's GTR2 and mammalian RAG C/D) are mutated, the FRQ-less rhythms of conidiation are abolished (Eskandari et al., 2021; Ratnayake et al., 2018). These findings collectively point to the potential role of TOR pathway in generating the FRQ-less oscillations in *Neurospora* (Lakin-Thomas, 2023).

To date, *Neurospora*'s TOR pathway is uncharacterized, therefore in order to investigate its role regulating the circadian rhythmicity of *N. crassa*, I first needed to identify the regulators of this pathway. In sections 3.1 and 3.2, I assessed the effects of different metabolites including amino acids and lipids on the TOR pathway activity of *Neurospora*. I found that the VTA protein plays an essential role in mediating amino acid signals to the TOR complex (Figure 3.2.3).

Additionally, I discovered that artificially elevating diacylglycerol (DAG) levels with a membrane-permeant DAG analog, DOG (1,2-dioctanoyl-*sn*-glycerol) in choline-starved, long-period *chol-1* mutant strain, inhibits the TOR pathway activity (Figure 3.6.3, B).

In this section, I focused on identifying the potential links between the TOR pathway and the circadian rhythmicity of *Neurospora* by analyzing the effects of TOR pathway regulators on the intrinsic clock property, the period of the conidiation rhythms. I reasoned that if the TOR pathway is indeed related to the clock, then its perturbation using the *vta* knockout strain and artificial elevation of DAG levels would affect the autonomous clock property, the length of period of conidiation. To complement these, I used two well-characterized TOR pathway inhibitors, Torin I and Torin II. Both compounds are selective ATP-competitive inhibitors that block the phosphorylation and activation of mTOR complexes, however, Torin II due to improved pharmacokinetics appears to be more potent than its predecessor, Torin I (Q. Liu et al., 2011). I hypothesized that if TOR pathway is involved in regulating the circadian rhythmicity of *Neurospora*, then its pharmacological inhibition using Torin compounds would affect the period of conidiation. Additionally, I sought to analyze whether the potential effects of elevated DAG on the clock is mediated through the TOR pathway by combining the DOG and Torin. Furthermore, the role of VTA protein in mediating the potential effects of Torin on the period of conidiation was analyzed.

To study the effects of the TOR modulators on the period of the conidiation rhythms, the race-tube assay was employed (Figure 3.7). The growth or “race tube” analysis permits the clear visualization of clock-driven asexual spore formation (conidiation) that can be easily seen as alternating zone between conidia (bands) and mycelia (interbands), when grown on a layer of solid agar media in long, hollow tubes, in constant darkness (DD) (Baker et al., 2012; Lakin-Thomas et al., 2011).



**Figure 3.7. The schematic diagram representing the race-tube assay used to study the circadian rhythms of conidiation of *Neurospora crassa* under laboratory conditions.** *Neurospora* grows linearly on hollow glass tubes filled with a layer of agar media, exhibiting periodic alternation between the conidia (bands) and mycelia (interbands) that happens every 22 hours in constant darkness (DD) (Adapted from Price-Lloyd et al., 2005).

### 3.3.2. Results & Discussion

#### 3.3.2.1 Effects of TOR Pathway Inhibitors on Rhythmicity in *chol-1* Strain of *Neurospora crassa*

Main Result: TOR pathway inhibitors alter the autonomous clock property, the length of period of conidiation of the *chol-1* strain of *Neurospora*

According to gene ontological (GO) analysis, lipid metabolism is highly enriched in clock output and alterations in lipid homeostasis can in turn feed back onto the core clock and affect the period length (Hurley et al., 2016; Ramsdale & Lakin-Thomas, 2000). The role of lipid metabolism in regulating the circadian oscillator is evident in the choline-requiring *chol-1* mutant strain where choline depletion alters membrane phospholipid composition such that the levels of PtdEtn (phosphatidylethanolamine) and PtdIns (phosphatidylinositol) increase while the levels of PtdCho (phosphatidylcholine) decrease (Lakin-Thomas & Brody, 2000). As shown in Figure 3.8.A and Table 3.1 (the control conditions), in choline-rich cultures, supplemented with 50 and 100  $\mu\text{M}$  choline, the periods of conidiation rhythm are  $21.26 \pm 0.01$  and  $20.34 \pm 2.04$  hours respectively, which resemble the wild-type conidiation period reported to be approximately 22 hours (Price-Lloyd et al., 2005). In choline-deprived cultures (below 50  $\mu\text{M}$  choline) the period length is inversely proportional to the choline concentration in the medium, reaching up to  $75.30 \pm 10.17$  hours at 0 choline concentration (Figure 3.8.A and Table 3.1). This strain is considered “leaky” because when grown without choline supplementation, the growth rate ( $0.40 \pm 0.04$ ) is half of the high choline cultures ( $0.92 \pm 0.02$  at 100  $\mu\text{M}$  choline) (Figure 3.8.B and Table 3.1).

In the absence of canonical clock components (i.e. absence of functional *frq*, or *wcc* genes), a cryptic oscillator (presumably a component of FRQ-Less Oscillator, FLO) in choline-starved *chol-1* strain, restores rhythmicity in these overtly arrhythmic backgrounds (Li et al., 2011). In attempts to identify the components of FLO, our laboratory has identified two mutations, namely the *vta* and *gtr2* that abolish the FRQ-less conidiation rhythm in the aforementioned genetic backgrounds; both of these proteins are components of the TOR pathway, suggesting a potential role of *Neurospora*’s TOR pathway in regulating the FLO (Eskandari et al., 2021; Ratnayake et al., 2018). To determine whether the TOR pathway is indeed involved in regulating circadian rhythmicity of *N. crassa*, I used two potent inhibitors of TOR complexes, Torin I and Torin II (1000 nM and 200 nM respectively, these concentrations were chosen based on the previous race tube assay performed in our laboratory) and tested their effects on the period length of *chol-1* strain

growing on different choline concentrations (0, 10, 50, and 100  $\mu$ M choline). I hypothesized that TOR pathway perturbation using artificial inhibitors would affect the period of conidiation and if the TOR pathway is responsible for the long-period phenotype seen in the absence of choline supplementation, then its inhibition would induce more pronounced effects in cultures growing on low-choline media. As seen in Figure 3.8.A, at 0 choline concentration, both Torin I and Torin II treatments significantly lengthened the period of conidiation up to  $167.14 \pm 16.23$  and  $159.52 \pm 12.54$  hours respectively, compared to Me<sub>2</sub>SO-containing samples ( $102.21 \pm 4.94$ ). Interestingly, at 10  $\mu$ M choline, only the Torin II treatment significantly increased the period length ( $57.79 \pm 3.59$ ) in relation to the DMSO controls ( $21.30 \pm 0.07$ ). At high choline concentrations (50 and 100  $\mu$ M choline) the period of conidiation rhythms appeared to be unaffected by the TOR inhibitors (Figure 3.8.A). At all of the tested choline concentrations, both Torin I and Torin II severely compromised the growth rate in comparison to their solvent-containing counterparts (Figure 3.8.B).

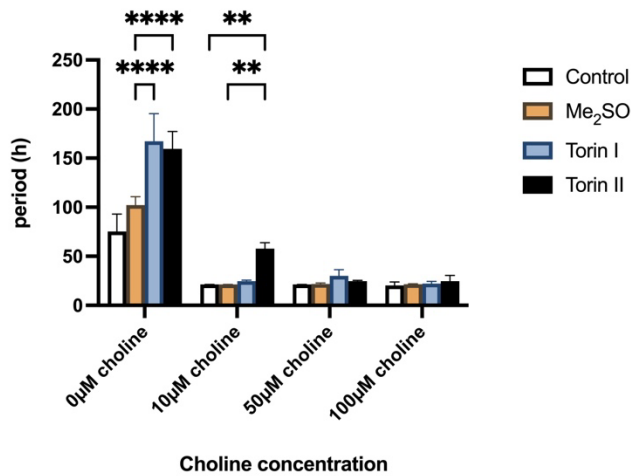
As seen in Figure 3.8.A, 200 nM concentration of Torin II lengthened the period at low-choline concentrations (0 and 10  $\mu$ M choline) but it was ineffective at high-choline (50 and 100  $\mu$ M) cultures. To verify whether the same relationship between choline sufficiency and Torin II-induced effects on period of the rhythmicity still holds true at a higher Torin II concentration, I used 500 nM of this inhibitor and tested its effects on cultures growing on 0 and 10 and 100  $\mu$ M choline. Of interest, when Torin II concentration was increased to 500 nM, in addition to low-choline cultures, the period of conidiation was significantly lengthened at high-choline tubes reaching up to  $50.80 \pm 8.09$  hours compared to Me<sub>2</sub>SO-containing cultures ( $23.04 \pm 0.60$  hours), suggesting a potential dose-dependent relationship between the choline sufficiency and Torin II's ability in affecting the period of conidiation (Figure 3.9.A and Table 3.2). Similar to the previous observation in Figure 3.8.B, Torin II treatment significantly inhibited the growth rate at all the tested choline concentrations (Figure 3.9.B).

The results obtained in this section suggest that TOR pathway inhibitors alter the autonomous clock property, the length of period of conidiation. The dramatic lengthening of the period at low choline concentrations treated with Torin compounds may imply that the mechanisms affected by changes in the phospholipid composition induced by choline depletion and TOR pathway are

interacting with each other to regulate the periodic rhythmicity. Therefore, at high choline concentrations when the lipid homeostasis is restored, higher concentration of Torin is required to exert a similar effect.

These findings are consistent with the previous observations that in both hepatocytes and adipocytes, mTOR pathway inhibitors such as rapamycin and Torin compounds lengthen the period and dampen the amplitude, while constitutive activation of mTOR shortens the period and augments the amplitude (Ramanathan et al., 2018). In addition to the pharmacological inhibition, genetic alterations of mTOR or its downstream effectors also impact the circadian rhythms in different organisms; for example, mTOR/4E-BP1-mediated translational control regulates the entrainment and synchrony of SCN (suprachiasmatic nucleus) neurons, and 4E-BP1 null mice exhibit accelerated re-entrainment to shifted light-dark cycles (R. Cao et al., 2013). Similarly, in *Drosophila*, silencing the *Tor* gene shortens the period of the locomotor activity rhythms of the fly (Kijak & Pyza, 2017). All of these observations support the role of the TOR pathway in the regulation of the rhythmic properties of circadian timekeeping systems.

3.8.A. A 200 nM concentration of Torin II significantly lengthens the period of conidiation rhythms at low choline concentrations, but it is ineffective at high choline concentrations



3.8.B. At all of the tested choline concentrations, both Torin I and Torin II significantly diminish the linear growth rate

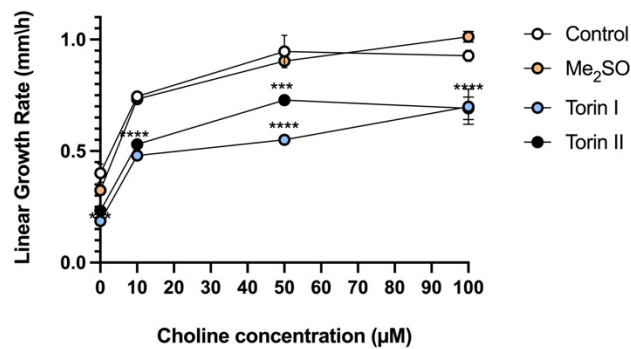


Table 3.1 Growth rate and period of *chol-1* strain growing on different choline concentrations. The values are reported as mean ± SEM of 3 race tubes in one experiment

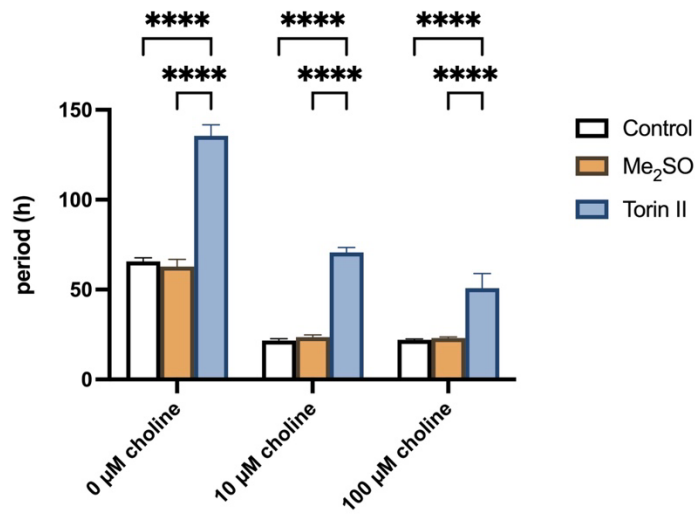
| Choline (μM) | Control       |                    | Me <sub>2</sub> SO |                    | Torin I        |                    | Torin II       |                    |
|--------------|---------------|--------------------|--------------------|--------------------|----------------|--------------------|----------------|--------------------|
|              | Period (h)    | Growth Rate (mm/h) | Period (h)         | Growth Rate (mm/h) | Period (h)     | Growth Rate (mm/h) | Period (h)     | Growth Rate (mm/h) |
| 0            | 75.30 ± 10.17 | 0.400 ± 0.04       | 102.21 ± 4.94      | 0.320 ± 0.01       | 167.14 ± 16.23 | 0.186 ± 0.00       | 159.51 ± 12.54 | 0.230 ± 0.00       |
| 10           | 21.26 ± 0.06  | 0.740 ± 0.06       | 21.30 ± 0.07       | 0.730 ± 0.01       | 24.68 ± 0.60   | 0.480 ± 0.01       | 57.71 ± 3.50   | 0.530 ± 0.00       |
| 50           | 21.26 ± 0.01  | 0.940 ± 0.07       | 21.81 ± 0.56       | 0.900 ± 0.01       | 30.10 ± 3.50   | 0.550 ± 0.01       | 24.63 ± 0.42   | 0.720 ± 0.01       |
| 100          | 20.34 ± 2.04  | 0.920 ± 0.025      | 21.53 ± 0.27       | 1.010 ± 0.02       | 22.28 ± 1.50   | 0.690 ± 0.07       | 24.64 ± 3.40   | 0.690 ± 0.05       |

**Figure 3.8. Effects of TOR pathway inhibitors on the period of conidiation rhythms and the linear growth rate of *chol-1* strain of *Neurospora crassa*.**

**3.8.A and B) A 200 nM concentration of Torin II significantly lengthens the period of conidiation rhythms at low choline concentrations, but it is ineffective at high choline concentrations, at all of the tested choline concentrations, both Torin I and Torin II significantly diminish the linear growth rate.** The *chol-1* (strain#667) was grown on maltose minimal media containing 0.5% maltose, 2% agar, 1x Vogel's salts, and various choline concentrations (0, 10, 50, and 100  $\mu$ M) in disposable race tubes. The TOR inhibitors were diluted in Me<sub>2</sub>SO and 1000 nM Torin I (from 1 mM stock solution) and 200 nM Torin II (from 5 mM stock solution) were added to the sterile MM prior to filling of the race tubes. The Me<sub>2</sub>SO tubes contained the highest treatment concentration, 1000 nM of the solvent. The cultures were incubated at 22°C at DD. The data are plotted as the mean  $\pm$  SEM of n=3 race tubes in one experiment. A two-way ANOVA followed by multiple comparison tests was performed to determine the significance of the data (for detailed statistical analysis, refer to Appendix B, Tables 3.8 A and B). Asterisks (\*) denote the statistically significant differences between conditions as follows: \*\* p < 0.01, \*\*\* p < 0.001, \*\*\*\* p < 0.0001. (In figure 3.8. B, smaller error bars are covered with the circle symbol).



3.9.A. A 500 nM concentration of Torin II significantly lengthens the period of conidiation rhythm at both low and high choline concentrations



3.9.B. A 500 nM concentration of Torin II significantly diminishes the growth rate at all of the tested choline concentrations

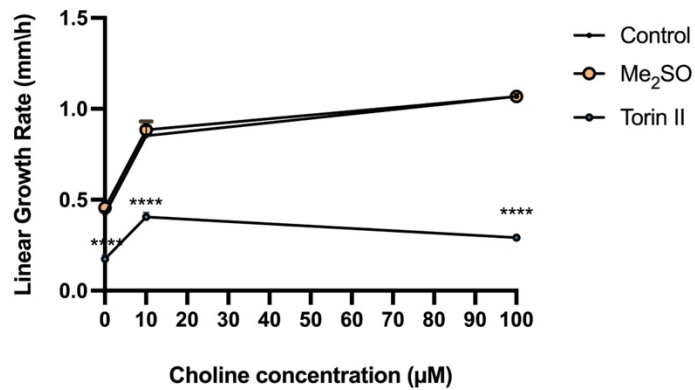


Table 3.2. Growth rate and period of *chol-1* strain growing on different choline concentrations supplemented with 500 nM of Torin II. The values are reported as mean ± SEM of n= 5 race tubes in one experiments.

| Choline (μM) | Control      |                    | Me <sub>2</sub> SO |                    | Torin II      |                    |
|--------------|--------------|--------------------|--------------------|--------------------|---------------|--------------------|
|              | Period (h)   | Growth Rate (mm/h) | Period (h)         | Growth Rate (mm/h) | Period (h)    | Growth Rate (mm/h) |
| 0            | 65.71 ± 1.97 | 0.42 ± 0.00        | 62.81 ± 3.99       | 0.45 ± 0.01        | 135.58 ± 6.10 | 0.17 ± 0.01        |
| 10           | 21.63 ± 1.11 | 0.85 ± 0.02        | 23.50 ± 1.28       | 0.88 ± 0.02        | 70.69 ± 2.69  | 0.40 ± 0.01        |
| 100          | 22.05 ± 0.55 | 1.06 ± 0.00        | 23.04 ± 0.60       | 1.06 ± 0.01        | 50.80 ± 8.09  | 0.29 ± 0.00        |

**Figure 3.9. Effects of 500 nM concentration of Torin II concentrations on the period of conidiation rhythms and the linear growth rate of *chol-1* strain of *Neurospora crassa* growing different choline concentrations. 3.9.A and B) A 500 nM concentration of Torin II significantly lengthens the period of conidiation rhythm at both low and high choline concentrations and significantly diminishes the growth rate at all of the tested choline concentrations.** The *chol-1* (strain#667) was grown on maltose minimal media containing 0.5% maltose, 2% agar, 1x Vogel's salts, and different choline concentrations: 0, 10, and 100  $\mu$ M choline in disposable race tubes. Torin II was diluted in Me<sub>2</sub>SO and 500 nM (from 5 mM stock solution) was added to the sterile MM prior to filling of the race tubes. The Me<sub>2</sub>SO tubes contained 500 nM of the solvent. The cultures were incubated at 22°C at DD. The data are plotted as the mean  $\pm$  SEM of n=5 race tubes in one experiment. A two-way ANOVA followed by multiple comparison tests was performed to determine the significance of the data (for detailed statistical analysis, refer to Appendix B, Tables 3.9. A and B). Asterisks (\*) denote the statistically significant differences between conditions as follows: \*\*\*\* p < 0.0001. (In figure 3.9. B, smaller error bars are covered with the circle symbol).

### 3.3.2.2 Effects of Elevated Diacylglycerol levels on Rhythmicity of *chol-1* Strain of *Neurospora crassa*

Main Result: The artificial elevation of diacylglycerol levels significantly lengthens the period of conidiation; however, whether these effects are exerted through the TOR pathway remains unclear

Blocks in the synthesis of phosphatidylcholine (PtdCho) in the choline-requiring *chol-1* mutant strain alter the levels of a number of important lipid metabolites, including membrane phospholipids and neutral lipids (Ramsdale & Lakin-Thomas, 2000). As seen in the previous section, when the *chol-1* strain is depleted of choline, the period of the conidiation rhythm lengthens dramatically up to approximately 60-75 hours (Figure 3.8.A). Although the direct biochemical basis for the lengthened period of choline-starved *chol-1* is unknown; it has been reported that the levels of *sn*-1,2-diacylglycerol (DAG) increase in proportion to the increase in period (Ramsdale & Lakin-Thomas, 2000). Additionally, the levels of DAG were found to be rhythmically modulated as an output of circadian clock (Ramsdale & Lakin-Thomas, 2000). These observations collectively point to the potential role of lipid metabolites in mediating the circadian rhythmicity of *N. crassa*.

As discussed in section 3.2.3.2, when endogenously elevated DAG levels in choline-depleted *chol-1* strain was further increased with an exogenous DAG analog, DOG (1,2-dioctanoyl-*sn*-glycerol), the excess diacylglycerol levels inhibited the TOR pathway activity (Figure 3.6.3.B). Therefore, to decipher the relationship between the TOR pathway and the clock properties, I analyzed the effects of increased DAG levels on the period of the conidiation using DOG. Furthermore, I asked whether the potential effects of elevated diacylglycerol on the clock is mediated through the TOR pathway, by combining the DOG with the TOR inhibitor, Torin II. I hypothesized that if indeed the long-period phenotype of choline-deficient *chol-1* is mediated through TOR pathway, then combination of DOG and Torin II would increase the length of the period more than DOG alone.

To address these questions, cultures of *chol-1* were grown on choline-deficient (to allow for elevation of endogenous DAG levels) race tubes. A 250  $\mu$ M of DOG that was previously shown to have maximum effect on the period of conidiation (Ramsdale & Lakin-Thomas, 2000) was

used. As expected, the artificial elevation of diacylglycerol levels using DOG significantly increased the period of conidiation rhythms to  $81.53 \pm 6.15$  hours in comparison to the Me<sub>2</sub>SO controls with the period length of  $62.81 \pm 3.99$  hours (Figure 3.10.A and Table 3.3). This may imply that indeed alterations in lipid homeostasis, in this case increased diacylglycerol level can in turn feed back onto the core clock and further affect the long-period of choline-starved *chol-1* strain.

Interestingly, combining DOG treatment with 500 nM concentration of Torin II ( $136.16 \pm 2.32$  hours) did not show an additive increase in the period compared to Torin II alone ( $135.58 \pm 6.10$  hours) (Figure 3.10.A). This may hint at the saturation of the system with this Torin II concentration, as no previous study has reported that the period of conidiation can be lengthened beyond this point. Therefore, a clearer depiction of potential additive effects of DOG + Torin II would be possible by lowering the concentration of the TOR inhibitor.

A similar trend was observed when comparing the linear growth rates. While both DOG ( $0.362 \pm 0.00$  mm/h) and Torin II ( $0.173 \pm 0.00$  mm/h) significantly diminished the growth rate in relation to the DMSO-containing tubes ( $0.457 \pm 0.01$  mm/h), the combined effects of DOG + Torin II ( $0.178 \pm 0.00$ ) did not exceed the individual Torin II's effects, which again raises the possibility of ceiling effect (Figure 3.10.B and Table 3.3).

Overall, these observations contribute to the existing knowledge of involvement of lipid molecules such as diacylglycerol in regulating the periodicity of *Neurospora*'s conidiation. It is noteworthy that accumulation of DAG as a membrane-bound lipid second messenger might alter the activity of many membrane-associated signaling pathways (Berridge & Irvine, 1989). Based on the previously observed inhibitory effects of increased diacylglycerol levels on the TOR pathway activity, it is tempting to speculate that TOR pathway is one of these cascades affected by changes in the level of this lipid metabolite (Section 3.2.3.2, Figure 3.6.3.B). However, whether the DAG-induced changes in the rhythmic properties of the clock are directed through the TOR pathway remains unclear.

Figure 3.10.A. In choline-deficient cultures, DOG significantly lengthens the period of conidiation, but in combination with Torin II, the observed effects do not exhibit an additive increase

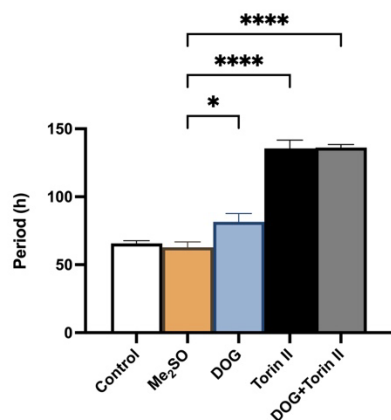


Figure 3.10.B. In choline-deficient cultures, DOG significantly diminishes the growth rate, but in combination with Torin II, the inhibitory effects are not additive

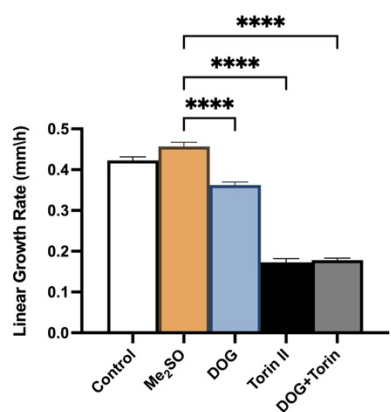


Table 3.3. Growth rate and period of *chol-1* strain growing on choline-deficient cultures supplemented with 250  $\mu$ M DOG and 500 nM Torin II. The data are presented as mean  $\pm$  SEM of  $n=5$  race tubes in one experiment.

| Treatment      | Period (h)        | Growth Rate (mm/h) |
|----------------|-------------------|--------------------|
| Control        | 65.71 $\pm$ 1.97  | 0.423 $\pm$ 0.00   |
| Me2SO          | 62.81 $\pm$ 3.90  | 0.457 $\pm$ 0.01   |
| DOG            | 81.53 $\pm$ 6.15  | 0.362 $\pm$ 0.00   |
| Torin II       | 135.58 $\pm$ 6.10 | 0.173 $\pm$ 0.00   |
| DOG + Torin II | 136.16 $\pm$ 2.35 | 0.178 $\pm$ 0.00   |

**Figure 3.10. Effects of Elevated Diacylglycerol levels on Rhythmicity of *chol-1* strain of *Neurospora crassa***  
**3.10.A and B) In choline-deficient cultures, DOG significantly lengthens the period of conidiation, but in combination with Torin II, the observed effects do not exhibit an additive increase, DOG significantly diminishes the growth rate, but in combination with Torin II, the inhibitory effects are not additive.** The *chol-1* (strain#667) was grown on maltose minimal media containing 0.5% maltose, 2% agar, 1x Vogel's salts, and no choline supplementation in disposable race tubes. A 250  $\mu$ M concentration of DOG (1,2-dioctanoyl-*sn*-glycerol) and a 500 nM (from 5 mM stock) concentration of Torin II was used. Both chemicals were diluted Me<sub>2</sub>SO and added to the sterile MM prior to filling of the race tubes. The Me<sub>2</sub>SO tubes contained final Me<sub>2</sub>SO concentration of 0.1% (v/v). The cultures were incubated at 22°C at DD. The data are plotted as the mean $\pm$  SEM of n=5 race tubes in one experiment. A one-way ANOVA followed by multiple comparison tests was performed to determine the significance of the data (for detailed statistical analysis, refer to Appendix B, Tables 3.10. A and B). Asterisks (\*) denote the statistically significant differences between conditions as follows: \*  $p < 0.05$ , \*\*\*\*  $p < 0.0001$ .

### 3.3.2.3 Role of the VTA Protein in Regulating the Rhythmicity of *Neurospora crassa*

Main Result: The VTA protein appears to be essential for maintaining the rhythmicity in both low and high choline cultures; the effects of the TOR inhibitor on the period are blocked in the absence of the VTA protein

VTA (vacuolar TOR-associated protein) is a component of the *Neurospora* TOR pathway which is anchored to the outer vacuolar membrane (Ratnayake et al., 2018). As shown in Section 3.1.2.3, the deletion of VTA significantly diminishes the S6 phosphorylation levels in response to glucose and amino acids (Figure 3.2.3). Our laboratory has identified and characterized the *vta* gene due to the observation that its deletion severely compromises the FRQ-less rhythms and damps out the FRQ protein rhythmicity (Li et al., 2011; Ratnayake et al., 2018). A similar pattern of compromised rhythmic conidiation was observed when Gtr2 (VTA binding partner, another component of *Neurospora*'s TOR pathway) was deleted, suggesting that these proteins are required to maintain the rhythmic properties in *Neurospora* (Eskandari et al., 2021).

The motivation for the investigations in this section was to establish potential connections between the TOR pathway components and the regulation of circadian rhythms. It was hypothesized that if the TOR pathway is required to maintain the endogenous rhythmic properties, then deletion mutation of one of its key components, namely the *vta* gene product, would alter the length of the conidiation period. To this aim, I compared the period length between the *chol-1* and  $\Delta vta$  strains growing on low (0 and 10  $\mu$ M) and high (100  $\mu$ M) choline concentrations. In choline-depleted cultures where the *chol-1* strain typically exhibits long period length, the *vta* knockout is arrhythmic (Table 3.4). This may imply that VTA protein function overlaps with the mechanisms responsible for the long-period phenotype observed in the absence of the choline supplementation. Interestingly, the growth rates of the two strains were not notably different at low-choline cultures (Table 3.4). In cultures growing on 100  $\mu$ M choline, *chol-1* has a period that resembles that of the wild-type strain ( $22.05 \pm 0.55$  hours). In the  $\Delta vta$  strain, the conidiation was initially rhythmic with a periodicity of  $27.06 \pm 0.53$  hours but the rhythms damped out after a few cycles, suggesting that even when lipid deficiencies are restored with high choline supplementation, the VTA protein is essential for maintaining the rhythmicity (Figure 3.11.A). The linear growth rate appeared to be similar between the two strains (Figure 3.11.B). The results obtained in this section on the effects of *vta* gene product on the period of

rhythmicity confirm the observations previously reported by Li & Lakin-Thomas (2011). The most striking results emerged when 500 nM concentration of Torin II was used to test whether knocking out the VTA protein would block the inhibitor's effects on the period length. As mentioned previously, on choline-rich tubes, this concentration of Torin II significantly lengthened the period up to  $65.71 \pm 1.97$  hours; remarkably, this Torin II-induced effect was blocked in the *vta* knockout strain, resulting in the period length of  $25.31 \pm 0.37$  hours (Figure 3.11.A). Torin II treatment diminished the growth rate of  $\Delta vta$  strain but not to the same extent as in the *chol-1* strain (Figure 3.11.B). Perhaps the most compelling findings of this section are that the VTA protein is essential for maintaining rhythmicity at both low and high choline concentrations and that the Torin II-induced effects on both the period and to a lesser extent on growth rate are blocked in the absence of this protein, suggesting the involvement of the VTA in Torin II's mechanism of action. (Davis, 1979)



Figure 3.11.A At 100  $\mu\text{M}$  choline, the  $\Delta\text{vta}$  strain is initially rhythmic but the rhythm dampens out after a few cycles; knocking out VTA blocks Torin II-induced effects on the period of conidiation

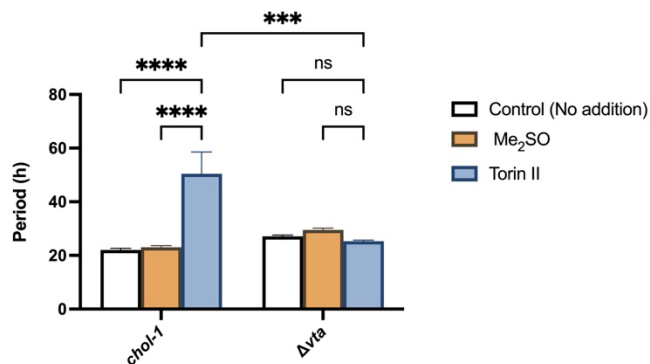


Figure 3.11.B Torin II treatment diminishes the linear growth rate in the  $\Delta\text{vta}$  strain but not to the same extent as in the *chol-1* strain

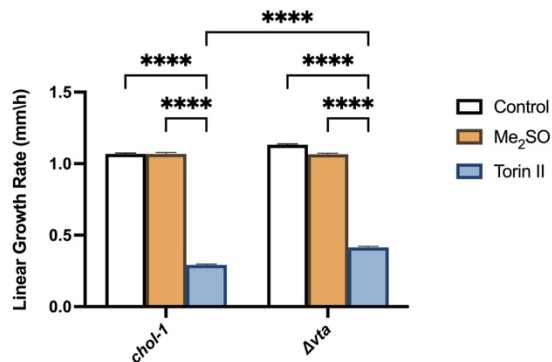


Table 3.4. Growth rate and period of *chol-1* and  $\Delta\text{vta}$  strains growing on different choline concentrations (0, 10, and 100  $\mu\text{M}$ ) supplemented with 500 nM concentration of Torin II. The data are presented as mean  $\pm$  SEM of n=5 race tubes from one experiment.

| [choline]<br>( $\mu\text{M}$ ) | <i>Chol-1</i>    |                    |                    |                    |                   |                    | $\Delta\text{vta}$ |                    |                    |                    |                  |                    |
|--------------------------------|------------------|--------------------|--------------------|--------------------|-------------------|--------------------|--------------------|--------------------|--------------------|--------------------|------------------|--------------------|
|                                | Control          |                    | Me <sub>2</sub> SO |                    | Torin II          |                    | Control            |                    | Me <sub>2</sub> SO |                    | Torin II         |                    |
|                                | Period (h)       | Growth Rate (mm/h) | Period (h)         | Growth Rate (mm/h) | Period (h)        | Growth Rate (mm/h) | Period (h)         | Growth Rate (mm/h) | Period (h)         | Growth Rate (mm/h) | Period (h)       | Growth Rate (mm/h) |
| 0                              | 65.71 $\pm$ 1.97 | 0.42 $\pm$ 0.00    | 62.81 $\pm$ 3.99   | 0.45 $\pm$ 0.01    | 135.58 $\pm$ 6.10 | 0.17 $\pm$ 0.01    | NR                 | 0.52 $\pm$ 0.03    | NR                 | 0.47 $\pm$ 0.01    | NR               | 0.40 $\pm$ 0.12    |
| 10                             | 21.63 $\pm$ 1.11 | 0.85 $\pm$ 0.02    | 23.50 $\pm$ 1.28   | 0.88 $\pm$ 0.02    | 70.69 $\pm$ 2.69  | 0.40 $\pm$ 0.01    | NR                 | 0.78 $\pm$ 0.00    | NR                 | 0.77 $\pm$ 0.00    | NR               | 0.50 $\pm$ 0.06    |
| 100                            | 22.05 $\pm$ 0.55 | 1.06 $\pm$ 0.00    | 23.04 $\pm$ 0.60   | 1.06 $\pm$ 0.01    | 50.80 $\pm$ 8.09  | 0.20 $\pm$ 0.00    | 27.06 $\pm$ 0.53   | 1.12 $\pm$ 0.01    | 29.5 $\pm$ 0.68    | 0.93 $\pm$ 0.13    | 25.31 $\pm$ 0.37 | 0.41 $\pm$ 0.00    |

NR: Not Rhythmic.

**Figure 3.11. role of the VTA protein in regulating Rhythmicity and growth rate of *Neurospora crassa*.**

**Figure 3.11.A and B) At 100  $\mu$ M choline, the  $\Delta$ *vta* strain is initially rhythmic but the rhythm dampens out after a few cycles; knocking out VTA blocks Torin II-induced effects on the period of conidiation, Torin II diminishes the linear growth rate in the  $\Delta$ *vta* strain but not to the same extent as in the *chol-1* strain.** The *chol-1*(strain#667) and  $\Delta$ *vta* (strain#694) were grown on maltose minimal media containing 0.5% maltose, 2% agar, 1x Vogel's salts, and 100  $\mu$ M choline in disposable race tubes. Torin II was diluted in Me<sub>2</sub>SO and from 5mM stock solution and 500 nM was added to the race tubes. The Me<sub>2</sub>SO tubes contained 500 nM of the solvent. The cultures were incubated at 22°C at DD. The data are plotted as the mean $\pm$  SEM of n=5 race tubes from one experiment. A two-way ANOVA followed by multiple comparison tests was performed to determine the significance of the data (for detailed statistical analysis, refer to Appendix B, Tables 3.11. A and B). Asterisks (\*) denote the statistically significant differences between conditions as follows: \*\*\* p < 0.001, \*\*\*\* p < 0.0001.

## CHAPTER 04: Discussion

### 4.1. Summary & Conclusion

Circadian time-keeping systems are precise yet plastic pacemakers that facilitate the temporal organization of a plethora of biological processes (Asher & Sassone-Corsi, 2015). In the filamentous fungus *Neurospora crassa*, the most accepted model of the core circadian oscillator is a transcription/translation feedback loop (TTFL) involving the interplay between the FREQUENCY (FRQ) and the White-Collar Complex (WCC) (Dunlap, 1999). However, partial rhythmicity can still be evoked in the absence of a bona fide FWO (FRQ-WCC Oscillator) (Lakin-Thomas et al., 2011). These rhythms that can arise under certain experimental conditions or defined genetic backgrounds are collectively coined as FLOs (FRQ-Less Oscillators) (Nsa et al., 2015). The presence of the FLO-based rhythms possibly hints to the existence of autonomous oscillators that may function in conjunction with the canonical TTFL and contribute to the robustness of the core pacemaker in *Neurospora* cells (Lakin-Thomas, 2006b). For many years, our laboratory's main agenda has been to identify the molecular components of the FLO and to depict a clearer picture of the *Neurospora* circadian network. To this aim, two mutations that abolish the FRQ-less rhythms have been identified, precisely, the *vta* and *gtr2* genes whose products were found to be the components of the TOR pathway (Eskandari et al., 2021; Ratnayake et al., 2018). These observations have redirected our attention to the potential connections between the TOR pathway and the circadian clock and have led to the speculation that TOR pathway, as one of the evolutionarily conserved metabolic pathways, is involved in the generation of FLO in *Neurospora*.

To date, *Neurospora*'s TOR pathway is uncharacterized, therefore, in order to evaluate its potential role as an oscillator, I first needed to define the regulators of this pathway and subsequently test whether those factors also affect the endogenous circadian clock properties. Since the main metabolites affecting the mammalian and the budding yeast's TOR pathway are amino acids and lipids molecules, I began by investigating the effects of amino acids on the phosphorylation state of endogenous S6 ribosomal protein as a marker of TOR pathway activation. In these experiments, I found that unlike mammalian cells where amino acids are essential for the maximal activation of mTORC1, *Neurospora* TOR pathway only becomes activated by amino acids after prolonged glucose starvation period (Figure 3.1.4), suggesting that

glucose alone is sufficient to activate the TOR kinase. The amino acid activation of mTORC1 and yeast TORC1 follows the same consensus models involving Rag GTPase-EGO complex (Alfatah et al., 2023; Powis & De Virgilio, 2016). To determine *Neurospora*'s cellular components involved in transmitting the amino acid signals to the TOR complex, I used *Neurospora* knockout strains  $\Delta gtr2$  (GTR2<sup>KO</sup>, equivalent of Gtr2 in budding yeast) and  $\Delta vta$  (VTA<sup>KO</sup>, homolog of Ego in yeast). Although both proteins appeared to be important in metabolite-induced TOR activation, the VTA protein is likely to play a more prominent role, as its deletion had a more pronounced effects on the S6 phosphorylation levels in response to both glucose and amino acids (Figure 3.2.3). In mammals, under amino acid-starved conditions, autophagy is initiated to restore the amino acid pool through increased protein degradation, and this autophagy-dependent elevation in the cytosolic amino acid pool can subsequently feed back and re-activate mTORC1 to upregulate the expression of genes essential for the survival of the organism under amino acid insufficiency (Condon & Sabatini, 2019). Because the wild-type *Neurospora* cells are capable of synthesizing all amino acids de novo, to determine whether amino acid starvation can increase the TOR pathway activity in *Neurospora*, I used amino acid auxotrophic mutants: the glutamine auxotroph (*gln-1*) and the arginine auxotroph (*arg-13*), which require glutamine and arginine supplementation for normal growth respectively. The results likely suggest that starving the amino acids auxotrophs for their required amino acid might increase TOR pathway activity; however, this study could benefit from more repeated trials (Figure 3.3.2).

In addition to amino acids, lipid second messengers such as phosphatidic acids regulate the activity of mTOR complexes (D. A. Foster, 2013). To explore the effects of lipid composition on *Neurospora*'s TOR pathway, I used choline-requiring *chol-1* mutant strain which in the absence of choline supplementation exhibits an altered lipid composition including the accumulation of neutral lipids such as diacylglycerols (DAG) in *Neurospora*'s membranes (Ramsdale & Lakin-Thomas, 2000). I found that choline starvation of the *chol-1* mutant strain increases TOR pathway activity only in choline-depleted cultures growing without glucose supplementation over an extended period of time (Figures 3.5.3 & 3.5.4). To complement this study, I combined the endogenously elevated DAG with the artificial DAG analog, DOG (1,2-dioctanoyl-*sn*-glycerol) and investigated whether these potential changes in the lipid composition would affect

the TOR pathway activity. The outcome of these experiments showed that in choline-starved, low-glucose cultures, an elevation in DAG levels inhibits P-S6 levels possibly due to lipid overload (Figure 3.6.3.B). Taken together, these observations suggest that there is an association between lipid homeostatic state and the TOR pathway of *Neurospora*, however, the precise mechanisms involved remain to be uncovered.

Based on the results obtained in Sections 3.1 and 3.2 regarding the characterization of *Neurospora*'s TOR pathway, it was shown that amino acids and lipids can affect *Neurospora*'s TOR pathway only in the absence of its main activator, glucose. This suggests that this easily-fermentable carbon-source is sufficient to activate TOR kinase in this organism. Additionally, the VTA protein plays an essential role in mediating amino acid signals to the TOR complex. In addition, artificially elevating diacylglycerol levels in choline-starved, long-period *chol-1* mutant strain inhibits the TOR pathway activity.

Moving forward, to establish a potential connection between the TOR pathway and circadian rhythms, I analyzed the effects of aforementioned TOR regulators on the endogenous clock property, the period of conidiation. These experiments began with a simple idea that if the eukaryotic circadian system is a network of multiple interconnected oscillators and the TOR pathway is one of them, then perturbation of this pathway would affect the periodicity of rhythmic conidiation. To test this, Torin II, a potent TOR inhibitor was employed. The results of these experiments indicated that Torin II dramatically lengthens the period of conidiation of *chol-1* strains growing on both at low and high choline cultures, and the magnitude of the response depends on the choline concentration in the medium with choline-deficient cultures being more susceptible to lower concentration of Torin II, while choline-rich cultures exhibiting a long-period phenotype only at higher Torin II doses (Figures 3.8.A & 3.9.A). The dose-dependent relationship between choline sufficiency and Torin-induced effects on period length may suggest an overlap between the cellular mechanisms responsible for maintaining lipid homeostasis, TOR pathway activity and the circadian time-keeping systems in *N. crassa*.

In order to investigate this intricate relationship in more detail, I combined the endogenously elevated diacylglycerol levels in the choline-starved *chol-1* mutant strain with an exogenous DAG analog, DOG. The results indicated that the artificial elevation of diacylglycerols levels

significantly lengthens the period of conidiation, emphasizing the correlation between membrane lipid composition and the periodicity of circadian rhythms. However, whether these effects are exerted through the TOR pathway remains an open question, as combining DOG with Torin II did not exhibit a synergistic response, possibly due to a ceiling effect induced by high Torin II concentration (Figure 3.10.A).

As shown previously, pharmacological inhibition of TOR pathway using Torin II significantly affected the periodic spore formation of *Neurospora*, in a similar approach, I sought to investigate whether a similar response would be observed if one of the key components of the TOR pathway, namely, the VTA protein is not functional. Interestingly, I found that this protein is essential for maintaining the rhythmicity in both low and high choline concentrations, as the VTA knockout strains growing on choline-deficient cultures were arrhythmic and the rhythms of those growing on choline-rich medium dampened rapidly (Figure 3.11.A). Another important finding that emerged from this study was that the deletion mutation of VTA appeared to block Torin II's effect on the period of conidiation, which hints at the potential role of this protein in the inhibitor's mechanism of action.

In conclusion, the goal of this thesis was to characterize the TOR pathway of the fungus *Neurospora crassa* to ultimately determine whether this pathway is involved in the regulation of circadian rhythmicity. The novel and preliminary insights obtained here regarding the factors influencing *Neurospora*'s TOR pathway activity such as non-functional VTA protein, lipid homeostasis altered by choline-starvation or excess diacylglycerol and its metabolic perturbation using the TOR inhibitors together with the previous research suggest a connection between the TOR pathway and the circadian machinery as factors affecting the TOR pathway were also found to influence the clock properties which collectively point toward a comprehensive network integrating metabolism and the circadian system.

## 4.2. Future Directions

Continued efforts are required to further elucidate the *Neurospora crassa* TOR pathway, since this pathway appears to be intermingled with many other vital signaling cascades essential for the survival of this organism. One of these pathways is likely to be the Carbon Catabolite Repression (CCR) through which favourable carbon sources such as glucose repress the enzymes required for the utilization of other less preferred compounds (Xiong et al., 2014). In budding yeast, glucose-mediated CCR dictates the activity of amino acid permeases; interestingly, the activity of amino acid transporters increases in the absence of glucose (Peter et al., 2006). Although the crosstalk between CCR and TOR pathway has not been elucidated yet, the results of the experiments conducted in this thesis align with the existence of such regulatory mechanisms in *Neurospora*. Therefore, monitoring the amino acid permease activities using fluorescently labelled amino acid analogs and visualizing their uptake using fluorescent microscopy or flow cytometry in the presence and absence of glucose can provide detailed description of whether glucose-induced CCR is responsible for the sensitivity or insensitivity of the TOR pathway to amino acids.

Starving the amino acid auxotrophs for their required amino acids appears to increase TOR pathway activity possibly due to an autophagy-induced increase in protein degradation. Further studies need to be carried out to statistically validate these observations. Moreover, if the number of autophagosomes induced by autophagy increases in the fungal hyphae, this would further indicate that indeed the protein degradation-mediated increase in the amino acid pool is responsible for the increase in the S6 phosphorylation.

Choline starvation of *chol-1* mutant strain increased the TOR pathway activity. However, it is unclear whether this is a result of the increased conversion of diacylglycerol (DAG) to phosphatidic acid (PA) or if other mechanisms are involved. Future research might explore this idea by using inhibitors of enzymes involved in phosphatidic acid synthesis such as diacylglycerol kinase inhibitor or phospholipase D inhibitor to establish whether the increase in the TOR pathway activity would be affected by abrogation of PA signaling.

It was previously proposed that when endogenously elevated diacylglycerol is combined with exogenous DAG analog, DOG, the TOR pathway activity is downregulated possibly due to lipotoxicity. In this thesis the tested concentration of DOG used was 250  $\mu$ M, it would be

informative to test the effects of lower doses of this compound, for example 100  $\mu$ M DOG (which was also effective in lengthening of the period of conidiation (Ramsdale & Lakin-Thomas, 2000), to assess whether the TOR inhibition magnitude changes; if at lower DOG concentrations, TOR inhibition is less pronounced that could possibly hint that this response is associated with lipid overload.

When analyzing the effects of artificial elevation of diacylglycerol on the period of conidiation, it was shown that excess diacylglycerol lengthens the period of the conidiation but the study was limited in that it could not determine whether these effects are mediated through the TOR pathway because of high Torin II concentration (500 nM) that potentially saturated the system. To gain a better understanding of the involvement of the TOR pathway in the DOG-mediated period increase, a more comprehensive study involving various lower concentrations of Torin II (200-300  $\mu$ M) could provide deeper insights into whether the DOG and Torin II treatments together exhibit a synergistic effect on the periodicity of the conidiation.



## References:

- Adamovich, Y., Roussio-Noori, L., Zwihaft, Z., Neufeld-Cohen, A., Golik, M., Kraut-Cohen, J., Wang, M., Han, X., & Asher, G. (2014). Circadian clocks and feeding time regulate the oscillations and levels of hepatic triglycerides. *Cell Metabolism*, 19(2). <https://doi.org/10.1016/j.cmet.2013.12.016>
- Adams, S., Manfield, I., Stockley, P., & Carré, I. A. (2015). Revised Morning Loops of the Arabidopsis Circadian Clock Based on Analyses of Direct Regulatory Interactions. *PLoS ONE*, 10(12). <https://doi.org/10.1371/journal.pone.0143943>
- Adhvaryu, K., Firoozi, G., Motavaze, K., & Lakin-Thomas, P. (2016). PRD-1, a Component of the Circadian System of Neurospora crassa, Is a Member of the DEAD-box RNA Helicase Family. *Journal of Biological Rhythms*, 31(3). <https://doi.org/10.1177/0748730416639717>
- Akhtari, G. (n.d.). *THE RELATIONSHIP BETWEEN TOR ACTIVITY AND THE CIRCADIAN CLOCK OF NEUROSPORA CRASSA*.
- Albert, V., & Hall, M. N. (2015). mTOR signaling in cellular and organismal energetics. In *Current Opinion in Cell Biology* (Vol. 33, Issue 1). <https://doi.org/10.1016/j.ceb.2014.12.001>
- Albrecht, U. (2012). Timing to Perfection: The Biology of Central and Peripheral Circadian Clocks. In *Neuron* (Vol. 74, Issue 2). <https://doi.org/10.1016/j.neuron.2012.04.006>
- Alfatah, M., Cui, L., Goh, C. J. H., Cheng, T. Y. N., Zhang, Y., Naaz, A., Wong, J. H., Lewis, J., Poh, W. J., & Arumugam, P. (2023). Metabolism of glucose activates TORC1 through multiple mechanisms in Saccharomyces cerevisiae. *Cell Reports*, 42(10). <https://doi.org/10.1016/j.celrep.2023.113205>
- Alfatah, M., Wong, J. H., Krishnan, V. G., Lee, Y. C., Sin, Q. F., Goh, C. J. H., Kong, K. W., Lee, W. T., Lewis, J., Hoon, S., & Arumugam, P. (2021). TORC1 regulates the transcriptional response to glucose and developmental cycle via the Tap42-Sit4-Rrd1/2 pathway in Saccharomyces cerevisiae. *BMC Biology*, 19(1). <https://doi.org/10.1186/s12915-021-01030-3>
- Allada, R., & Bass, J. (2021). Circadian Mechanisms in Medicine. *New England Journal of Medicine*, 384(6), 550–561. <https://doi.org/10.1056/nejmra1802337>
- Allada, R., & Chung, B. Y. (2009). Circadian organization of behavior and physiology in Drosophila. In *Annual Review of Physiology* (Vol. 72). <https://doi.org/10.1146/annurev-physiol-021909-135815>
- Amemiya, Y., Maki, M., Shibata, H., & Takahara, T. (2023). New Insights into the Regulation of mTOR Signaling via Ca<sup>2+</sup>-Binding Proteins. In *International Journal of Molecular Sciences* (Vol. 24, Issue 4). <https://doi.org/10.3390/ijms24043923>
- Aramayo, R., & Selker, E. U. (2013). Neurospora crassa, a model system for epigenetics research. *Cold Spring Harbor Perspectives in Biology*, 5(10). <https://doi.org/10.1101/cshperspect.a017921>
- Asher, G., & Sassone-Corsi, P. (2015). Time for food: The intimate interplay between nutrition, metabolism, and the circadian clock. In *Cell* (Vol. 161, Issue 1). <https://doi.org/10.1016/j.cell.2015.03.015>
- Asiimwe, O. H., Rubaihayo, J., Sulaiman, S. O., Osuwat, L. O., & Kasozi, K. I. (2023). A protein restricted diet induces a stable increased fat storage phenotype in flies. *Toxicology Reports*, 10. <https://doi.org/10.1016/j.toxrep.2023.06.003>
- Atan, Y. S., Subaşı, M., Özdemir, P. G., & Batur, M. (2023). The Effect of Blindness on Biological Rhythms and the Consequences of Circadian Rhythm Disorder. In *Turkish*

- Journal of Ophthalmology* (Vol. 53, Issue 2).  
<https://doi.org/10.4274/tjo.galenos.2022.59296>
- Avruch, J., Long, X., Ortiz-Vega, S., Rapley, J., Papageorgiou, A., & Dai, N. (2009). Amino acid regulation of TOR complex 1. In *American Journal of Physiology - Endocrinology and Metabolism* (Vol. 296, Issue 4). <https://doi.org/10.1152/ajpendo.90645.2008>
- Baker, C. L., Loros, J. J., & Dunlap, J. C. (2012). The circadian clock of *Neurospora crassa*. In *FEMS Microbiology Reviews* (Vol. 36, Issue 1). <https://doi.org/10.1111/j.1574-6976.2011.00288.x>
- Barnea, M., Haviv, L., Gutman, R., Chapnik, N., Madar, Z., & Froy, O. (2012). Metformin affects the circadian clock and metabolic rhythms in a tissue-specific manner. *Biochimica et Biophysica Acta - Molecular Basis of Disease*, 1822(11).  
<https://doi.org/10.1016/j.bbadis.2012.08.005>
- Bar-Peled, L., Schweitzer, L. D., Zoncu, R., & Sabatini, D. M. (2012). Ragulator is a GEF for the rag GTPases that signal amino acid levels to mTORC1. *Cell*, 150(6).  
<https://doi.org/10.1016/j.cell.2012.07.032>
- Barrett, R. K., & Takahashi, J. S. (1995). Temperature compensation and temperature entrainment of the chick pineal cell circadian clock. *Journal of Neuroscience*, 15(8).  
<https://doi.org/10.1523/jneurosci.15-08-05681.1995>
- Bass, J. (2012). Circadian topology of metabolism. In *Nature* (Vol. 491, Issue 7424).  
<https://doi.org/10.1038/nature11704>
- Bass, J., & Takahashi, J. S. (2010). Circadian integration of metabolism and energetics. In *Science* (Vol. 330, Issue 6009). <https://doi.org/10.1126/science.1195027>
- Basse, A. L., Nielsen, K. N., Karavaeva, I., Ingerslev, L. R., Ma, T., Havelund, J. F., Nielsen, T. S., Frost, M., Peics, J., Dalbram, E., Dall, M., Zierath, J. R., Barrès, R., Færgeman, N. J., Treebak, J. T., & Gerhart-Hines, Z. (2023). NAMPT-dependent NAD<sup>+</sup> biosynthesis controls circadian metabolism in a tissue-specific manner. *Proceedings of the National Academy of Sciences of the United States of America*, 120(14). <https://doi.org/10.1073/pnas.2220102120>
- Bell-Pedersen, D., Cassone, V. M., Earnest, D. J., Golden, S. S., Hardin, P. E., Thomas, T. L., & Zoran, M. J. (2005). Circadian rhythms from multiple oscillators: Lessons from diverse organisms. In *Nature Reviews Genetics* (Vol. 6, Issue 7). <https://doi.org/10.1038/nrg1633>
- Ben-Sahra, I., & Manning, B. D. (2017). mTORC1 signaling and the metabolic control of cell growth. In *Current Opinion in Cell Biology* (Vol. 45).  
<https://doi.org/10.1016/j.ceb.2017.02.012>
- Bernfeld, E., Menon, D., Vaghela, V., Zerlin, I., Faruque, P., Frias, M. A., & Foster, D. A. (2018). Phospholipase D-dependent mTOR complex 1 (mTORC1) activation by glutamine. *Journal of Biological Chemistry*, 293(42). <https://doi.org/10.1074/jbc.RA118.004972>
- Berridge, M. J., & Irvine, R. F. (1989). Inositol phosphates and cell signalling. In *Nature* (Vol. 341, Issue 6239). <https://doi.org/10.1038/341197a0>
- Bhadra, U., Patra, P., & Pal-Bhadra, M. (2018). Cardinal Epigenetic Role of non-coding Regulatory RNAs in Circadian Rhythm. In *Molecular Neurobiology* (Vol. 55, Issue 4).  
<https://doi.org/10.1007/s12035-017-0573-8>
- Binda, M., Péli-Gulli, M. P., Bonfils, G., Panchaud, N., Urban, J., Sturgill, T. W., Loewith, R., & De Virgilio, C. (2009). The Vam6 GEF Controls TORC1 by Activating the EGO Complex. *Molecular Cell*, 35(5). <https://doi.org/10.1016/j.molcel.2009.06.033>

- Blume, C., Garbazza, C., & Spitschan, M. (2019). Effects of light on human circadian rhythms, sleep and mood. In *Somnologie* (Vol. 23, Issue 3). <https://doi.org/10.1007/s11818-019-00215-x>
- Borkovich, K. A., Alex, L. A., Yarden, O., Freitag, M., Turner, G. E., Read, N. D., Seiler, S., Bell-Pedersen, D., Paietta, J., Plesofsky, N., Plamann, M., Goodrich-Tanrikulu, M., Schulte, U., Mannhaupt, G., Nargang, F. E., Radford, A., Selitrennikoff, C., Galagan, J. E., Dunlap, J. C., ... Pratt, R. (2004). Lessons from the Genome Sequence of *Neurospora crassa* : Tracing the Path from Genomic Blueprint to Multicellular Organism . *Microbiology and Molecular Biology Reviews*, 68(1). <https://doi.org/10.1128/mmbr.68.1.1-108.2004>
- Botman, D., Kanagasabapathi, S., Savakis, P., & Teusink, B. (2023). Using the AKAR3-EV biosensor to assess Sch9p- and PKA-signalling in budding yeast. *FEMS Yeast Research*, 23. <https://doi.org/10.1093/femsyr/foad029>
- Broach, J. R. (2012). Nutritional control of growth and development in yeast. In *Genetics* (Vol. 192, Issue 1). <https://doi.org/10.1534/genetics.111.135731>
- Bröer, S., & Bröer, A. (2017). Amino acid homeostasis and signalling in mammalian cells and organisms. In *Biochemical Journal* (Vol. 474, Issue 12). <https://doi.org/10.1042/BCJ20160822>
- Buhr, E. D., & Takahashi, J. S. (n.d.-a). *Molecular components of the mammalian circadian clock*.
- Buhr, E. D., & Takahashi, J. S. (n.d.-b). *Molecular components of the mammalian circadian clock*.
- Burkart, G. M., & Brandizzi, F. (2021). A Tour of TOR Complex Signaling in Plants. In *Trends in Biochemical Sciences* (Vol. 46, Issue 5). <https://doi.org/10.1016/j.tibs.2020.11.004>
- Cam, H., Easton, J. B., High, A., & Houghton, P. J. (2010). mTORC1 signaling under hypoxic conditions is controlled by atm-dependent phosphorylation of HIF-1 $\alpha$ . *Molecular Cell*, 40(4). <https://doi.org/10.1016/j.molcel.2010.10.030>
- Cangelosi, A. L., Puszynska, A. M., Roberts, J. M., Armani, A., Nguyen, T. P., Spinelli, J. B., Kunchok, T., Wang, B., Chan, S. H., Lewis, C. A., Comb, W. C., Bell, G. W., Helman, A., & Sabatini, D. M. (2022). Zonated leucine sensing by Sestrin-mTORC1 in the liver controls the response to dietary leucine. *Science*, 377(6601). <https://doi.org/10.1126/science.abi9547>
- Cao, P., Kim, S. J., Xing, A., Schenck, C. A., Liu, L., Jiang, N., Wang, J., Last, R. L., & Brandizzi, F. (2019). Homeostasis of branched-chain amino acids is critical for the activity of TOR signaling in Arabidopsis. *ELife*, 8. <https://doi.org/10.7554/eLife.50747>
- Cao, R., Robinson, B., Xu, H., Gkogkas, C., Khoutorsky, A., Alain, T., Yanagiya, A., Nevarko, T., Liu, A. C., Amir, S., & Sonenberg, N. (2013). Translational control of entrainment and synchrony of the suprachiasmatic circadian clock by mTOR/4E-BP1 signaling. *Neuron*, 79(4). <https://doi.org/10.1016/j.neuron.2013.06.026>
- Cervela-Cardona, L., Alary, B., & Mas, P. (2021). The Arabidopsis Circadian Clock and Metabolic Energy: A Question of Time. In *Frontiers in Plant Science* (Vol. 12). <https://doi.org/10.3389/fpls.2021.804468>
- Cha, J., Zhou, M., & Liu, Y. (2015). Mechanism of the *Neurospora* circadian clock, a FREQUENCY-centric view. *Biochemistry*, 54(2). <https://doi.org/10.1021/bi5005624>
- Chang, Y. G., Cohen, S. E., Phong, C., Myers, W. K., Kim, Y. I., Tseng, R., Lin, J., Zhang, L., Boyd, J. S., Lee, Y., Kang, S., Lee, D., Li, S., Britt, R. D., Rust, M. J., Golden, S. S., & Li Wang, A. (2015). A protein fold switch joins the circadian oscillator to clock output in cyanobacteria. *Science*, 349(6245). <https://doi.org/10.1126/science.1260031>

- Chantranupong, L., Scaria, S. M., Saxton, R. A., Gygi, M. P., Shen, K., Wyant, G. A., Wang, T., Harper, J. W., Gygi, S. P., & Sabatini, D. M. (2016). The CASTOR Proteins Are Arginine Sensors for the mTORC1 Pathway. *Cell*, 165(1). <https://doi.org/10.1016/j.cell.2016.02.035>
- Chatterjee, A., Tanoue, S., Houl, J. H., & Hardin, P. E. (2010). Regulation of Gustatory Physiology and Appetitive Behavior by the Drosophila Circadian Clock. *Current Biology*, 20(4). <https://doi.org/10.1016/j.cub.2009.12.055>
- Chen, G. H., Liu, M. J., Xiong, Y., Sheen, J., & Wu, S. H. (2018). TOR and RPS6 transmit light signals to enhance protein translation in deetiolating Arabidopsis seedlings. *Proceedings of the National Academy of Sciences of the United States of America*, 115(50). <https://doi.org/10.1073/pnas.1809526115>
- Chen, Z., Yoo, S. H., & Takahashi, J. S. (2018). Development and Therapeutic Potential of Small-Molecule Modulators of Circadian Systems. In *Annual Review of Pharmacology and Toxicology* (Vol. 58). <https://doi.org/10.1146/annurev-pharmtox-010617-052645>
- Cockrell, A. L., Pirlo, R. K., Babson, D. M., Cusick, K. D., Soto, C. M., Petersen, E. R., Davis, M. J., Hong, C. I., Lee, K., Fitzgerald, L. A., & Biffinger, J. C. (2015). Suppressing the *Neurospora crassa* circadian clock while maintaining light responsiveness in continuous stirred tank reactors. *Scientific Reports*, 5. <https://doi.org/10.1038/srep10691>
- Cohen, S. E., & Golden, S. S. (2015). Circadian Rhythms in Cyanobacteria. *Microbiology and Molecular Biology Reviews*, 79(4). <https://doi.org/10.1128/mmb.00036-15>
- Condon, K. J., & Sabatini, D. M. (2019). Nutrient regulation of mTORC1 at a glance. *Journal of Cell Science*, 132(21). <https://doi.org/10.1242/JCS.222570>
- Conlon, B. H., Aanen, D. K., Beemelmans, C., de Beer, Z. W., De Fine Licht, H. H., Gunde-Cimerman, N., Schiøtt, M., & Poulsen, M. (2019). Reviewing the taxonomy of Podaxis: Opportunities for understanding extreme fungal lifestyles. In *Fungal Biology* (Vol. 123, Issue 3). <https://doi.org/10.1016/j.funbio.2019.01.001>
- Corral-Ramos, C., Barrios, R., Ayté, J., & Hidalgo, E. (2022). TOR and MAP kinase pathways synergistically regulate autophagy in response to nutrient depletion in fission yeast. *Autophagy*, 18(2). <https://doi.org/10.1080/15548627.2021.1935522>
- Crespo, M., Leiva, M., & Sabio, G. (2021). Circadian clock and liver cancer. In *Cancers* (Vol. 13, Issue 14). <https://doi.org/10.3390/cancers13143631>
- Creux, N., & Harmer, S. (2019). Circadian rhythms in plants. *Cold Spring Harbor Perspectives in Biology*, 11(9). <https://doi.org/10.1101/cshperspect.a034611>
- Davis, R. H. (1979). Genetics of arginine biosynthesis in *Neurospora crassa*. *Genetics*, 93(3). <https://doi.org/10.1093/genetics/93.3.557>
- Davis, R. H., & de Serres, F. J. (1970). Genetic and Microbiological Research Techniques for *Neurospora crassa*. *Methods in Enzymology*, 17. [https://doi.org/10.1016/0076-6879\(71\)17168-6](https://doi.org/10.1016/0076-6879(71)17168-6)
- Davis, R. H., & Perkins, D. D. (2002). *Neurospora*: A model of model microbes. In *Nature Reviews Genetics* (Vol. 3, Issue 5). <https://doi.org/10.1038/nrg797>
- Di Como, C. J., & Jiang, Y. (2006). The association of Tap42 phosphatase complexes with TORC1: Another level of regulation in Tor signaling. In *Cell Cycle* (Vol. 5, Issue 23). <https://doi.org/10.4161/cc.5.23.3516>
- Dibner, C., & Gachon, F. (2015). Circadian dysfunction and obesity: Is leptin the missing link? In *Cell Metabolism* (Vol. 22, Issue 3). <https://doi.org/10.1016/j.cmet.2015.08.008>
- Dibner, C., & Schibler, U. (2015). Circadian timing of metabolism in animal models and humans. *Journal of Internal Medicine*, 277(5). <https://doi.org/10.1111/joim.12347>

- Diernfellner, A. C. R., Lauinger, L., Shostak, A., & Brunner, M. (2019). A pathway linking translation stress to checkpoint kinase 2 signaling in *Neurospora crassa*. *Proceedings of the National Academy of Sciences of the United States of America*, 116(35). <https://doi.org/10.1073/pnas.1815396116>
- Diernfellner, A., Colot, H. V., Dintsis, O., Loros, J. J., Dunlap, J. C., & Brunner, M. (2007). Long and short isoforms of *Neurospora* clock protein FRQ support temperature-compensated circadian rhythms. *FEBS Letters*, 581(30). <https://doi.org/10.1016/j.febslet.2007.11.043>
- Dong, G., Kim, Y. I., & Golden, S. S. (2010). Simplicity and complexity in the cyanobacterial circadian clock mechanism. In *Current Opinion in Genetics and Development* (Vol. 20, Issue 6). <https://doi.org/10.1016/j.gde.2010.09.002>
- Dubowy, C., & Sehgal, A. (2017). Circadian rhythms and sleep in *Drosophila melanogaster*. *Genetics*, 205(4). <https://doi.org/10.1534/genetics.115.185157>
- Dunlap, J. C. (1999). Molecular bases for circadian clocks. In *Cell* (Vol. 96, Issue 2). [https://doi.org/10.1016/S0092-8674\(00\)80566-8](https://doi.org/10.1016/S0092-8674(00)80566-8)
- Dunlap, J. C., Borkovich, K. A., Henn, M. R., Turner, G. E., Sachs, M. S., Glass, N. L., McCluskey, K., Plamann, M., Galagan, J. E., Birren, B. W., Weiss, R. L., Townsend, J. P., Loros, J. J., Nelson, M. A., Lambreghts, R., Colot, H. V., Park, G., Collopy, P., Ringelberg, C., ... Xu, J. (2007). Enabling a Community to Dissect an Organism: Overview of the *Neurospora* Functional Genomics Project. In *Advances in Genetics* (Vol. 57). [https://doi.org/10.1016/S0065-2660\(06\)57002-6](https://doi.org/10.1016/S0065-2660(06)57002-6)
- Dunlap, J. C., & Loros, J. J. (2017). Making Time: Conservation of Biological Clocks from Fungi to Animals. *Microbiology Spectrum*, 5(3). <https://doi.org/10.1128/microbiolspec.funk-0039-2016>
- Dunlap, J. C., Loros, J. J., Colot, H. V., Mehra, A., Belden, W. J., Shi, M., Hong, C. I., Larrondo, L. F., Baker, C. L., Chen, C. H., Schwerdtfeger, C., Collopy, P. D., Gamsby, J. J., & Lambreghts, R. (2007). A circadian clock in *Neurospora*: How genes and proteins cooperate to produce a sustained, entrainable, and compensated biological oscillator with a period of about a day. *Cold Spring Harbor Symposia on Quantitative Biology*, 72. <https://doi.org/10.1101/sqb.2007.72.072>
- Eckel-Mahan, K. L., & Storm, D. R. (2009). Circadian rhythms and memory: Not so simple as cogs and gears. In *EMBO Reports* (Vol. 10, Issue 6). <https://doi.org/10.1038/embor.2009.123>
- Edgar, R. S., Green, E. W., Zhao, Y., Van Ooijen, G., Olmedo, M., Qin, X., Xu, Y., Pan, M., Valekunja, U. K., Feeney, K. A., Maywood, E. S., Hastings, M. H., Baliga, N. S., Merrow, M., Millar, A. J., Johnson, C. H., Kyriacou, C. P., O'Neill, J. S., & Reddy, A. B. (2012). Peroxiredoxins are conserved markers of circadian rhythms. *Nature*, 485(7399). <https://doi.org/10.1038/nature11088>
- Efeyan, A., Zoncu, R., Chang, S., Gumper, I., Snitkin, H., Wolfson, R. L., Kirak, O., Sabatini, D. D., & Sabatini, D. M. (2013). Regulation of mTORC1 by the Rag GTPases is necessary for neonatal autophagy and survival. *Nature*, 493(7434). <https://doi.org/10.1038/nature11745>
- Eltschinger, S., & Loewith, R. (2016). TOR Complexes and the Maintenance of Cellular Homeostasis. In *Trends in Cell Biology* (Vol. 26, Issue 2). <https://doi.org/10.1016/j.tcb.2015.10.003>

- Eskandari, R., Ratnayake, L., & Lakin-Thomas, P. L. (2021). Shared Components of the FRQ-Less Oscillator and TOR Pathway Maintain Rhythmicity in *Neurospora*. *Journal of Biological Rhythms*, 36(4). <https://doi.org/10.1177/0748730421999948>
- Evans, J. A., & Gorman, M. R. (2016). In synch but not in step: Circadian clock circuits regulating plasticity in daily rhythms. In *Neuroscience* (Vol. 320). <https://doi.org/10.1016/j.neuroscience.2016.01.072>
- Fang, Y., Vilella-Bach, M., Bachmann, R., Flanigan, A., & Chen, J. (2001). Phosphatidic acid-mediated mitogenic activation of mTOR signaling. *Science*, 294(5548). <https://doi.org/10.1126/science.1066015>
- Fingar, D. C., & Blenis, J. (2004). Target of rapamycin (TOR): An integrator of nutrient and growth factor signals and coordinator of cell growth and cell cycle progression. In *Oncogene* (Vol. 23, Issue 18). <https://doi.org/10.1038/sj.onc.1207542>
- Finger, A. M., & Kramer, A. (2021). Mammalian circadian systems: Organization and modern life challenges. In *Acta Physiologica* (Vol. 231, Issue 3). <https://doi.org/10.1111/apha.13548>
- Fishbein, A. B., Knutson, K. L., & Zee, P. C. (2021). Circadian disruption and human health. In *Journal of Clinical Investigation* (Vol. 131, Issue 19). <https://doi.org/10.1172/JCI148286>
- Fisher, S. P., Foster, R. G., & Peirson, S. N. (2013). The circadian control of sleep. *Handbook of Experimental Pharmacology*, 217. [https://doi.org/10.1007/978-3-642-25950-0\\_7](https://doi.org/10.1007/978-3-642-25950-0_7)
- Foster, D. A. (2013). Phosphatidic acid and lipid-sensing by mTOR. In *Trends in Endocrinology and Metabolism* (Vol. 24, Issue 6). <https://doi.org/10.1016/j.tem.2013.02.003>
- Foster, D. A., Salloum, D., Menon, D., & Frias, M. A. (2014). Phospholipase D and the maintenance of phosphatidic acid levels for regulation of mammalian target of rapamycin (mTOR). In *Journal of Biological Chemistry* (Vol. 289, Issue 33). <https://doi.org/10.1074/jbc.R114.566091>
- Foster, R. G., & Kreitzman, L. (2009). Seasons of life: The biological rhythms that enable living things to thrive and survive. In *Seasons of Life: The Biological Rhythms that Enable Living Things to Thrive and Survive*. <https://doi.org/10.5860/choice.47-1401>
- Foster, R. G., & Kreitzman, L. (2017a). 3. When timing goes wrong. In *Circadian Rhythms: A Very Short Introduction*. <https://doi.org/10.1093/actrade/9780198717683.003.0003>
- Foster, R. G., & Kreitzman, L. (2017b). 5. The tick-tock of the molecular clock. In *Circadian Rhythms: A Very Short Introduction*. <https://doi.org/10.1093/actrade/9780198717683.003.0005>
- Foster, R. G., & Kreitzman, L. (2017c). 9. Evolution and another look at the clock. In *Circadian Rhythms: A Very Short Introduction*. <https://doi.org/10.1093/actrade/9780198717683.003.0009>
- Frias, M. A., Hatipoglu, A., & Foster, D. A. (2023). Regulation of mTOR by phosphatidic acid. In *Trends in Endocrinology and Metabolism* (Vol. 34, Issue 3). <https://doi.org/10.1016/j.tem.2023.01.004>
- Frias, M. A., Mukhopadhyay, S., Lehman, E., Walasek, A., Utter, M., Menon, D., & Foster, D. A. (2020). Phosphatidic acid drives mTORC1 lysosomal translocation in the absence of amino acids. *Journal of Biological Chemistry*, 295(1). <https://doi.org/10.1074/jbc.RA119.010892>
- Fu, W., & Hall, M. N. (2020). Regulation of MTORC2 signaling. In *Genes* (Vol. 11, Issue 9). <https://doi.org/10.3390/genes11091045>
- Galagan, J. E., Calvo, S. E., Borkovich, K. A., Selker, E. U., Read, N. O., Jaffe, D., FitzHugh, W., Ma, L. J., Smirnov, S., Purcell, S., Rehman, B., Elkins, T., Engels, R., Wang, S.,

- Nielsen, C. B., Butler, J., Endrizzi, M., Qui, D., Ianakiev, P., ... Birren, B. (2003). The genome sequence of the filamentous fungus *Neurospora crassa*. *Nature*, 422(6934). <https://doi.org/10.1038/nature01554>
- Gnocchi, D., Pedrelli, M., Pedrelli, M., Hurt-Camejo, E., & Parini, P. (2015). Lipids around the clock: Focus on circadian rhythms and lipid metabolism. In *Biology* (Vol. 4, Issue 1). <https://doi.org/10.3390/biology4010104>
- Golden, S. S. (2020). Principles of rhythmicity emerging from cyanobacteria. In *European Journal of Neuroscience* (Vol. 51, Issue 1). <https://doi.org/10.1111/ejn.14434>
- Golombek, D. A., & Rosenstein, R. E. (2010). Physiology of circadian entrainment. In *Physiological Reviews* (Vol. 90, Issue 3). <https://doi.org/10.1152/physrev.00009.2009>
- González, A., & Hall, M. N. (2017). Nutrient sensing and TOR signaling in yeast and mammals. *The EMBO Journal*, 36(4). <https://doi.org/10.15252/embj.201696010>
- Gooley, J. J. (2016). Circadian regulation of lipid metabolism. *Proceedings of the Nutrition Society*, 75(4). <https://doi.org/10.1017/S0029665116000288>
- Greco, C. M., & Sassone-Corsi, P. (2019). Circadian blueprint of metabolic pathways in the brain. In *Nature Reviews Neuroscience* (Vol. 20, Issue 2). <https://doi.org/10.1038/s41583-018-0096-y>
- Greenham, K., & McClung, C. R. (2015). Integrating circadian dynamics with physiological processes in plants. In *Nature Reviews Genetics* (Vol. 16, Issue 10). <https://doi.org/10.1038/nrg3976>
- Grimaldi, B., Bellet, M. M., Katada, S., Astarita, G., Hirayama, J., Amin, R. H., Granneman, J. G., Piomelli, D., Leff, T., & Sassone-Corsi, P. (2010). PER2 controls lipid metabolism by direct regulation of PPAR $\gamma$ . *Cell Metabolism*, 12(5). <https://doi.org/10.1016/j.cmet.2010.10.005>
- Guerra, P., Vuilleminot, L. A. P. E., van Oppen, Y. B., Been, M., & Miliás-Argeitis, A. (2022). TORC1 and PKA activity towards ribosome biogenesis oscillates in synchrony with the budding yeast cell cycle. *Journal of Cell Science*, 135(18). <https://doi.org/10.1242/jcs.260378>
- Hanks, D. L., & Sussman, A. S. (1969). Control of trehalase synthesis in *Neurospora crassa*. *American Journal of Botany*, 56(10). <https://doi.org/10.2307/2440777>
- Hardin, P. E. (2011). Molecular genetic analysis of circadian timekeeping in *Drosophila*. In *Advances in Genetics* (Vol. 74). <https://doi.org/10.1016/B978-0-12-387690-4.00005-2>
- Harmer, S. L. (2009). The circadian system in higher plants. *Annual Review of Plant Biology*, 60. <https://doi.org/10.1146/annurev.arplant.043008.092054>
- Harmer, S. L., Panda, S., & Kay, S. A. (2001). Molecular bases of circadian rhythms. *Annual Review of Cell and Developmental Biology*, 17. <https://doi.org/10.1146/annurev.cellbio.17.1.215>
- He, Q., Cheng, P., Yang, Y., He, Q., Yu, H., & Liu, Y. (2003). FWD1-mediated degradation of FREQUENCY in *Neurospora* establishes a conserved mechanism for circadian clock regulation. *EMBO Journal*, 22(17). <https://doi.org/10.1093/emboj/cdg425>
- Hickey, P. C., Jacobson, D. J., Read, N. D., & Louise Glass, N. (2002). Live-cell imaging of vegetative hyphal fusion in *Neurospora crassa*. *Fungal Genetics and Biology*, 37(1). [https://doi.org/10.1016/S1087-1845\(02\)00035-X](https://doi.org/10.1016/S1087-1845(02)00035-X)
- Honma, S. (2018). The mammalian circadian system: a hierarchical multi-oscillator structure for generating circadian rhythm. In *Journal of Physiological Sciences* (Vol. 68, Issue 3). <https://doi.org/10.1007/s12576-018-0597-5>

- Hsu, P. Y., & Harmer, S. L. (2014). Wheels within wheels: The plant circadian system. In *Trends in Plant Science* (Vol. 19, Issue 4). <https://doi.org/10.1016/j.tplants.2013.11.007>
- Huang, R. C. (2018). The discoveries of molecular mechanisms for the circadian rhythm: The 2017 Nobel Prize in Physiology or Medicine. In *Biomedical Journal* (Vol. 41, Issue 1). <https://doi.org/10.1016/j.bj.2018.02.003>
- Hughes Hallett, J. E., Luo, X., & Capaldi, A. P. (2014). State transitions in the TORC1 signaling pathway and information processing in *Saccharomyces cerevisiae*. *Genetics*, 198(2). <https://doi.org/10.1534/genetics.114.168369>
- Hurley, J., Loros, J. J., & Dunlap, J. C. (2015). Dissecting the Mechanisms of the Clock in *Neurospora*. In *Methods in Enzymology* (Vol. 551). <https://doi.org/10.1016/bs.mie.2014.10.009>
- Hurley, J. M., Loros, J. J., & Dunlap, J. C. (2016). The circadian system as an organizer of metabolism. *Fungal Genetics and Biology*, 90. <https://doi.org/10.1016/j.fgb.2015.10.002>
- Jeon, S. M. (2016). Regulation and function of AMPK in physiology and diseases. In *Experimental and Molecular Medicine* (Vol. 48, Issue 7). <https://doi.org/10.1038/EMM.2016.81>
- Jewell, J. L., Russell, R. C., & Guan, K. L. (2013). Amino acid signalling upstream of mTOR. *Nature Reviews Molecular Cell Biology*, 14(3). <https://doi.org/10.1038/nrm3522>
- Jing, Y., Chen, Y., Wang, S., Ouyang, J., Hu, L., Yang, Q., Wang, M., Zhang, B., & Loo, J. J. (2021). Circadian gene *per2* silencing downregulates *pparg* and *srebf1* and suppresses lipid synthesis in bovine mammary epithelial cells. *Biology*, 10(12). <https://doi.org/10.3390/biology10121226>
- Johansson, M., & Köster, T. (2019). On the move through time – a historical review of plant clock research. In *Plant Biology* (Vol. 21). <https://doi.org/10.1111/plb.12729>
- Johnson, C. H., Stewart, P. L., & Egli, M. (2011). The cyanobacterial circadian system: From biophysics to bioevolution. *Annual Review of Biophysics*, 40(1). <https://doi.org/10.1146/annurev-biophys-042910-155317>
- Jordan, S. D., & Lamia, K. A. (2013). AMPK at the crossroads of circadian clocks and metabolism. In *Molecular and Cellular Endocrinology* (Vol. 366, Issue 2). <https://doi.org/10.1016/j.mce.2012.06.017>
- Jung, J., Genau, H. M., & Behrends, C. (2015). Amino Acid-Dependent mTORC1 Regulation by the Lysosomal Membrane Protein SLC38A9. *Molecular and Cellular Biology*, 35(14). <https://doi.org/10.1128/mcb.00125-15>
- Kaneko, H., Head, L. M., Ling, J., Tang, X., Liu, Y., Hardin, P. E., Emery, P., & Hamada, F. N. (2012). Circadian rhythm of temperature preference and its neural control in *Drosophila*. *Current Biology*, 22(19). <https://doi.org/10.1016/j.cub.2012.08.006>
- Karpowicz, P., Zhang, Y., Hogenesch, J. B., Emery, P., & Perrimon, N. (2013). The Circadian Clock Gates the Intestinal Stem Cell Regenerative State. *Cell Reports*, 3(4). <https://doi.org/10.1016/j.celrep.2013.03.016>
- Kayikci, Ö., & Nielsen, J. (2015). Glucose repression in *Saccharomyces cerevisiae*. In *FEMS Yeast Research* (Vol. 15, Issue 6). <https://doi.org/10.1093/femsyr/fov068>
- Kelliher, C. M., Loros, J. J., & Dunlap, J. C. (2020). Evaluating the circadian rhythm and response to glucose addition in dispersed growth cultures of *Neurospora crassa*. *Fungal Biology*, 124(5). <https://doi.org/10.1016/j.funbio.2019.11.004>



- Khatun, R., & Lakin-Thomas, P. (2011). Activation and localization of protein kinase C in *Neurospora crassa*. *Fungal Genetics and Biology*, 48(4).  
<https://doi.org/10.1016/j.fgb.2010.11.002>
- Kijak, E., & Pyza, E. (2017). TOR signaling pathway and autophagy are involved in the regulation of circadian rhythms in behavior and plasticity of L2 interneurons in the brain of *Drosophila melanogaster*. *PLoS ONE*, 12(2). <https://doi.org/10.1371/journal.pone.0171848>
- King, D. P., & Takahashi, J. S. (2000). Molecular genetics of circadian rhythms in mammals. In *Annual Review of Neuroscience* (Vol. 23). <https://doi.org/10.1146/annurev.neuro.23.1.713>
- Ko, C. H., & Takahashi, J. S. (2006). Molecular components of the mammalian circadian clock. In *Human Molecular Genetics* (Vol. 15, Issue SUPPL. 2).  
<https://doi.org/10.1093/hmg/ddl207>
- Kola, B. (2008). Role of AMP-activated protein kinase in the control of appetite. *Journal of Neuroendocrinology*, 20(7). <https://doi.org/10.1111/j.1365-2826.2008.01745.x>
- Koritala, B. S. C., & Lee, K. (2017). Natural Variation of the Circadian Clock in *Neurospora*. In *Advances in Genetics* (Vol. 99). <https://doi.org/10.1016/bs.adgen.2017.09.001>
- Kudo, T., Loh, D. H., Truong, D., Wu, Y., & Colwell, C. S. (2011). Circadian dysfunction in a mouse model of Parkinson's disease. *Experimental Neurology*, 232(1).  
<https://doi.org/10.1016/j.expneurol.2011.08.003>
- Kumar Jha, P., Challet, E., & Kalsbeek, A. (2015). Circadian rhythms in glucose and lipid metabolism in nocturnal and diurnal mammals. In *Molecular and Cellular Endocrinology* (Vol. 418). <https://doi.org/10.1016/j.mce.2015.01.024>
- Kuo, H. C., Hui, S., Choi, J., Asiegbu, F. O., Valkonen, J. P. T., & Lee, Y. H. (2014). Secret lifestyles of *Neurospora crassa*. *Scientific Reports*, 4. <https://doi.org/10.1038/srep05135>
- Lakin-Thomas, P. (2019). Circadian rhythms, metabolic oscillators, and the target of rapamycin (TOR) pathway: the *Neurospora* connection. In *Current Genetics* (Vol. 65, Issue 2).  
<https://doi.org/10.1007/s00294-018-0897-6>
- Lakin-Thomas, P. (2023). The Case for the Target of Rapamycin Pathway as a Candidate Circadian Oscillator. In *International Journal of Molecular Sciences* (Vol. 24, Issue 17).  
<https://doi.org/10.3390/ijms241713307>
- Lakin-Thomas, P. L. (1996). Effects of choline depletion on the circadian rhythm in *Neurospora crassa*. *Biological Rhythm Research*, 27(1). <https://doi.org/10.1076/brhm.27.1.12.12933>
- Lakin-Thomas, P. L. (1998). Choline Depletion, frq Mutations, and Temperature Compensation of the Circadian Rhythm in *Neurospora crassa*. *Journal of Biological Rhythms*, 13(4).  
<https://doi.org/10.1177/074873098129000101>
- Lakin-Thomas, P. L. (2000). Circadian rhythms - New functions for old clock genes. In *Trends in Genetics* (Vol. 16, Issue 3). [https://doi.org/10.1016/S0168-9525\(99\)01945-9](https://doi.org/10.1016/S0168-9525(99)01945-9)
- Lakin-Thomas, P. L. (2006a). New models for circadian systems in microorganisms. In *FEMS Microbiology Letters* (Vol. 259, Issue 1). <https://doi.org/10.1111/j.1574-6968.2006.00211.x>
- Lakin-Thomas, P. L. (2006b). Transcriptional feedback oscillators: Maybe, maybe not... In *Journal of Biological Rhythms* (Vol. 21, Issue 2).  
<https://doi.org/10.1177/0748730405286102>
- Lakin-Thomas, P. L., Bell-Pedersen, D., & Brody, S. (2011). The genetics of circadian rhythms in *Neurospora*. In *Advances in Genetics* (Vol. 74). <https://doi.org/10.1016/B978-0-12-387690-4.00003-9>
- Lakin-Thomas, P. L., & Brody, S. (2000). Circadian rhythms in *Neurospora crassa*: Lipid deficiencies restore robust rhythmicity to null frequency and white-collar mutants.

- Proceedings of the National Academy of Sciences of the United States of America*, 97(1).  
<https://doi.org/10.1073/pnas.97.1.256>
- Lakin-Thomas, P. L., & Brody, S. (2004). Circadian rhythms in microorganisms: New complexities. In *Annual Review of Microbiology* (Vol. 58).  
<https://doi.org/10.1146/annurev.micro.58.030603.123744>
- Lakin-Thomas, P. L., Brody, S., & Côté, G. G. (1997). Temperature compensation and membrane composition in *Neurospora crassa*. In *Chronobiology International* (Vol. 14, Issue 5). <https://doi.org/10.3109/07420529709001467>
- Laplane, M., & Sabatini, D. M. (2012). mTOR signaling in growth control and disease. In *Cell* (Vol. 149, Issue 2). <https://doi.org/10.1016/j.cell.2012.03.017>
- Legerton, T. L., & Weiss, R. L. (1984). Mobilization of vacuolar arginine in *Neurospora crassa*. Mechanism and role of glutamine. *Journal of Biological Chemistry*, 259(14).  
[https://doi.org/10.1016/s0021-9258\(17\)47235-8](https://doi.org/10.1016/s0021-9258(17)47235-8)
- Leprivier, G., & Rotblat, B. (2020). How does mTOR sense glucose starvation? AMPK is the usual suspect. *Cell Death Discovery*, 6(1). <https://doi.org/10.1038/s41420-020-0260-9>
- Li, L., Liu, K. H., & Sheen, J. (2021). Dynamic Nutrient Signaling Networks in Plants. In *Annual Review of Cell and Developmental Biology* (Vol. 37).  
<https://doi.org/10.1146/annurev-cellbio-010521-015047>
- Li, M. D., Xin, H., Yuan, Y., Yang, X., Li, H., Tian, D., Zhang, H., Zhang, Z., Han, T. L., Chen, Q., Duan, G., Ju, D., Chen, K., Deng, F., & He, W. (2021). Circadian Clock-Controlled Checkpoints in the Pathogenesis of Complex Disease. In *Frontiers in Genetics* (Vol. 12).  
<https://doi.org/10.3389/fgene.2021.721231>
- Li, S., & Lakin-Thomas, P. (2010). Effects of prd circadian clock mutations on FRQ-less rhythms in *neurospora*. *Journal of Biological Rhythms*, 25(2).  
<https://doi.org/10.1177/0748730409360889>
- Li, S., Motavaze, K., Kafes, E., Suntharalingam, S., & Lakin-Thomas, P. (2011). A new mutation affecting frq-less rhythms in the circadian system of *neurospora crassa*. *PLoS Genetics*, 7(6). <https://doi.org/10.1371/journal.pgen.1002151>
- Linde-Garelli, K. Y., & Rogala, K. B. (2023). Structural mechanisms of the mTOR pathway. In *Current Opinion in Structural Biology* (Vol. 82). <https://doi.org/10.1016/j.sbi.2023.102663>
- Liu, P., Gan, W., Chin, Y. R., Ogura, K., Guo, J., Zhang, J., Wang, B., Blenis, J., Cantley, L. C., Toker, A., Su, B., & Wei, W. (2015). Ptdins(3,4,5) P3 -dependent activation of the mTORC2 kinase complex. *Cancer Discovery*, 5(11). <https://doi.org/10.1158/2159-8290.CD-15-0460>
- Liu, Q., Wang, J., Kang, S. A., Thoreen, C. C., Hur, W., Ahmed, T., Sabatini, D. M., & Gray, N. S. (2011). Discovery of 9-(6-aminopyridin-3-yl)-1-(3-(trifluoromethyl)phenyl)benzo[ h ][1,6]naphthyridin-2(1 H)-one (torin2) as a potent, selective, and orally available mammalian target of rapamycin (mTOR) inhibitor for treatment of cancer. *Journal of Medicinal Chemistry*, 54(5). <https://doi.org/10.1021/jm101520v>
- Liu, Y., & Bell-Pedersen, D. (2006). Circadian rhythms in *Neurospora crassa* and other filamentous fungi. In *Eukaryotic Cell* (Vol. 5, Issue 8). <https://doi.org/10.1128/EC.00133-06>
- Loewith, R., & Hall, M. N. (2011). Target of rapamycin (TOR) in nutrient signaling and growth control. In *Genetics* (Vol. 189, Issue 4). <https://doi.org/10.1534/genetics.111.133363>
- Loizides-Mangold, U., Perrin, L., Vandereycken, B., Betts, J. A., Walhin, J. P., Templeman, I., Chanon, S., Weger, B. D., Durand, C., Robert, M., Montoya, J. P., Moniatte, M., Karagounis, L. G., Johnston, J. D., Gachon, F., Lefai, E., Riezman, H., & Dibner, C. (2017).

- Lipidomics reveals diurnal lipid oscillations in human skeletal muscle persisting in cellular myotubes cultured in vitro. *Proceedings of the National Academy of Sciences of the United States of America*, 114(41). <https://doi.org/10.1073/pnas.1705821114>
- Lowrey, P. L., & Takahashi, J. S. (2004). Mammalian circadian biology: Elucidating genome-wide levels of temporal organization. In *Annual Review of Genomics and Human Genetics* (Vol. 5). <https://doi.org/10.1146/annurev.genom.5.061903.175925>
- Lutt, N., & Brunkard, J. O. (2022). Amino Acid Signaling for TOR in Eukaryotes: Sensors, Transducers, and a Sustainable Agricultural fuTORe. In *Biomolecules* (Vol. 12, Issue 3). <https://doi.org/10.3390/biom12030387>
- Markson, J. S., Piechura, J. R., Puszyńska, A. M., & O'Shea, E. K. (2013). Circadian control of global gene expression by the cyanobacterial master regulator RpaA. *Cell*, 155(6). <https://doi.org/10.1016/j.cell.2013.11.005>
- Masri, S., Rigor, P., Cervantes, M., Ceglia, N., Sebastian, C., Xiao, C., Roqueta-Rivera, M., Deng, C., Osborne, T. F., Mostoslavsky, R., Baldi, P., & Sassone-Corsi, P. (2014). Partitioning circadian transcription by SIRT6 leads to segregated control of cellular metabolism. *Cell*, 158(3). <https://doi.org/10.1016/j.cell.2014.06.050>
- McClung, C. R. (2006). Plant circadian rhythms. In *Plant Cell* (Vol. 18, Issue 4). <https://doi.org/10.1105/tpc.106.040980>
- McClung, C. R. (2019). The plant circadian oscillator. In *Biology* (Vol. 8, Issue 1). <https://doi.org/10.3390/biology8010014>
- McClung, C. R., Fox, B. A., & Dunlap, J. C. (1989). The Neurospora clock gene frequency shares a sequence element with the Drosophila clock gene period. *Nature*, 339(6225). <https://doi.org/10.1038/339558a0>
- McClung, C. R., Salomé, P. A., & Michael, T. P. (2002). The Arabidopsis Circadian System. *The Arabidopsis Book*, 1. <https://doi.org/10.1199/tab.0044>
- McKeon, T. A., Goodrich-Tanrikulu, M., Lin, J. T., & Stafford, A. (1997). Pathways for fatty acid elongation and desaturation in Neurospora crassa. *Lipids*, 32(1). <https://doi.org/10.1007/s11745-997-0001-8>
- Melick, C. H., & Jewell, J. L. (2020). Regulation of mtorc1 by upstream stimuli. In *Genes* (Vol. 11, Issue 9). <https://doi.org/10.3390/genes11090989>
- Menet, J. S., Abruzzi, K. C., Desrochers, J., Rodriguez, J., & Rosbash, M. (2010). Dynamic PER repression mechanisms in the Drosophila circadian clock: From on-DNA to off-DNA. *Genes and Development*, 24(4). <https://doi.org/10.1101/gad.1883910>
- Meng, D., Yang, Q., Wang, H., Melick, C. H., Navlani, R., Frank, A. R., & Jewell, J. L. (2020). Glutamine and asparagine activate mTORC1 independently of Rag GTPases. In *Journal of Biological Chemistry* (Vol. 295, Issue 10). <https://doi.org/10.1074/jbc.AC119.011578>
- Meng, Y., Zhang, N., Li, J., Shen, X., Sheen, J., & Xiong, Y. (2022). TOR kinase, a GPS in the complex nutrient and hormonal signaling networks to guide plant growth and development. In *Journal of Experimental Botany* (Vol. 73, Issue 20). <https://doi.org/10.1093/jxb/erac282>
- Menon, D., Salloum, D., Bernfeld, E., Gorodetsky, E., Akselrod, A., Frias, M. A., Sudderth, J., Chen, P. H., DeBerardinis, R., & Foster, D. A. (2017). Lipid sensing by mTOR complexes via de novo synthesis of phosphatidic acid. *Journal of Biological Chemistry*, 292(15). <https://doi.org/10.1074/jbc.M116.772988>
- Mijaljica, D., Prescott, M., & Devenish, R. J. (2012). The intriguing life of Autophagosomes. In *International Journal of Molecular Sciences* (Vol. 13, Issue 3). <https://doi.org/10.3390/ijms13033618>

- Mohawk, J. A., Green, C. B., & Takahashi, J. S. (2012). Central and peripheral circadian clocks in mammals. In *Annual Review of Neuroscience* (Vol. 35). <https://doi.org/10.1146/annurev-neuro-060909-153128>
- Money, N. P. (2017). Fungal mutualisms. In *Fungi: A Very Short Introduction*. <https://doi.org/10.1093/actrade/9780199688784.003.0004>
- Montenegro-Montero, A., Canessa, P., & Larrondo, L. F. (2015). Around the Fungal Clock: Recent Advances in the Molecular Study of Circadian Clocks in *Neurospora* and Other Fungi. *Advances in Genetics*, 92. <https://doi.org/10.1016/bs.adgen.2015.09.003>
- Montenegro-Montero John Wiley, A., & Larrondo, L. F. (n.d.). *The Neurospora Circadian System: From Genes to Proteins and Back in Less Than 24 hours*. <https://www.researchgate.net/publication/279791091>
- Morozumi, Y., & Shiozaki, K. (2021). Conserved and divergent mechanisms that control torc1 in yeasts and mammals. *Genes*, 12(1). <https://doi.org/10.3390/genes12010088>
- Moruno, F., Pérez-Jiménez, E., & Knecht, E. (2012). Regulation of autophagy by glucose in mammalian cells. *Cells*, 1(3). <https://doi.org/10.3390/cells1030372>
- Musiek, E. S., & Holtzman, D. M. (2016). Mechanisms linking circadian clocks, sleep, and neurodegeneration. In *Science* (Vol. 354, Issue 6315, pp. 1004–1008). American Association for the Advancement of Science. <https://doi.org/10.1126/science.aah4968>
- Nakahata, Y., & Bessho, Y. (2016). The circadian NAD<sup>+</sup> metabolism: Impact on chromatin remodeling and aging. In *BioMed Research International* (Vol. 2016). <https://doi.org/10.1155/2016/3208429>
- Noël, D., Nguyen, D. V., & Sormani, R. (2021). How to rot: A role for TOR. Interplay between carbon catabolite repression and TOR signaling pathway. In *Advances in Botanical Research* (Vol. 99). <https://doi.org/10.1016/bs.abr.2021.05.002>
- Novarina, D., Guerra, P., & Miliás-Argeitis, A. (2021). Vacuolar Localization via the N-terminal Domain of Sch9 is Required for TORC1-dependent Phosphorylation and Downstream Signal Transduction. *Journal of Molecular Biology*, 433(24). <https://doi.org/10.1016/j.jmb.2021.167326>
- Nsa, I. Y., Karunarathna, N., Liu, X. G., Huang, H., Boettger, B., & Bell-Pedersen, D. (2015). A novel cryptochrome-dependent oscillator in *Neurospora crassa*. *Genetics*, 199(1). <https://doi.org/10.1534/genetics.114.169441>
- Oakenfull, R. J., & Davis, S. J. (2017). Shining a light on the Arabidopsis circadian clock. In *Plant Cell and Environment* (Vol. 40, Issue 11). <https://doi.org/10.1111/PCE.13033>
- O'Neill, J. S., Maywood, E. S., & Hastings, M. H. (2013). Cellular mechanisms of circadian pacemaking: Beyond transcriptional loops. *Handbook of Experimental Pharmacology*, 217. [https://doi.org/10.1007/978-3-642-25950-0\\_4](https://doi.org/10.1007/978-3-642-25950-0_4)
- Ono, D. (2022). Neural circuits in the central circadian clock and their regulation of sleep and wakefulness in mammals. In *Neuroscience Research* (Vol. 182). <https://doi.org/10.1016/j.neures.2022.05.005>
- Panwar, V., Singh, A., Bhatt, M., Tonk, R. K., Azizov, S., Raza, A. S., Sengupta, S., Kumar, D., & Garg, M. (2023). Multifaceted role of mTOR (mammalian target of rapamycin) signaling pathway in human health and disease. In *Signal transduction and targeted therapy* (Vol. 8, Issue 1). <https://doi.org/10.1038/s41392-023-01608-z>
- Paranjpe, D. A., & Sharma, V. K. (2005). Evolution of temporal order in living organisms. In *Journal of Circadian Rhythms* (Vol. 3). <https://doi.org/10.1186/1740-3391-3-7>

- Park, G., Servin, J. A., Turner, G. E., Altamirano, L., Colot, H. V., Collopy, P., Litvinkova, L., Li, L., Jones, C. A., Diala, F. G., Dunlap, J. C., & Borkovich, K. A. (2011). Global analysis of serine-threonine protein kinase genes in *Neurospora crassa*. *Eukaryotic Cell*, 10(11). <https://doi.org/10.1128/EC.05140-11>
- Partch, C. L., Green, C. B., & Takahashi, J. S. (2014). Molecular architecture of the mammalian circadian clock. In *Trends in Cell Biology* (Vol. 24, Issue 2). <https://doi.org/10.1016/j.tcb.2013.07.002>
- Patel, P. V., Wong, J. L., & Arora, R. (2008). The morning blood pressure surge: Therapeutic implications. In *Journal of Clinical Hypertension* (Vol. 10, Issue 2). <https://doi.org/10.1111/j.1751-7176.2008.07427.x>
- Patke, A., Young, M. W., & Axelrod, S. (2020). Molecular mechanisms and physiological importance of circadian rhythms. In *Nature Reviews Molecular Cell Biology* (Vol. 21, Issue 2). <https://doi.org/10.1038/s41580-019-0179-2>
- Paul, J. R., Davis, J. A., Goode, L. K., Becker, B. K., Fusilier, A., Meador-Woodruff, A., & Gamble, K. L. (2020). Circadian regulation of membrane physiology in neural oscillators throughout the brain. In *European Journal of Neuroscience* (Vol. 51, Issue 1). <https://doi.org/10.1111/ejn.14343>
- Peter, G. J., Düring, L., & Ahmed, A. (2006). Carbon catabolite repression regulates amino acid permeases in *Saccharomyces cerevisiae* via the TOR signaling pathway. *Journal of Biological Chemistry*, 281(9). <https://doi.org/10.1074/jbc.M513842200>
- Pittendrigh, C. S. (1954). ON TEMPERATURE INDEPENDENCE IN THE CLOCK SYSTEM CONTROLLING EMERGENCE TIME IN *DROSOPHILA*. *Proceedings of the National Academy of Sciences*, 40(10). <https://doi.org/10.1073/pnas.40.10.1018>
- Pittendrigh, C. S. (1993). Temporal organization: Reflections of a Darwinian clock-watcher. In *Annual Review of Physiology* (Vol. 55). <https://doi.org/10.1146/annurev.ph.55.030193.000313>
- Popova, N. V., & Jücker, M. (2021). The role of mTOR signaling as a therapeutic target in cancer. In *International Journal of Molecular Sciences* (Vol. 22, Issue 4). <https://doi.org/10.3390/ijms22041743>
- Powis, K., & De Virgilio, C. (2016). Conserved regulators of Rag GTPases orchestrate amino acid-dependent TORC1 signaling. In *Cell Discovery* (Vol. 2). <https://doi.org/10.1038/celldisc.2015.49>
- Price-Lloyd, N., Elvin, M., & Heintzen, C. (2005). Synchronizing the *Neurospora crassa* circadian clock with the rhythmic environment. In *Biochemical Society Transactions* (Vol. 33, Issue 5). <https://doi.org/10.1042/BST20050949>
- Qin, X., Byrne, M., Xu, Y., Mori, T., & Johnson, C. H. (2010). Coupling of a core post-translational pacemaker to a slave transcription/translation feedback loop in a circadian system. *PLoS Biology*, 8(6). <https://doi.org/10.1371/journal.pbio.1000394>
- Rabanal-Ruiz, Y., & Korolchuk, V. I. (2018). mTORC1 and nutrient homeostasis: The central role of the lysosome. In *International Journal of Molecular Sciences* (Vol. 19, Issue 3). <https://doi.org/10.3390/ijms19030818>
- Rabanal-Ruiz, Y., Otten, E. G., & Korolchuk, V. I. (2017). MTORC1 as the main gateway to autophagy. In *Essays in Biochemistry* (Vol. 61, Issue 6). <https://doi.org/10.1042/EBC20170027>

- Raja, A. A., Dandare, A., Khan, M. J., & Khan, M. J. (2022). Free Fatty Acid Overload Targets Mitochondria: Gene Expression Analysis of Palmitic Acid-Treated Endothelial Cells. *Genes*, 13(10). <https://doi.org/10.3390/genes13101704>
- Ralph, M. R., & Menaker, M. (1988). A mutation of the circadian system in golden hamsters. *Science*, 241(4870). <https://doi.org/10.1126/science.3413487>
- Ramanathan, C., Kathale, N. D., Liu, D., Lee, C., Freeman, D. A., Hogenesch, J. B., Cao, R., & Liu, A. C. (2018). mTOR signaling regulates central and peripheral circadian clock function. *PLoS Genetics*, 14(5). <https://doi.org/10.1371/journal.pgen.1007369>
- Ramsdale, M., & Lakin-Thomas, P. L. (2000). sn-1,2-diacylglycerol levels in the fungous *Neurospora crassa* display circadian rhythmicity. *Journal of Biological Chemistry*, 275(36). <https://doi.org/10.1074/jbc.M002911200>
- Ratnayake, L., Adhvaryu, K. K., Kafes, E., Motavaze, K., & Lakin-Thomas, P. (2018). A component of the TOR (Target Of Rapamycin) nutrient-sensing pathway plays a role in circadian rhythmicity in *Neurospora crassa*. *PLoS Genetics*, 14(6). <https://doi.org/10.1371/journal.pgen.1007457>
- Reddy, A. B., & O'Neill, J. S. (2010). Healthy clocks, healthy body, healthy mind. In *Trends in Cell Biology* (Vol. 20, Issue 1). <https://doi.org/10.1016/j.tcb.2009.10.005>
- Reinke, H., & Asher, G. (2019). Crosstalk between metabolism and circadian clocks. In *Nature Reviews Molecular Cell Biology* (Vol. 20, Issue 4). <https://doi.org/10.1038/s41580-018-0096-9>
- Reppert, S. M., & Weaver, D. R. (2002). Coordination of circadian timing in mammals. In *Nature* (Vol. 418, Issue 6901). <https://doi.org/10.1038/nature00965>
- Rijo-Ferreira, F., & Takahashi, J. S. (2019). Genomics of circadian rhythms in health and disease. In *Genome Medicine* (Vol. 11, Issue 1). <https://doi.org/10.1186/s13073-019-0704-0>
- Robinson, I., & Reddy, A. B. (2014). Molecular mechanisms of the circadian clockwork in mammals. In *FEBS Letters* (Vol. 588, Issue 15). <https://doi.org/10.1016/j.febslet.2014.06.005>
- Robles, M. S., Humphrey, S. J., & Mann, M. (2017). Phosphorylation Is a Central Mechanism for Circadian Control of Metabolism and Physiology. *Cell Metabolism*, 25(1). <https://doi.org/10.1016/j.cmet.2016.10.004>
- Roche, C. M., Loros, J. J., McCluskey, K., & Louise Glass, N. (2014). *Neurospora crassa*: Looking back and looking forward at a model microbe. In *American Journal of Botany* (Vol. 101, Issue 12, pp. 2022–2035). Botanical Society of America Inc. <https://doi.org/10.3732/ajb.1400377>
- Rockenfeller, P., Smolnig, M., Diessl, J., Bashir, M., Schmiedhofer, V., Knittelfelder, O., Ring, J., Franz, J., Foessler, I., Khan, M. J., Rost, R., Graier, W. F., Kroemer, G., Zimmermann, A., Carmona-Gutierrez, D., Eisenberg, T., Büttner, S., Sigrist, S. J., Kühnlein, R. P., ... Madeo, F. (2018). Diacylglycerol triggers Rim101 pathway-dependent necrosis in yeast: A model for lipotoxicity. *Cell Death and Differentiation*, 25(4). <https://doi.org/10.1038/s41418-017-0014-2>
- Roelants, F. M., Leskoske, K. L., Marshall, M. N. M., Locke, M. N., & Thorner, J. (2017). The TORC2-dependent signaling network in the yeast *Saccharomyces cerevisiae*. In *Biomolecules* (Vol. 7, Issue 3). <https://doi.org/10.3390/biom7030066>
- Roertneberg, T., & Mellow, M. (1998). Molecular Circadian Oscillators: An Alternative Hypothesis. *Journal of Biological Rhythms*, 13(2). <https://doi.org/10.1177/074873098129000011>

- Ruan, W., Yuan, X., & Eltzschig, H. K. (2021). Circadian rhythm as a therapeutic target. In *Nature Reviews Drug Discovery* (Vol. 20, Issue 4). <https://doi.org/10.1038/s41573-020-00109-w>
- Sahar, S., & Sassone-Corsi, P. (2012). Regulation of metabolism: The circadian clock dictates the time. In *Trends in Endocrinology and Metabolism* (Vol. 23, Issue 1). <https://doi.org/10.1016/j.tem.2011.10.005>
- Saini, R., Jaskolski, M., & Davis, S. J. (2019a). Circadian oscillator proteins across the kingdoms of life: Structural aspects. In *BMC Biology* (Vol. 17, Issue 1). BioMed Central Ltd. <https://doi.org/10.1186/s12915-018-0623-3>
- Saini, R., Jaskolski, M., & Davis, S. J. (2019b). Circadian oscillator proteins across the kingdoms of life: Structural aspects. In *BMC Biology* (Vol. 17, Issue 1). <https://doi.org/10.1186/s12915-018-0623-3>
- Salva, M. A. Q., Hartley, S., Léger, D., & Dauvilliers, Y. A. (2017). Non-24-hour sleep-wake rhythm disorder in the totally blind: diagnosis and management. In *Frontiers in Neurology* (Vol. 8, Issue DEC). <https://doi.org/10.3389/fneur.2017.00686>
- Sancak, Y., Bar-Peled, L., Zoncu, R., Markhard, A. L., Nada, S., & Sabatini, D. M. (2010). Ragulator-rag complex targets mTORC1 to the lysosomal surface and is necessary for its activation by amino acids. *Cell*, 141(2). <https://doi.org/10.1016/j.cell.2010.02.024>
- Sancak, Y., Peterson, T. R., Shaul, Y. D., Lindquist, R. A., Thoreen, C. C., Bar-Peled, L., & Sabatini, D. M. (2008). The rag GTPases bind raptor and mediate amino acid signaling to mTORC1. *Science*, 320(5882). <https://doi.org/10.1126/science.1157535>
- Sanchez, S. E., & Kay, S. A. (2016). The plant circadian clock: From a simple timekeeper to a complex developmental manager. *Cold Spring Harbor Perspectives in Biology*, 8(12). <https://doi.org/10.1101/cshperspect.a027748>
- Sartor, F., Eelderink-Chen, Z., Aronson, B., Bosman, J., Hibbert, L. E., Dodd, A. N., Kovács, Á. T., & Mellow, M. (2019). Are there circadian clocks in non-photosynthetic bacteria? *Biology*, 8(2). <https://doi.org/10.3390/biology8020041>
- Saxton, R. A., & Sabatini, D. M. (2017). mTOR Signaling in Growth, Metabolism, and Disease. In *Cell* (Vol. 168, Issue 6). <https://doi.org/10.1016/j.cell.2017.02.004>
- Schmutz, I., Ripperger, J. A., Baeriswyl-Aebischer, S., & Albrecht, U. (2010). The mammalian clock component PERIOD2 coordinates circadian output by interaction with nuclear receptors. *Genes and Development*, 24(4). <https://doi.org/10.1101/gad.564110>
- Seo, P. J., & Mas, P. (2014). Multiple layers of posttranslational regulation refine circadian clock activity in Arabidopsis. In *Plant Cell* (Vol. 26, Issue 1). <https://doi.org/10.1105/tpc.113.119842>
- Serin, Y., & Acar Tek, N. (2019). Effect of Circadian Rhythm on Metabolic Processes and the Regulation of Energy Balance. *Annals of Nutrition and Metabolism*, 74(4). <https://doi.org/10.1159/000500071>
- Shertz, C. A., Bastidas, R. J., Li, W., Heitman, J., & Cardenas, M. E. (2010). Conservation, duplication, and loss of the Tor signaling pathway in the fungal kingdom. *BMC Genomics*, 11(1). <https://doi.org/10.1186/1471-2164-11-510>
- Shimobayashi, M., & Hall, M. N. (2014). Making new contacts: The mTOR network in metabolism and signalling crosstalk. *Nature Reviews Molecular Cell Biology*, 15(3). <https://doi.org/10.1038/nrm3757>
- Skene, D. J., & Arendt, J. (2007). Circadian rhythm sleep disorders in the blind and their treatment with melatonin. *Sleep Medicine*, 8(6). <https://doi.org/10.1016/j.sleep.2006.11.013>

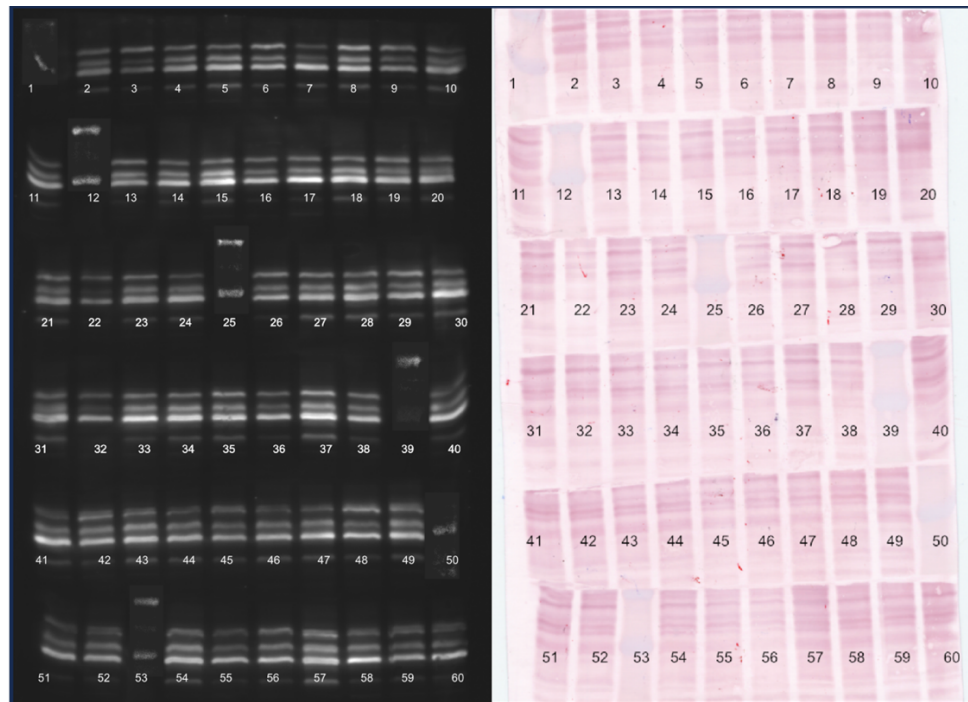
- Sollars, P. J., & Pickard, G. E. (2015). The Neurobiology of Circadian Rhythms. In *Psychiatric Clinics of North America* (Vol. 38, Issue 4). <https://doi.org/10.1016/j.psc.2015.07.003>
- Sullivan, A., Wallace, R. L., Wellington, R., Luo, X., & Capaldi, A. P. (2019). Multilayered regulation of TORC1-body formation in budding yeast. *Molecular Biology of the Cell*, 30(3). <https://doi.org/10.1091/mbc.E18-05-0297>
- Sun, J., Tian, C., Diamond, S., & Louise Glassa, N. (2012). Deciphering transcriptional regulatory mechanisms associated with hemicellulose degradation in *Neurospora crassa*. *Eukaryotic Cell*, 11(4). <https://doi.org/10.1128/EC.05327-11>
- Sun, S., Liu, Z., Feng, Y., Shi, L., Cao, X., Cai, Y., & Liu, B. (2019). Sirt6 deacetylase activity regulates circadian rhythms via Per2. *Biochemical and Biophysical Research Communications*, 511(2). <https://doi.org/10.1016/j.bbrc.2019.01.143>
- Sundar, I. K., Yao, H., Sellix, M. T., & Rahman, I. (2015). Circadian molecular clock in lung pathophysiology. In *American Journal of Physiology - Lung Cellular and Molecular Physiology* (Vol. 309, Issue 10). <https://doi.org/10.1152/ajplung.00152.2015>
- Swan, J. A., Golden, S. S., LiWang, A., & Partch, C. L. (2018). Structure, function, and mechanism of the core circadian clock in cyanobacteria. In *Journal of Biological Chemistry* (Vol. 293, Issue 14). <https://doi.org/10.1074/jbc.TM117.001433>
- Tafur, L., Kefauver, J., & Loewith, R. (2020). Structural insights into TOR signaling. In *Genes* (Vol. 11, Issue 8). <https://doi.org/10.3390/genes11080885>
- Takahara, T., Amemiya, Y., Sugiyama, R., Maki, M., & Shibata, H. (2020). Amino acid-dependent control of mTORC1 signaling: A variety of regulatory modes. In *Journal of Biomedical Science* (Vol. 27, Issue 1). <https://doi.org/10.1186/s12929-020-00679-2>
- Takahashi, J. S. (2017). Transcriptional architecture of the mammalian circadian clock. In *Nature Reviews Genetics* (Vol. 18, Issue 3). <https://doi.org/10.1038/nrg.2016.150>
- Tan, H. W. S., Sim, A. Y. L., & Long, Y. C. (2017). Glutamine metabolism regulates autophagy-dependent mTORC1 reactivation during amino acid starvation. *Nature Communications*, 8(1). <https://doi.org/10.1038/s41467-017-00369-y>
- Tataroglu, O., & Emery, P. (2014). Studying circadian rhythms in *Drosophila melanogaster*. *Methods*, 68(1). <https://doi.org/10.1016/j.ymeth.2014.01.001>
- Tatebe, H., & Shiozaki, K. (2017). Evolutionary conservation of the components in the tor signaling pathways. In *Biomolecules* (Vol. 7, Issue 4). <https://doi.org/10.3390/biom7040077>
- Taton, A., Erikson, C., Yang, Y., Rubin, B. E., Rifkin, S. A., Golden, J. W., & Golden, S. S. (2020). The circadian clock and darkness control natural competence in cyanobacteria. *Nature Communications*, 11(1). <https://doi.org/10.1038/s41467-020-15384-9>
- Thorner, J. (2022). TOR complex 2 is a master regulator of plasma membrane homeostasis. In *Biochemical Journal* (Vol. 479, Issue 18). <https://doi.org/10.1042/BCJ20220388>
- Toledo, M., Batista-Gonzalez, A., Merheb, E., Aoun, M. L., Tarabra, E., Feng, D., Sarparanta, J., Merlo, P., Botrè, F., Schwartz, G. J., Pessin, J. E., & Singh, R. (2018). Autophagy Regulates the Liver Clock and Glucose Metabolism by Degrading CRY1. *Cell Metabolism*, 28(2). <https://doi.org/10.1016/j.cmet.2018.05.023>
- Tsang, A. H., Barclay, J. L., & Oster, H. (2013). Interactions between endocrine and circadian systems. In *Journal of Molecular Endocrinology* (Vol. 52, Issue 1). <https://doi.org/10.1530/JME-13-0118>
- Tseng, R., Goularte, N. F., Chavan, A., Luu, J., Cohen, S. E., Chang, Y. G., Heisler, J., Li, S., Michael, A. K., Tripathi, S., Golden, S. S., LiWang, A., & Partch, C. L. (2017). Structural



- basis of the day-night transition in a bacterial circadian clock. *Science*, 355(6330).  
<https://doi.org/10.1126/science.aag2516>
- Valbuena, N., Guan, K. L., & Moreno, S. (2012). The Vam6 and Gtr1-Gtr2 pathway activates TORC1 in response to amino acids in fission yeast. *Journal of Cell Science*, 125(8).  
<https://doi.org/10.1242/jcs.094219>
- Van Meer, G., Voelker, D. R., & Feigenson, G. W. (2008). Membrane lipids: Where they are and how they behave. In *Nature Reviews Molecular Cell Biology* (Vol. 9, Issue 2).  
<https://doi.org/10.1038/nrm2330>
- Vetter, C. (2020). Circadian disruption: What do we actually mean? In *European Journal of Neuroscience* (Vol. 51, Issue 1). <https://doi.org/10.1111/ejn.14255>
- Vijayan, V., Zuzow, R., & O'Shea, E. K. (2009). Oscillations in supercoiling drive circadian gene expression in cyanobacteria. *Proceedings of the National Academy of Sciences of the United States of America*, 106(52). <https://doi.org/10.1073/pnas.0912673106>
- Vollmers, C., Gill, S., DiTacchio, L., Pulivarthy, S. R., Le, H. D., & Panda, S. (2009). Time of feeding and the intrinsic circadian clock drive rhythms in hepatic gene expression. *Proceedings of the National Academy of Sciences of the United States of America*, 106(50).  
<https://doi.org/10.1073/pnas.0909591106>
- Vujović, N., Davidson, A. J., & Menaker, M. (2008). Sympathetic input modulates, but does not determine, phase of peripheral circadian oscillators. *American Journal of Physiology - Regulatory Integrative and Comparative Physiology*, 295(1).  
<https://doi.org/10.1152/ajpregu.00498.2007>
- Walker, W. H., Walton, J. C., DeVries, A. C., & Nelson, R. J. (2020). Circadian rhythm disruption and mental health. In *Translational Psychiatry* (Vol. 10, Issue 1).  
<https://doi.org/10.1038/s41398-020-0694-0>
- Wang, B., & Cheng, K. K. Y. (2018). Hypothalamic AMPK as a mediator of hormonal regulation of energy balance. In *International Journal of Molecular Sciences* (Vol. 19, Issue 11). <https://doi.org/10.3390/ijms19113552>
- Wang, S., Tsun, Z.-Y., Wolfson, R. L., Shen, K., Wyant, G. A., Plovanich, M. E., Yuan, E. D., Jones, T. D., Chantranupong, L., Comb, W., Wang, T., Bar-Peled, L., Zoncu, R., Straub, C., Kim, C., Park, J., Sabatini, B. L., & Sabatini, D. M. (2015). The amino acid transporter SLC38A9 is a key component of a lysosomal membrane complex that signals arginine sufficiency to mTORC1. *Science*, 347(6218).
- Wang, X., & Ma, L. (2013). Unraveling the circadian clock in Arabidopsis. In *Plant Signaling and Behavior* (Vol. 8, Issue 2). <https://doi.org/10.4161/psb.23014>
- Wang, X., Xu, Z., Cai, Y., Zeng, S., Peng, B., Ren, X., Yan, Y., & Gong, Z. (2020). Rheostatic Balance of Circadian Rhythm and Autophagy in Metabolism and Disease. In *Frontiers in Cell and Developmental Biology* (Vol. 8). <https://doi.org/10.3389/fcell.2020.616434>
- Watterson, K. R., Bestow, D., Gallagher, J., Hamilton, D. L., Ashford, F. B., Meakin, P. J., & Ashford, M. L. J. (2013). Anorexigenic and orexigenic hormone modulation of mammalian target of rapamycin complex 1 activity and the regulation of hypothalamic agouti-related protein mRNA expression. *NeuroSignals*, 21(1–2). <https://doi.org/10.1159/000334144>
- Wollnik, F. (1989). Physiology and regulation of biological rhythms in laboratory animals: An overview. *Laboratory Animals*, 23(2). <https://doi.org/10.1258/002367789780863538>
- Wu, Y., Shi, L., Li, L., Fu, L., Liu, Y., Xiong, Y., & Sheen, J. (2019). Integration of nutrient, energy, light, and hormone signalling via TOR in plants. In *Journal of Experimental Botany* (Vol. 70, Issue 8). <https://doi.org/10.1093/jxb/erz028>

- Wullschleger, S., Loewith, R., & Hall, M. N. (2006). TOR signaling in growth and metabolism. In *Cell* (Vol. 124, Issue 3). <https://doi.org/10.1016/j.cell.2006.01.016>
- Xiong, Y., Sun, J., & Glass, N. L. (2014). VIB1, a Link between Glucose Signaling and Carbon Catabolite Repression, Is Essential for Plant Cell Wall Degradation by *Neurospora crassa*. *PLoS Genetics*, 10(8). <https://doi.org/10.1371/journal.pgen.1004500>
- Yamazaki, S., Straume, M., Tei, H., Sakaki, Y., Menaker, M., & Block, G. D. (2002). Effects of aging on central and peripheral mammalian clocks. *Proceedings of the National Academy of Sciences of the United States of America*, 99(16). <https://doi.org/10.1073/pnas.152318499>
- Yan, G., Lai, Y., & Jiang, Y. (2012). TOR under stress: Targeting TORC1 by Rho1 GTPase. In *Cell Cycle* (Vol. 11, Issue 18). <https://doi.org/10.4161/cc.21461>
- Yan, G., Shen, X., & Jiang, Y. (2006). Rapamycin activates Tap42-associated phosphatases by abrogating their association with Tor complex 1. *EMBO Journal*, 25(15). <https://doi.org/10.1038/sj.emboj.7601239>
- Yerlikaya, S., Meusburger, M., Kumari, R., Huber, A., Anrather, D., Costanzo, M., Boone, C., Ammerer, G., Baranov, P. V., & Loewith, R. (2016). TORC1 and TORC2 work together to regulate ribosomal protein S6 phosphorylation in *Saccharomyces cerevisiae*. *Molecular Biology of the Cell*, 27(2). <https://doi.org/10.1091/mbc.E15-08-0594>
- Yildirim, E., Curtis, R., & Hwangbo, D. S. (2022). Roles of peripheral clocks: lessons from the fly. In *FEBS Letters* (Vol. 596, Issue 3). <https://doi.org/10.1002/1873-3468.14251>
- You, J. S., Lincoln, H. C., Kim, C. R., Frey, J. W., Goodman, C. A., Zhong, X. P., & Hornberger, T. A. (2014). The role of diacylglycerol kinase  $\zeta$  and phosphatidic acid in the mechanical activation of mammalian target of rapamycin (mTOR) signaling and skeletal muscle hypertrophy. *Journal of Biological Chemistry*, 289(3). <https://doi.org/10.1074/jbc.M113.531392>
- Young, M. W., & Kay, S. A. (2001). Time zones: A comparative genetics of circadian clocks. *Nature Reviews Genetics*, 2(9). <https://doi.org/10.1038/35088576>
- Zhang, D., Tong, X., Arthurs, B., Guha, A., Rui, L., Kamath, A., Inoki, K., & Yin, L. (2014). Liver clock protein BMAL1 promotes de novo lipogenesis through insulin-mTORC2-AKT signaling. *Journal of Biological Chemistry*, 289(37). <https://doi.org/10.1074/jbc.M114.567628>
- Zou, H., Zhou, H., Yan, R., Yao, Z., & Lu, Q. (2022). Chronotype, circadian rhythm, and psychiatric disorders: Recent evidence and potential mechanisms. In *Frontiers in Neuroscience* (Vol. 16). <https://doi.org/10.3389/fnins.2022.811771>

## Appendix A: Sample Immunoblot



**Figure 2.1. Sample Western blot analysis of *Neurospora crassa* S6 ribosomal protein separated on a 12.5% acrylamide gel.** The experiment analyzes the effects of leucine supplementation (with/without 0.5 mM Leu) on the S6 phosphorylation levels on low-glucose shaking culture subjected to a short-term (20 and 60 minutes) starvation period. The wild-type (WT, strain#667),  $\Delta$ vt<sub>a</sub> (strain#694), and  $\Delta$ gtr2 (strain#737) were grown in liquid media with 2% glucose, 1x Vogel's salt, 100  $\mu$ M choline, and 0.2 % Tween 80 and the amino acid treatments +Leu (0.5 mM) or -Leu (without leucine) for 48h at 22°C under LL. Following this, the mycelial pads were incubated in low-glucose media containing 0.08% glucose, 1x Vogel's salt, and 100 $\mu$ M choline with the corresponding amino acid treatments as mentioned above. The flasks were shaken at 150 r.p.m on an orbital shaker and the samples were harvested after 20- and 60-minutes incubation period. The S6 phosphorylation levels were monitored by western blotting. The immunoblot was stained with Colloidal Gold to measure the total protein levels.

| Lane                               | Sample                                                  |
|------------------------------------|---------------------------------------------------------|
| 1, 12, 25, 39, 50, 53              | BLUelf protein ladder                                   |
| 2, 21, 41 (trial1, trial2, trial3) | Wild-type + Leu, 48h Initial Growth                     |
| 3, 22, 42                          | Wild-type + Leu, 20 min in shaking media                |
| 4, 23, 43                          | Wild-type + Leu, 60 min in shaking media                |
| 5, 24, 44                          | Wild-type - Leu, 48h Initial Growth                     |
| 6, 26, 45                          | Wild-type - Leu, 20 min in shaking media                |
| 7, 27, 46                          | Wild-type - Leu, 60 min in shaking media                |
| 8, 28, 47                          | $\Delta$ gtr2 + Leu, 48h Initial Growth                 |
| 9, 29, 48                          | $\Delta$ gtr2 + Leu, 20 min in shaking media            |
| 10, 30, 49                         | $\Delta$ gtr2 + Leu, 60 min in shaking media            |
| 11, 31, 51                         | $\Delta$ gtr2 - Leu, 48h Initial Growth                 |
| 13, 32, 52                         | $\Delta$ gtr2 - Leu, 20 min in shaking media            |
| 14, 33, 54                         | $\Delta$ gtr2 - Leu, 60 min in shaking media            |
| 15, 34, 55                         | $\Delta$ vt <sub>a</sub> + Leu, 48h Initial Growth      |
| 16, 35, 56                         | $\Delta$ vt <sub>a</sub> + Leu, 20 min in shaking media |
| 17, 36, 57                         | $\Delta$ vt <sub>a</sub> + Leu, 60 min in shaking media |
| 18, 37, 58                         | $\Delta$ vt <sub>a</sub> - Leu, 48h Initial Growth      |
| 19, 38, 59                         | $\Delta$ vt <sub>a</sub> - Leu, 20 min in shaking media |
| 20, 40, 60                         | $\Delta$ vt <sub>a</sub> - Leu, 60 min in shaking media |

## Appendix B: Statistical Analysis

### 3.1.1) Leucine

| Comparison                                          | Mean Diff. | 95% CI of diff.      | Below threshold? | Summary | Adjusted P Value |
|-----------------------------------------------------|------------|----------------------|------------------|---------|------------------|
| 48h Initial Growth- Leu vs. 48h Initial Growth+ Leu | 0.02225    | [-0.1051 to 0.1496]  | No               | ns      | 0.9993           |
| 4h Recovery- Leu vs. 4h Recovery+ Leu               | -0.01571   | [-0.1431 to 0.1117]  | No               | ns      | >0.9999          |
| 20 min - Leu vs. 20 min + Leu                       | -0.03139   | [-0.1588 to 0.09599] | No               | ns      | 0.9940           |
| 60 min- Leu vs. 60 min+ Leu                         | -0.04020   | [-0.1676 to 0.08718] | No               | ns      | 0.9745           |

### 3.1.1) Arginine

| Comparison                          | Mean Diff. | 95% CI of diff. | Below threshold? | Summary | Adjusted P Value |
|-------------------------------------|------------|-----------------|------------------|---------|------------------|
| 48h Initial Growth: - Arg vs. + Arg | -0.044 45  | -0.2477to0.1588 | No               | ns      | 0.9932           |
| 4h Recovery: - Arg vs. + Arg        | -0.077 56  | -0.2088to0.1257 | No               | ns      | 0.8780           |
| 20 min: - Arg vs. + Arg             | -0.080 48  | -0.2638to0.1228 | No               | ns      | 0.8575           |
| 60 min: - Arg vs. + Arg             | 0.003 52   | -0.1999to0.2066 | No               | ns      | >0.9999          |

### 3.1.1) Glutamine

| Comparison                                            | Mean Diff.   | 95.00% CI of diff. | Below threshold? | Summary | Adjusted P Value |
|-------------------------------------------------------|--------------|--------------------|------------------|---------|------------------|
| 48h Initial Growth:- Gln vs. 48h Initial Growth:+ Gln | -0.1045      | -0.3583 to 0.1494  | No               | ns      | >0.8335          |
| 4h Recovery:- Gln vs. 4h Recovery:+ Gln               | 0.030 16     | -0.2237 to 0.2840  | No               | ns      | >0.9999          |
| 20 min:- Gln vs. 20 min:+ Gln                         | -0.003 955   | -0.2578 to 0.2499  | No               | ns      | >0.9999          |
| 60 min:- Gln vs. 60 min:+ Gln                         | -0.000 641 9 | -0.2545 to 0.2532  | No               | ns      | >0.9999          |

### 3.1.2)

| Comparison        | Mean Diff. | 95.00% CI of diff. | Below threshold? | Summary | Adjusted P Value |
|-------------------|------------|--------------------|------------------|---------|------------------|
| Control vs. + Leu | 0.01401    | -0.2206 to 0.2486  | No               | ns      | 0.9961           |
| Control vs. + Arg | -0.01032   | -0.2450 to 0.2243  | No               | ns      | 0.9984           |
| Control vs. + Gln | -0.02864   | -0.2633 to 0.2060  | No               | ns      | 0.9699           |

### 3.1.3)

| Comparison        | Mean Diff. | 95.00% CI of diff. | Below threshold? | Summary | Adjusted P Value |
|-------------------|------------|--------------------|------------------|---------|------------------|
| Control vs. + Leu | -0.02142   | -0.1497 to 0.1068  | No               | ns      | 0.9301           |
| Control vs. + Arg | -0.07849   | -0.2067 to 0.04975 | No               | ns      | 0.2555           |
| Control vs. + Gln | -0.07259   | -0.2008 to 0.05565 | No               | ns      | 0.3059           |

### 3.1.4)

| Comparison            | Mean Diff. | 95.00% CI of diff.    | Below threshold? | Summary | Adjusted P Value |
|-----------------------|------------|-----------------------|------------------|---------|------------------|
| Control vs. + Glucose | -0.1152    | [-0.1621 to -0.06831] | Yes              | ****    | <0.0001          |
| Control vs. + Leu     | -0.05702   | [-0.1039 to -0.01013] | Yes              | *       | 0.0139           |
| Control vs. + Arg     | -0.08609   | [-0.1330 to -0.03919] | Yes              | ***     | 0.0002           |
| Control vs. + Gln     | -0.06885   | [-0.1158 to -0.02195] | Yes              | **      | 0.0028           |

### 3.2.1) Leucine

| Comparison                                                     | Mean Diff. | 95.00% CI of diff.   | Below threshold? | Summary | Adjusted P Value |
|----------------------------------------------------------------|------------|----------------------|------------------|---------|------------------|
| 48h Initial Growth:WT + Leu vs. 48h Initial Growth: vta + Leu  | -0.05129   | [-0.1595 to 0.05689] | No               | ns      | 0.7210           |
| 4h Recovery :WT + Leu vs. 4h Recovery : vta + Leu              | 0.02865    | [-0.07952 to 0.1368] | No               | ns      | 0.9798           |
| 20 min:WT + Leu vs. 20 min: vta + Leu                          | -0.02264   | [-0.1308 to 0.08554] | No               | ns      | 0.9948           |
| 60 min:WT + Leu vs. 60 min: vta + Leu                          | 0.03016    | [-0.07802 to 0.1383] | No               | ns      | 0.9733           |
| 48h Initial Growth:WT + Leu vs. 48h Initial Growth: gtr2 + Leu | -0.05611   | [-0.2248 to 0.1126]  | No               | ns      | 0.9346           |
| 4h Recovery :WT + Leu vs. 4h Recovery : gtr2 + Leu             | 0.02052    | [-0.1482 to 0.1893]  | No               | ns      | 0.9998           |
| 20 min:WT + Leu vs. 20 min: gtr2 + Leu                         | -0.01259   | [-0.1813 to 0.1561]  | No               | ns      | >0.9999          |
| 60 min:WT + Leu vs. 60 min: gtr2 + Leu                         | -0.02086   | [-0.1896 to 0.1479]  | No               | ns      | 0.9998           |

### 3.2.1) Arginine

| Comparison                                                     | Mean Diff. | 95% CI of diff.    | Below threshold? | Summary | Adjusted P Value |
|----------------------------------------------------------------|------------|--------------------|------------------|---------|------------------|
| 48h Initial Growth:WT + Arg vs. 48h Initial Growth: vta + Arg  | 0.0004482  | -0.1889 to 0.1898  | No               | ns      | >0.9999          |
| 4h Recovery :WT + Arg vs. 4h Recovery : vta + Arg              | 0.1343     | -0.05500 to 0.3237 | No               | ns      | 0.2800           |
| 20 min:WT + Arg vs. 20 min: vta + Arg                          | 0.0003163  | -0.1890 to 0.1896  | No               | ns      | >0.9999          |
| 60 min:WT + Arg vs. 60 min: vta + Arg                          | -0.1035    | -0.2929 to 0.08579 | No               | ns      | 0.5733           |
| 48h Initial Growth:WT + Arg vs. 48h Initial Growth: gtr2 + Arg | 0.02639    | -0.1578 to 0.2106  | No               | ns      | 0.9995           |
| 4h Recovery :WT + Arg vs. 4h Recovery : gtr2 + Arg             | 0.1308     | -0.05335 to 0.3150 | No               | ns      | 0.2788           |
| 20 min:WT + Arg vs. 20 min: gtr2 + Arg                         | 0.03878    | -0.1454 to 0.2230  | No               | ns      | 0.9946           |
| 60 min:WT + Arg vs. 60 min: gtr2 + Arg                         | -0.1287    | -0.3129 to 0.05552 | No               | ns      | 0.2960           |

### 3.2.1) Glutamine

| Comparisons                                                    | Mean Diff. | 95.00% CI of diff. | Below threshold? | Summary | Adjusted P Value |
|----------------------------------------------------------------|------------|--------------------|------------------|---------|------------------|
| 48h Initial Growth:WT + Gln vs. 48h Initial Growth: vta + Gln  | -0.01157   | -0.1499 to 0.1268  | No               | ns      | >0.9999          |
| 4h Recovery :WT + Gln vs. 4h Recovery : vta + Gln              | -0.05352   | -0.1919 to 0.08483 | No               | ns      | 0.8706           |
| 20 min:WT + Gln vs. 20 min: vta + Gln                          | -0.01764   | -0.1560 to 0.1207  | No               | ns      | 0.9998           |
| 60 min:WT + Gln vs. 60 min: vta + Gln                          | 0.05097    | -0.08738 to 0.1893 | No               | ns      | 0.8952           |
| 48h Initial Growth:WT + Gln vs. 48h Initial Growth: gtr2 + Gln | -0.007698  | -0.1982 to 0.1828  | No               | ns      | >0.9999          |
| 4h Recovery :WT + Gln vs. 4h Recovery : gtr2 + Gln             | 0.03680    | -0.1537 to 0.2273  | No               | ns      | 0.9968           |
| 20 min:WT + Gln vs. 20 min: gtr2 + Gln                         | 0.02384    | -0.1667 to 0.2144  | No               | ns      | 0.9998           |
| 60 min:WT + Gln vs. 60 min: gtr2 + Gln                         | 0.06631    | -0.1242 to 0.2568  | No               | ns      | 0.9190           |

### 3.2.2) Leucine

| Comparison                                                              | Mean Diff. | 95.00% CI of diff. | Below threshold? | Summary | Adjusted P Value |
|-------------------------------------------------------------------------|------------|--------------------|------------------|---------|------------------|
| 48h Initial Growth:WT + Leu vs. 48h Initial Growth: $\Delta$ vta + Leu  | 0.04900    | -0.06959 to 0.1676 | No               | ns      | 0.7333           |
| 20 min:WT + Leu vs. 20 min: $\Delta$ vta + Leu                          | 0.1235     | 0.004854 to 0.2421 | Yes              | *       | 0.0397           |
| 60 min:WT + Leu vs. 60 min: $\Delta$ vta + Leu                          | 0.007619   | -0.1110 to 0.1262  | No               | ns      | >0.9999          |
| 48h Initial Growth:WT + Leu vs. 48h Initial Growth: $\Delta$ gtr2 + Leu | 0.01548    | -0.1194 to 0.1503  | No               | ns      | 0.9986           |
| 20 min:WT + Leu vs. 20 min: $\Delta$ gtr2 + Leu                         | 0.05904    | -0.07580 to 0.1939 | No               | ns      | 0.6874           |
| 60 min:WT + Leu vs. 60 min: $\Delta$ gtr2 + Leu                         | 0.03912    | -0.09572 to 0.1740 | No               | ns      | 0.9176           |

### 3.2.2) Glutamine

| Comparison                                                              | Mean Diff. | 95.00% CI of diff. | Below threshold? | Summary | Adjusted P Value |
|-------------------------------------------------------------------------|------------|--------------------|------------------|---------|------------------|
| 48h Initial Growth:WT + Leu vs. 48h Initial Growth: $\Delta$ vta + Leu  | 0.04900    | -0.06959 to 0.1676 | No               | ns      | 0.7333           |
| 20 min:WT + Leu vs. 20 min: $\Delta$ vta + Leu                          | 0.1235     | 0.004854 to 0.2421 | Yes              | *       | 0.0397           |
| 60 min:WT + Leu vs. 60 min: $\Delta$ vta + Leu                          | 0.007619   | -0.1110 to 0.1262  | No               | ns      | >0.9999          |
| 48h Initial Growth:WT + Leu vs. 48h Initial Growth: $\Delta$ gtr2 + Leu | 0.01548    | -0.1194 to 0.1503  | No               | ns      | 0.9986           |
| 20 min:WT + Leu vs. 20 min: $\Delta$ gtr2 + Leu                         | 0.05904    | -0.07580 to 0.1939 | No               | ns      | 0.6874           |
| 60 min:WT + Leu vs. 60 min: $\Delta$ gtr2 + Leu                         | 0.03912    | -0.09572 to 0.1740 | No               | ns      | 0.9176           |

### 3.2.3) $\Delta$ vta

| Comparison                        | Mean Diff. | 95.00% CI of diff.  | Below threshold? | Summary | Individual P Value |
|-----------------------------------|------------|---------------------|------------------|---------|--------------------|
| Control<br>WT vs. $\Delta$ gtr2   | -0.02347   | -0.06959 to 0.02265 | No               | ns      | 0.3069             |
| + Glucose<br>WT vs. $\Delta$ gtr2 | 0.02436    | -0.02176 to 0.07048 | No               | ns      | 0.2894             |
| + Leu<br>WT vs. $\Delta$ gtr2     | 0.04899    | 0.002873 to 0.09511 | Yes              | *       | 0.0381             |
| + Arg<br>WT vs. $\Delta$ gtr2     | 0.02255    | -0.02357 to 0.06867 | No               | ns      | 0.3259             |
| + Gln<br>WT vs. $\Delta$ gtr2     | 0.03094    | -0.01518 to 0.07706 | No               | ns      | 0.1808             |

### 3.2.3) $\Delta$ gtr2

| Comparison          | Mean Diff. | 95.00% CI of diff.   | Below threshold? | Summary | Individual P Value |
|---------------------|------------|----------------------|------------------|---------|--------------------|
| Control             |            |                      |                  |         |                    |
| WT vs. $\Delta vta$ | -0.027 38  | -0.070 20 to 0.01544 | No               | ns      | 0.2015             |
| + Glucose           |            |                      |                  |         |                    |
| WT vs. $\Delta vta$ | 0.050 86   | 0.008 043 to 0.09368 | Yes              | *       | 0.0215             |
| + Leu               |            |                      |                  |         |                    |
| WT vs. $\Delta vta$ | 0.053 84   | 0.011 02 to 0.09666  | Yes              | *       | 0.0154             |
| + Arg               |            |                      |                  |         |                    |
| WT vs. $\Delta vta$ | 0.029 10   | -0.013 72 to 0.07192 | No               | ns      | 0.1754             |
| + Gln               |            |                      |                  |         |                    |
| WT vs. $\Delta vta$ | 0.073 30   | 0.030 48 to 0.1161   | Yes              | **      | 0.0015             |

### 3.3.1

#### Arginine auxotroph

| Comparison           | Mean Diff. | 95.00% CI of diff.  | Below threshold? | Summary | Individual P Value |
|----------------------|------------|---------------------|------------------|---------|--------------------|
| 48h Initial Growth   |            |                     |                  |         |                    |
| WT vs. Arg auxotroph | -0.1557    | -0.2788 to -0.03264 | Yes              | *       | 0.0164             |
| 4h recovery          |            |                     |                  |         |                    |
| WT vs. Arg auxotroph | 0.03696    | -0.08610 to 0.1600  | No               | ns      | 0.5333             |
| 20 min               |            |                     |                  |         |                    |
| WT vs. Arg auxotroph | 0.03738    | -0.08568 to 0.1604  | No               | ns      | 0.5287             |
| 60 min               |            |                     |                  |         |                    |
| WT vs. Arg auxotroph | -0.004555  | -0.1276 to 0.1185   | No               | ns      | 0.9384             |

#### Glutamine auxotroph

| Comparison           | Mean Diff. | 95% CI of diff.      | Below threshold? | Summary | Individual P Value |
|----------------------|------------|----------------------|------------------|---------|--------------------|
| 48h Initial Growth   |            |                      |                  |         |                    |
| WT vs. Gln auxotroph | 0.02412    | [-0.05663, 0.1049]   | No               | ns      | 0.5528             |
| 4h recovery          |            |                      |                  |         |                    |
| WT vs. Gln auxotroph | -0.01212   | [-0.09287, 0.06862]  | No               | ns      | 0.7652             |
| 20 min               |            |                      |                  |         |                    |
| WT vs. Gln auxotroph | -0.08226   | [-0.1630, -0.001512] | Yes              | *       | 0.0460             |
| 60 min               |            |                      |                  |         |                    |
| WT vs. Gln auxotroph | -0.05493   | [-0.1357, 0.02581]   | No               | ns      | 0.1789             |

### 3.3.2) Arginine auxotroph

| Comparison                                      | Mean Diff. | 95.00% CI of diff. | Below threshold? | Summary | Individual P Value |
|-------------------------------------------------|------------|--------------------|------------------|---------|--------------------|
| Inhibitory [Arg] WT vs. Arg auxotroph           | -0.1787    | -0.4666 to 0.1091  | No               | ns      | 0.1901             |
| Rescue [Arg] WT vs. Arg auxotroph               | -0.06469   | -0.3525 to 0.2232  | No               | ns      | 0.6183             |
| WT Inhibitory [Arg] vs. Rescue [Arg]            | -0.01364   | -0.3015 to 0.2742  | No               | ns      | 0.9157             |
| Arg auxotroph Inhibitory [Arg] vs. Rescue [Arg] | 0.1004     | -0.1875 to 0.3882  | No               | ns      | 0.4445             |

### 3.3.2) Glutamine auxotroph

| Comparison                                            | Mean Diff. | 95.00% CI of diff.      | Below threshold? | Summary | Individual P Value |
|-------------------------------------------------------|------------|-------------------------|------------------|---------|--------------------|
| Inhibitory [Gln]<br>WT vs. Gln<br>auxotroph           | -0.056 46  | -0.2315 to 0.1186<br>to | No               | ns      | 0.4782             |
| Rescue [Gln]<br>WT vs. Gln<br>auxotroph               | -0.040 66  | -0.2157 to 0.1344<br>to | No               | ns      | 0.6067             |
| WT<br>Inhibitory [Gln]<br>vs. Rescue [Gln]            | -0.036 76  | -0.2118 to 0.1383<br>to | No               | ns      | 0.6411             |
| Gln auxotroph<br>Inhibitory [Gln]<br>vs. Rescue [Gln] | -0.020 96  | -0.1960 to 0.1541<br>to | No               | ns      | 0.7894             |

### 3.3.3.A) Arginine auxotroph Biomass accumulation

| Comparison                                         | Mean Diff. | 95.00% CI of diff. | Below threshold? | Summary | Individual P Value |
|----------------------------------------------------|------------|--------------------|------------------|---------|--------------------|
| Inhibitory [Arg]<br>WT vs. Arg auxotroph           | 133.7      | 86.62 to 180.8     | Yes              | ***     | 0.0002             |
| Rescue [Arg]<br>WT vs. Arg auxotroph               | 62.17      | 15.08 to 109.2     | Yes              | *       | 0.0159             |
| WT<br>Inhibitory [Arg] vs. Rescue [Arg]            | -43.07     | -90.15 to 4.017    | No               | ns      | 0.0679             |
| Arg auxotroph<br>Inhibitory [Arg] vs. Rescue [Arg] | -114.6     | -161.7 to -67.52   | Yes              | ***     | 0.0005             |

### Linear growth rate

| Comparison                                         | Mean Diff. | 95.00% CI of diff.   | Below threshold? | Summary | Individual P Value |
|----------------------------------------------------|------------|----------------------|------------------|---------|--------------------|
| Inhibitory [Arg]<br>WT vs. Arg auxotroph           | 0.1407     | 0.041 30 to 0.2401   | Yes              | *       | 0.0115             |
| Rescue [Arg]<br>WT vs. Arg auxotroph               | 0.058 67   | -0.040 74 to 0.1581  | No               | ns      | 0.2106             |
| WT<br>Inhibitory [Arg] vs. Rescue [Arg]            | -0.018 32  | -0.1177 to 0.08108   | No               | ns      | 0.6820             |
| Arg auxotroph<br>Inhibitory [Arg] vs. Rescue [Arg] | -0.1004    | -0.1998 to -0.009509 | Yes              | *       | 0.0483             |

### 3.3.3.B) Glutamine auxotroph Biomass accumulation



| Comparison                        | Mean Diff. | 95% CI of diff.  | Below threshold? | Summary | Individual P Value |
|-----------------------------------|------------|------------------|------------------|---------|--------------------|
| <b>Inhibitory [Gln]</b>           |            |                  |                  |         |                    |
| WT vs. Gln auxotroph              | 96.00      | 3.284to 188.7    | Yes              | *       | 0.0440             |
| <b>Rescue [Gln]</b>               |            |                  |                  |         |                    |
| WT vs. Gln auxotroph              | -43.53     | -136.2 to 49.18  | No               | ns      | 0.3105             |
| <b>WT</b>                         |            |                  |                  |         |                    |
| Inhibitory [Gln] vs. Rescue [Gln] | 19.33      | -73.38to 112.0   | No               | ns      | 0.6435             |
| <b>Gln auxotroph</b>              |            |                  |                  |         |                    |
| Inhibitory [Gln] vs. Rescue [Gln] | -120.2     | -212.9 to -27.48 | Yes              | *       | 0.0173             |

## Linear growth rate

| Comparison                        | Mean Diff. | 95.00% CI of diff. | Below threshold? | Summary | Individual P Value |
|-----------------------------------|------------|--------------------|------------------|---------|--------------------|
| <b>Inhibitory [Gln]</b>           |            |                    |                  |         |                    |
| WT vs. Gln auxotroph              | 0.1194     | 0.02730 to 0.2115  | Yes              | *       | 0.0174             |
| <b>Rescue [Gln]</b>               |            |                    |                  |         |                    |
| WT vs. Gln auxotroph              | 0.04650    | -0.04563 to 0.1386 | No               | ns      | 0.2780             |
| <b>WT</b>                         |            |                    |                  |         |                    |
| Inhibitory [Gln] vs. Rescue [Gln] | 0.04910    | -0.04302 to 0.1412 | No               | ns      | 0.2539             |
| <b>Gln auxotroph</b>              |            |                    |                  |         |                    |
| Inhibitory [Gln] vs. Rescue [Gln] | -0.02382   | -0.1159 to 0.06831 | No               | ns      | 0.5675             |

3.4. Note: Due to the large size of the table, only significant comparisons are included. The rest of the comparative analysis is not significant.

| Comparison                           | Mean Diff. | 95.00% CI of diff.   | Below threshold? | Summary | Adjusted P Value |
|--------------------------------------|------------|----------------------|------------------|---------|------------------|
| Control:1h vs. Glucose + Choline:1h  | -0.1222    | -0.2400 to -0.004415 | Yes              | *       | 0.0362           |
| Control:1h vs. Glucose + Choline:4h  | -0.1520    | -0.2698 to -0.03426  | Yes              | **      | 0.0034           |
| Control:1h vs. Glucose:4h            | -0.1776    | -0.2954 to -0.05979  | Yes              | ***     | 0.0004           |
| Control:4h vs. Glucose + Choline:4h  | -0.1410    | -0.2588 to -0.02325  | Yes              | **      | 0.0084           |
| Control:4h vs. Glucose:4h            | -0.1666    | -0.2844 to -0.04879  | Yes              | **      | 0.0010           |
| Control:24h vs. Glucose + Choline:4h | -0.1437    | -0.2615 to -0.02591  | Yes              | **      | 0.0068           |
| Control:24h vs. Glucose:4h           | -0.1692    | -0.2870 to -0.05144  | Yes              | ***     | 0.0008           |
| Glucose + Choline:1h vs. Choline:24h | 0.1452     | 0.02739 to 0.2630    | Yes              | *       | 0.0335           |
| Glucose + Choline:4h vs. Choline:1h  | 0.1232     | 0.005430 to 0.2410   | Yes              | *       | 0.0335           |
| Glucose + Choline:4h vs. Choline:24h | 0.1750     | 0.05723 to 0.2928    | Yes              | ***     | 0.0005           |
| Glucose + Choline:4h vs. Water:1h    | 0.1433     | 0.02554 to 0.2611    | Yes              | **      | 0.0070           |
| Glucose + Choline:4h vs. Water:4h    | 0.1340     | 0.01624 to 0.2518    | Yes              | *       | 0.0146           |
| Glucose + Choline:4h vs. Water:24h   | 0.1360     | 0.01819 to 0.2538    | Yes              | *       | 0.0125           |
| Glucose:4h vs. Glucose:24h           | 0.1203     | 0.002510 to 0.2381   | Yes              | *       | 0.0416           |
| Glucose:4h vs. Choline:1h            | 0.1487     | 0.03097 to 0.2665    | Yes              | **      | 0.0045           |
| Glucose:4h vs. Choline:24h           | 0.2005     | 0.08277 to 0.3183    | Yes              | ****    | <0.0001          |
| Glucose:4h vs. Water:1h              | 0.1689     | 0.05107 to 0.2866    | Yes              | ***     | 0.0008           |
| Glucose:4h vs. Water:4h              | 0.1596     | 0.04178 to 0.2773    | Yes              | **      | 0.0018           |
| Glucose:4h vs. Water:24h             | 0.1615     | 0.04372 to 0.2793    | Yes              | **      | 0.0015           |

### 3.5.1

| Comparison                | Mean Diff. | 95.00% CI of diff.  | Below threshold? | Summary | Individual P Value |
|---------------------------|------------|---------------------|------------------|---------|--------------------|
| <b>48h Initial Growth</b> |            |                     |                  |         |                    |
| + Choline vs. - Choline   | -0.01974   | -0.09909 to 0.05961 | No               | ns      | 0.6124             |
| <b>4h recovery</b>        |            |                     |                  |         |                    |
| + Choline vs. - Choline   | 0.09997    | 0.02063 to 0.1793   | Yes              | *       | 0.0157             |
| <b>5min</b>               |            |                     |                  |         |                    |
| + Choline vs. - Choline   | 0.06585    | -0.01350 to 0.1452  | No               | ns      | 0.0997             |
| <b>10min</b>              |            |                     |                  |         |                    |
| + Choline vs. - Choline   | 0.02657    | -0.05278 to 0.1059  | No               | ns      | 0.4962             |
| <b>20min</b>              |            |                     |                  |         |                    |
| + Choline vs. - Choline   | 0.003367   | -0.07598 to 0.08271 | No               | ns      | 0.9309             |
| <b>60min</b>              |            |                     |                  |         |                    |
| + Choline vs. - Choline   | 0.006170   | -0.07318 to 0.08552 | No               | ns      | 0.8738             |

### 3.5.2

| Comparison                                                    | 95.00% CI of diff.   | Below threshold? | Summary | Adjusted P Value |
|---------------------------------------------------------------|----------------------|------------------|---------|------------------|
| 48h Initial Growth:+ Choline vs. 48h Initial Growth:- Choline | -0.1730 to 0.08919   | No               | ns      | 0.8826           |
| 48h Initial Growth:+ Choline vs. 20min:+ Choline              | -0.2720 to -0.009765 | Yes              | *       | 0.0328           |
| 48h Initial Growth:+ Choline vs. 20min:- Choline              | -0.2711 to -0.008891 | Yes              | *       | 0.0341           |
| 48h Initial Growth:+ Choline vs. 60min :+ Choline             | -0.2914 to -0.02917  | Yes              | *       | 0.0142           |
| 48h Initial Growth:+ Choline vs. 60min :- Choline             | -0.3002 to -0.03796  | Yes              | **      | 0.0097           |
| 48h Initial Growth:- Choline vs. 20min:+ Choline              | -0.2301 to 0.03215   | No               | ns      | 0.1883           |
| 48h Initial Growth:- Choline vs. 20min:- Choline              | -0.2292 to 0.03303   | No               | ns      | 0.1947           |
| 48h Initial Growth:- Choline vs. 60min :+ Choline             | -0.2495 to 0.01275   | No               | ns      | 0.0859           |
| 48h Initial Growth:- Choline vs. 60min :- Choline             | -0.2583 to 0.003960  | No               | ns      | 0.0592           |
| 20min:+ Choline vs. 20min:- Choline                           | -0.1302 to 0.1320    | No               | ns      | >0.9999          |
| 20min:+ Choline vs. 60min :+ Choline                          | -0.1505 to 0.1117    | No               | ns      | 0.9953           |
| 20min:+ Choline vs. 60min :- Choline                          | -0.1593 to 0.1029    | No               | ns      | 0.9753           |
| 20min:- Choline vs. 60min :+ Choline                          | -0.1514 to 0.1108    | No               | ns      | 0.9943           |
| 20min:- Choline vs. 60min :- Choline                          | -0.1602 to 0.1020    | No               | ns      | 0.9719           |
| 60min :+ Choline vs. 60min :- Choline                         | -0.1399 to 0.1223    | No               | ns      | 0.9999           |

### 3.5.3

| Comparison                                                    | Mean Diff. | 95.00% CI of diff.  | Below threshold? | Summary | Adjusted P Value |
|---------------------------------------------------------------|------------|---------------------|------------------|---------|------------------|
| 48h Initial Growth:+ Choline vs. 48h Initial Growth:- Choline | -0.02122   | -0.1088 to 0.06635  | No               | ns      | 0.8632           |
| 48h Initial Growth:+ Choline vs. 24h :+ Choline               | 0.1459     | 0.05837 to 0.2335   | Yes              | **      | 0.0031           |
| 48h Initial Growth:- Choline vs. 24h :- Choline               | 0.006993   | -0.08058 to 0.09457 | No               | ns      | 0.9937           |
| 24h :+ Choline vs. 24h :- Choline                             | -0.1602    | -0.2477 to -0.07259 | Yes              | **      | 0.0017           |

### 3.6.1.A)

| Comparison                           | Mean Diff. | 95.00% CI of diff.  | Below threshold? | Summary | Individual P Value |
|--------------------------------------|------------|---------------------|------------------|---------|--------------------|
| <b>10min</b><br>+ DOG vs. + $Me_2SO$ | 0.005717   | -0.04376 to 0.05520 | No               | ns      | 0.8120             |
| <b>20min</b><br>+ DOG vs. + $Me_2SO$ | -0.03013   | -0.07961 to 0.01935 | No               | ns      | 0.2186             |
| <b>60min</b><br>+ DOG vs. + $Me_2SO$ | -0.01765   | -0.06713 to 0.03183 | No               | ns      | 0.4656             |
| <b>4h</b><br>+ DOG vs. + $Me_2SO$    | -0.03509   | -0.08457 to 0.01439 | No               | ns      | 0.1546             |

### 3.6.1.B)

| Comparison                           | Mean Diff. | 95.00% CI of diff. |            | Below thresh | Summary | Individual P Value |
|--------------------------------------|------------|--------------------|------------|--------------|---------|--------------------|
|                                      |            | Lower              | Upper      |              |         |                    |
| 10min + DOG vs. + Me <sub>2</sub> SO | 0.004 090  | -0.086 85          | 0.095 03   | No           | ns      | 0.9262             |
| 20min + DOG vs. + Me <sub>2</sub> SO | -0.009 424 | -0.1004            | 0.081 51   | No           | ns      | 0.8310             |
| 60min + DOG vs. + Me <sub>2</sub> SO | -0.094 11  | -0.1850            | -0.003 178 | Yes          | *       | 0.0432             |
| 4h + DOG vs. + Me <sub>2</sub> SO    | -0.066 18  | -0.1571            | 0.024 76   | No           | ns      | 0.1447             |

### 3.6.2.A)

| Comparison                           | Mean Diff. | 95.00% CI of diff. | Below threshold? | Summary | Individual P Value |
|--------------------------------------|------------|--------------------|------------------|---------|--------------------|
| 5min + DOG vs. + Me <sub>2</sub> SO  | -0.032 89  | -0.1572 to 0.09139 | No               | ns      | 0.5900             |
| 10min + DOG vs. + Me <sub>2</sub> SO | 0.073 53   | -0.05075 to 0.1978 | No               | ns      | 0.2339             |
| 20min + DOG vs. + Me <sub>2</sub> SO | -0.016 20  | -0.1405 to 0.1081  | No               | ns      | 0.7902             |
| 60min + DOG vs. + Me <sub>2</sub> SO | 0.025 46   | -0.09882 to 0.1497 | No               | ns      | 0.6762             |

### 3.6.2.B)

| Comparison                              | Mean Diff. | 95% CI of diff.           | Below threshold? | Summary | Individual P Value |
|-----------------------------------------|------------|---------------------------|------------------|---------|--------------------|
| 5min<br>+ DOG vs. + Me <sub>2</sub> SO  | 0.037 99   | -0.093 47 to 0.1695<br>to | No               | ns      | 0.5565             |
| 10min<br>+ DOG vs. + Me <sub>2</sub> SO | 0.033 32   | -0.098 15 to 0.1648<br>to | No               | ns      | 0.6057             |
| 20min<br>+ DOG vs. + Me <sub>2</sub> SO | 0.1027     | -0.028 80 to 0.2341<br>to | No               | ns      | 0.1201             |
| 60min<br>+ DOG vs. + Me <sub>2</sub> SO | -0.070 82  | -0.2023 to 0.060 65<br>to | No               | ns      | 0.2772             |

### 3.6.3.A)

| Comparison                                                | Mean diff. | 95.00% CI of diff. | Below threshold? | Summary | Adjusted P Value |
|-----------------------------------------------------------|------------|--------------------|------------------|---------|------------------|
| 48h Initial Growth: vs. 20min:+ DOG                       | -0.1269    | -0.2955 0.041 59   | No               | ns      | 0.1849           |
| 48h Initial Growth: vs. 20min:+ Me <sub>2</sub> SO        | -0.1880    | -0.3565 -0.019 42  | Yes              | *       | 0.0266           |
| 48h Initial Growth: vs. 60min:+ DOG                       | -0.055 64  | -0.2242 0.1129     | No               | ns      | 0.8610           |
| 48h Initial Growth: vs. 60min:+ Me <sub>2</sub> SO        | -0.1167    | -0.2852 0.051 88   | No               | ns      | 0.2491           |
| 20min:+ DOG vs. 20min:+ Me <sub>2</sub> SO                | -0.061 02  | -0.1844 0.066 38   | No               | ns      | 0.5957           |
| 20min:+ DOG vs. 60min:+ DOG                               | 0.071 30   | -0.056 10 0.1987   | No               | ns      | 0.4452           |
| 20min:+ DOG vs. 60min:+ Me <sub>2</sub> SO                | 0.010 28   | -0.1699 0.1905     | No               | ns      | >0.9999          |
| 20min:+ Me <sub>2</sub> SO vs. 60min:+ DOG                | 0.1323     | -0.047 85 0.3125   | No               | ns      | 0.2030           |
| 20min:+ Me <sub>2</sub> SO vs. 60min:+ Me <sub>2</sub> SO | 0.071 30   | -0.056 10 0.1987   | No               | ns      | 0.4452           |
| 60min:+ DOG vs. 60min:+ Me <sub>2</sub> SO                | -0.061 02  | -0.1884 0.066 38   | No               | ns      | 0.5957           |

### 3.6.3.B)

| Comparison                                                | Mean diff. | 95.00% CI of diff.  | Below threshold? | Summary | Adjusted P Value |
|-----------------------------------------------------------|------------|---------------------|------------------|---------|------------------|
| 48h Initial Growth: vs. 20min:+ DOG                       | 0.01729    | -0.04613 to 0.0807  | No               | ns      | 0.9305           |
| 48h Initial Growth: vs. 20min:+ Me <sub>2</sub> SO        | -0.04630   | -0.1097 to 0.01712  | No               | ns      | 0.2074           |
| 48h Initial Growth: vs. 60min:+ DOG                       | 0.04474    | -0.01868 to 0.1082  | No               | ns      | 0.2337           |
| 48h Initial Growth: vs. 60min:+ Me <sub>2</sub> SO        | -0.01884   | -0.08226 to 0.04451 | No               | ns      | 0.9041           |
| 20min:+ DOG vs. 20min:+ Me <sub>2</sub> SO                | -0.06358   | -0.1115 to -0.01564 | Yes              | **      | 0.0085           |
| 20min:+ DOG vs. 60min:+ DOG                               | 0.02746    | -0.02048 to 0.0754  | No               | ns      | 0.4225           |
| 20min:+ DOG vs. 60min:+ Me <sub>2</sub> SO                | -0.03613   | -0.1039 to 0.03167  | No               | ns      | 0.4934           |
| 20min:+ Me <sub>2</sub> SO vs. 60min:+ DOG                | 0.09104    | 0.02324 to 0.1588   | Yes              | **      | 0.0078           |
| 20min:+ Me <sub>2</sub> SO vs. 60min:+ Me <sub>2</sub> SO | 0.02746    | -0.02048 to 0.0754  | No               | ns      | 0.4225           |
| 60min:+ DOG vs. 60min:+ Me <sub>2</sub> SO                | -0.06358   | -0.1115 to -0.01564 | Yes              | **      | 0.0085           |

### 3.8.A)

| Comparison                                                  | Mean diff. | 95.00% CI of diff. | Below threshold? | Summary | Adjusted P Value |
|-------------------------------------------------------------|------------|--------------------|------------------|---------|------------------|
| 0µM choline:Control vs. 0µM choline:Me <sub>2</sub> SO      | -26.91     | -57.54to3.718      | No               | ns      | 0.1332           |
| 0µM choline:Control vs. 0µM choline:Torin I                 | -91.84     | -122.5 to-61.21    | Yes              | ****    | <0.0001          |
| 0µM choline:Control vs. 0µM choline:Torin II                | -84.21     | -118.5 to-49.97    | Yes              | ****    | <0.0001          |
| 0µM choline:Me <sub>2</sub> SO vs. 0µM choline:Torin I      | -64.93     | -95.56to-34.30     | Yes              | ****    | <0.0001          |
| 0µM choline:Me <sub>2</sub> SO vs. 0µM choline:Torin II     | -57.30     | -91.54to-23.06     | Yes              | ****    | <0.0001          |
| 10µM choline:Control vs. 10µM choline:Me <sub>2</sub> SO    | -0.038 17  | -30.66to30.59      | No               | ns      | >0.9999          |
| 10µM choline:Control vs. 10µM choline:Torin I               | -3.414     | -37.66to30.83      | No               | ns      | >0.9999          |
| 10µM choline:Control vs. 10µM choline:Torin II              | -36.45     | -67.08to-5.824     | Yes              | **      | 0.0089           |
| 10µM choline:Me <sub>2</sub> SO vs. 10µM choline:Torin I    | -3.375     | -37.62to30.87      | No               | ns      | >0.9999          |
| 10µM choline:Me <sub>2</sub> SO vs. 10µM choline:Torin II   | -36.41     | -67.04to-5.786     | Yes              | **      | 0.0090           |
| 50µM choline:Control vs. 50µM choline:Me <sub>2</sub> SO    | -0.5457    | -31.17to30.08      | No               | ns      | >0.9999          |
| 50µM choline:Control vs. 50µM choline:Torin I               | -8.837     | -39.46to21.79      | No               | ns      | 0.9989           |
| 50µM choline:Control vs. 50µM choline:Torin II              | -3.366     | -33.99to27.26      | No               | ns      | >0.9999          |
| 50µM choline:Me <sub>2</sub> SO vs. 50µM choline:Torin I    | -8.291     | -38.92to22.34      | No               | ns      | 0.9995           |
| 50µM choline:Me <sub>2</sub> SO vs. 50µM choline:Torin II   | -2.821     | -33.45to27.81      | No               | ns      | >0.9999          |
| 100µM choline:Control vs. 100µM choline:Me <sub>2</sub> SO  | -1.183     | -31.81to29.44      | No               | ns      | >0.9999          |
| 100µM choline:Control vs. 100µM choline:Torin I             | -1.935     | -36.18to32.31      | No               | ns      | >0.9999          |
| 100µM choline:Control vs. 100µM choline:Torin II            | -4.300     | -34.93to26.33      | No               | ns      | >0.9999          |
| 100µM choline:Me <sub>2</sub> SO vs. 100µM choline:Torin I  | -0.7522    | -34.99to33.49      | No               | ns      | >0.9999          |
| 100µM choline:Me <sub>2</sub> SO vs. 100µM choline:Torin II | -3.118     | -33.74to27.51      | No               | ns      | >0.9999          |

3.8.B)

| Comparison           | Mean Diff. | 95.00% CI of diff.  | Below threshold | Summary | Individual P Value |
|----------------------|------------|---------------------|-----------------|---------|--------------------|
| <b>0μM choline</b>   |            |                     |                 |         |                    |
| control vs. DMSO     | 0.07708    | -0.02070 to 0.1749  | No              | ns      | 0.1182             |
| control vs. Torin1   | 0.2148     | 0.1171 to 0.3126    | Yes             | ****    | <0.0001            |
| control vs. Torin 2  | 0.1691     | 0.07132 to 0.2669   | Yes             | **      | 0.0013             |
| DMSO vs. Torin1      | 0.1378     | 0.03997 to 0.2355   | Yes             | **      | 0.0072             |
| DMSO vs. Torin 2     | 0.09202    | -0.005761 to 0.1898 | No              | ns      | 0.0642             |
| Torin1 vs. Torin 2   | -0.04573   | -0.1435 to 0.05205  | No              | ns      | 0.3479             |
| <b>10μM choline</b>  |            |                     |                 |         |                    |
| control vs. DMSO     | 0.01184    | -0.08594 to 0.1096  | No              | ns      | 0.8068             |
| control vs. Torin1   | 0.2653     | 0.1675 to 0.3630    | Yes             | ****    | <0.0001            |
| control vs. Torin 2  | 0.2142     | 0.1164 to 0.3120    | Yes             | ****    | <0.0001            |
| DMSO vs. Torin1      | 0.2534     | 0.1556 to 0.3512    | Yes             | ****    | <0.0001            |
| DMSO vs. Torin 2     | 0.2023     | 0.1046 to 0.3001    | Yes             | ***     | 0.0002             |
| Torin1 vs. Torin 2   | -0.05107   | -0.1489 to 0.04671  | No              | ns      | 0.2954             |
| <b>50μM choline</b>  |            |                     |                 |         |                    |
| control vs. DMSO     | 0.40302    | -0.05476 to 0.1408  | No              | ns      | 0.3768             |
| control vs. Torin1   | 0.3963     | 0.2985 to 0.4941    | Yes             | ****    | <0.0001            |
| control vs. Torin 2  | 0.2184     | 0.1206 to 0.3161    | Yes             | ****    | <0.0001            |
| DMSO vs. Torin1      | 0.3533     | 0.2555 to 0.4511    | Yes             | ****    | <0.0001            |
| DMSO vs. Torin 2     | 0.1753     | 0.07756 to 0.2731   | Yes             | ***     | 0.0009             |
| Torin1 vs. Torin 2   | -0.1779    | -0.2757 to -0.08015 | Yes             | ***     | 0.0008             |
| <b>100μM choline</b> |            |                     |                 |         |                    |
| control vs. DMSO     | -0.08605   | -0.1838 to 0.01173  | No              | ns      | 0.0825             |
| control vs. Torin1   | 0.2277     | 0.1299 to 0.3255    | Yes             | ****    | <0.0001            |
| control vs. Torin 2  | 0.2352     | 0.1374 to 0.3330    | Yes             | ****    | <0.0001            |
| DMSO vs. Torin1      | 0.3138     | 0.2160 to 0.4116    | Yes             | ****    | <0.0001            |
| DMSO vs. Torin 2     | 0.3213     | 0.2235 to 0.4191    | Yes             | ****    | <0.0001            |
| Torin1 vs. Torin 2   | 0.007496   | -0.09029 to 0.1053  | No              | ns      | 0.8769             |

3.9.A)

|                       |                                 | Mean Diff. | 95.00% CI of diff. | Below threshold? | Summary | Individual P Value |
|-----------------------|---------------------------------|------------|--------------------|------------------|---------|--------------------|
| <b>0 μM choline</b>   | Control vs. Me <sub>2</sub> SO  | 2.894      | -8.154 to 13.94    | No               | ns      | 0.5985             |
|                       | Control vs. Torin II            | -69.87     | -80.92 to -58.82   | Yes              | ****    | <0.0001            |
|                       | Me <sub>2</sub> SO vs. Torin II | -72.76     | -83.81 to -61.71   | Yes              | ****    | <0.0001            |
| <b>10 μM choline</b>  | Control vs. Me <sub>2</sub> SO  | -1.870     | -12.92 to 9.178    | No               | ns      | 0.7334             |
|                       | Control vs. Torin II            | -49.06     | -60.10 to -38.01   | Yes              | ****    | <0.0001            |
|                       | Me <sub>2</sub> SO vs. Torin II | -47.19     | -58.23 to -36.14   | Yes              | ****    | <0.0001            |
| <b>100 μM choline</b> | Control vs. Me <sub>2</sub> SO  | -0.9920    | -12.04 to 10.06    | No               | ns      | 0.8565             |
|                       | Control vs. Torin II            | -28.75     | -39.80 to -17.71   | Yes              | ****    | <0.0001            |
|                       | Me <sub>2</sub> SO vs. Torin II | -27.76     | -38.81 to -16.71   | Yes              | ****    | <0.0001            |

## 3.9.B)

| Comparison                      | Mean Diff. | 95.00% CI of diff.   | Below threshold? | Summary | Individual P Value |
|---------------------------------|------------|----------------------|------------------|---------|--------------------|
| 0 $\mu$ M choline               |            |                      |                  |         |                    |
| Control vs. Me <sub>2</sub> SO  | -0.03400   | -0.07132 to 0.003323 | No               | ns      | 0.0729             |
| Control vs. Torin II            | 0.2480     | 0.2107 to 0.2853     | Yes              | ****    | <0.0001            |
| Me <sub>2</sub> SO vs. Torin II | 0.2820     | 0.2447 to 0.3193     | Yes              | ****    | <0.0001            |
| 10 $\mu$ M choline              |            |                      |                  |         |                    |
| Control vs. Me <sub>2</sub> SO  | -0.03200   | -0.06932 to 0.005323 | No               | ns      | 0.0906             |
| Control vs. Torin II            | 0.4460     | 0.4087 to 0.4833     | Yes              | ****    | <0.0001            |
| Me <sub>2</sub> SO vs. Torin II | 0.4780     | 0.4407 to 0.5153     | Yes              | ****    | <0.0001            |
| 100 $\mu$ M choline             |            |                      |                  |         |                    |
| Control vs. Me <sub>2</sub> SO  | 0.000      | -0.03732 to 0.03732  | No               | ns      | >0.9999            |
| Control vs. Torin II            | 0.7760     | 0.7387 to 0.8133     | Yes              | ****    | <0.0001            |
| Me <sub>2</sub> SO vs. Torin II | 0.7760     | 0.7387 to 0.8133     | Yes              | ****    | <0.0001            |

## 3.10.A)

| Comparison                          | Mean Diff. | 95.00% CI of diff. | Below threshold? | Summary | Adjusted P Value |
|-------------------------------------|------------|--------------------|------------------|---------|------------------|
| Me <sub>2</sub> SO vs. Control      | -2.891     | -19.70 to 13.92    | No               | ns      | 0.9732           |
| Me <sub>2</sub> SO vs. DOG          | -18.72     | -35.53 to -1.909   | Yes              | *       | 0.0265           |
| Me <sub>2</sub> SO vs. Torin II     | -72.76     | -89.57 to -55.95   | Yes              | ****    | <0.0001          |
| Me <sub>2</sub> SO vs. DOG+Torin II | -73.35     | -90.16 to -56.54   | Yes              | ****    | <0.0001          |

## 3.10.B)

| Comparison                            | Mean Diff. | 95.00% CI of diff.  | Below threshold? | Summary | Adjusted P Value |
|---------------------------------------|------------|---------------------|------------------|---------|------------------|
| <i>Me<sub>2</sub>SO vs. Control</i>   | 0.03402    | 0.002643 to 0.06540 | Yes              | *       | 0.0313           |
| <i>Me<sub>2</sub>SO vs. DOG</i>       | 0.09458    | 0.06320 to 0.1260   | Yes              | ****    | <0.0001          |
| <i>Me<sub>2</sub>SO vs. Torin II</i>  | 0.2843     | 0.2529 to 0.3157    | Yes              | ****    | <0.0001          |
| <i>Me<sub>2</sub>SO vs. DOG+Torin</i> | 0.2789     | 0.2475 to 0.3103    | Yes              | ****    | <0.0001          |



## 3.11.A)

| Comparison                                                              | Mean Diff. | 95.00% CI of diff. | Below threshold? | Summary | Adjusted P Value |
|-------------------------------------------------------------------------|------------|--------------------|------------------|---------|------------------|
| <i>chol-1</i> :Control vs. <i>chol-1</i> : Me <sub>2</sub> SO           | -0.920     | -15.74 to13.75     | No               | ns      | >0.9999          |
| <i>chol-1</i> :Control vs. <i>chol-1</i> :Torin II                      | -28.36     | -43.10 to-13.61    | Yes              | ****    | <0.0001          |
| <i>chol-1</i> :Control vs. <i>Avta</i> :Control                         | -5.014     | -19.76 to9.732     | No               | ns      | 0.8954           |
| <i>chol-1</i> :Control vs. <i>Avta</i> : Me <sub>2</sub> SO             | -7.456     | -22.20 to7.290     | No               | ns      | 0.6289           |
| <i>chol-1</i> :Torin II vs. <i>Avta</i> :Torin II                       | -3.260     | -18.01 to11.49     | No               | ns      | 0.9821           |
| <i>chol-1</i> : Me <sub>2</sub> SO vs. <i>chol-1</i> :Torin II          | -27.436    | -42.11 to-12.62    | Yes              | ****    | <0.0001          |
| <i>chol-1</i> : Me <sub>2</sub> SO vs. <i>Avta</i> :Control             | -4.022     | -18.77 to10.72     | No               | ns      | 0.9561           |
| <i>chol-1</i> : Me <sub>2</sub> SO vs. <i>Avta</i> : Me <sub>2</sub> SO | -6.464     | -21.21 to8.282     | No               | ns      | 0.7520           |
| <i>chol-1</i> : Me <sub>2</sub> SO vs. <i>Avta</i> :Torin II            | -2.268     | -17.01 to12.48     | No               | ns      | 0.9966           |
| <i>chol-1</i> :Torin II vs. <i>Avta</i> :Control                        | 23.384     | 8.596to38.09       | Yes              | ***     | 0.0007           |
| <i>chol-1</i> :Torin II vs. <i>Avta</i> : Me <sub>2</sub> SO            | 20.900     | 6.154to35.65       | Yes              | **      | 0.0024           |
| <i>chol-1</i> :Torin II vs. <i>Avta</i> :Torin II                       | 25.100     | 10.35 to39.84      | Yes              | ***     | 0.0003           |
| <i>Avta</i> :Control vs. <i>Avta</i> : Me <sub>2</sub> SO               | -2.484     | -17.19 to12.30     | No               | ns      | 0.9952           |
| <i>Avta</i> :Control vs. <i>Avta</i> :Torin II                          | 1.754      | -12.99 to16.50     | No               | ns      | 0.9990           |
| <i>Avta</i> : Me <sub>2</sub> SO vs. <i>Avta</i> :Torin II              | 4.196      | -10.55 to18.94     | No               | ns      | 0.9478           |

## 3.11.B)

| Comparison                           | Mean Diff. | 95% CI of diff.     | Below threshold? | Summary | Adjusted P Value |
|--------------------------------------|------------|---------------------|------------------|---------|------------------|
| chol-1::Control vs. chol-1::Me2SO    | 0.0000     | -0.03183 to0.03183  | No               | ns      | >0.9999          |
| chol-1::Control vs. chol-1::Torin II | 0.7760     | 0.7442 to0.8078     | Yes              | * * *   | <0.0001          |
| chol-1::Control vs. Δvta::Control    | -0.0640    | -0.09583 to-0.03217 | Yes              | * * *   | <0.0001          |
| chol-1::Control vs. Δvta::Me2SO      | 0.0020     | -0.02983 to0.03383  | No               | ns      | >0.9999          |
| chol-1::Control vs. Δvta::Torin II   | 0.6540     | 0.6222 to0.6858     | Yes              | * * *   | <0.0001          |
| chol-1::Me2SO vs. chol-1::Torin II   | 0.7760     | 0.7442 to0.8078     | Yes              | * * *   | <0.0001          |
| chol-1::Me2SO vs. Δvta::Control      | -0.0640    | -0.09583 to-0.03217 | Yes              | * * *   | <0.0001          |
| chol-1::Me2SO vs. Δvta::Me2SO        | 0.0020     | -0.02983 to0.03383  | No               | ns      | >0.9999          |
| chol-1::Me2SO vs. Δvta::Torin II     | 0.6540     | 0.6222 to0.6858     | Yes              | * * *   | <0.0001          |
| chol-1::Torin II vs. Δvta::Control   | -0.8400    | -0.8718 to-0.8082   | Yes              | * * *   | <0.0001          |
| chol-1::Torin II vs. Δvta::Me2SO     | -0.7740    | -0.8058 to-0.7422   | Yes              | * * *   | <0.0001          |
| chol-1::Torin II vs. Δvta::Torin II  | -0.1220    | -0.1538 to-0.09017  | Yes              | * * *   | <0.0001          |
| Δvta::Control vs. Δvta::Me2SO        | 0.0660     | 0.03417 to0.09783   | Yes              | * * *   | <0.0001          |
| Δvta::Control vs. Δvta::Torin II     | 0.7180     | 0.6862 to0.7498     | Yes              | * * *   | <0.0001          |
| Δvta::Me2SO vs. Δvta::Torin II       | 0.6520     | 0.6202 to0.6838     | Yes              | * * *   | <0.0001          |

## Appendix C: Growth media, buffers and solutions

| Liquid minimal medium (MM): |        |
|-----------------------------|--------|
| Reagent                     | Amount |
| Vogel's 50X                 | 2 mL   |
| Glucose                     | 2 g    |
| 10 mM Choline               | 1 mL   |
| ddH <sub>2</sub> O          | 100 mL |

| Minimal agar medium: |        |
|----------------------|--------|
| Reagent              | Amount |
| Vogel's 50X          | 2 mL   |
| 10 mM Choline        | 1 mL   |
| Glucose              | 0.5 g  |
| Agar                 | 2 g    |
| ddH <sub>2</sub> O   | 100 mL |

| 0.5% Maltose gar medium: |        |
|--------------------------|--------|
| Reagent                  | Amount |
| Vogel's 50X              | 2 mL   |
| 10 mM Choline            | 1 mL   |
| Maltose                  | 0.5 g  |
| Agar                     | 2 g    |
| ddH <sub>2</sub> O       | 100 mL |

| Vogel's salt 50x:                                                             |        |
|-------------------------------------------------------------------------------|--------|
| Reagents                                                                      | amount |
| Na <sub>3</sub> citrate-5 H <sub>2</sub> O                                    | 150 g  |
| KH <sub>2</sub> PO <sub>4</sub>                                               | 250 g  |
| NH <sub>4</sub> NO <sub>3</sub>                                               | 100 g  |
| MgSO <sub>4</sub> -7 H <sub>2</sub> O                                         | 10 g   |
| CaCl <sub>2</sub> -2 H <sub>2</sub> O (predissolve in 20 mL H <sub>2</sub> O) | 5 g    |
| Biotin solution                                                               | 5 mL   |
| Trace element solution                                                        | 5 mL   |

| Protein Extraction Buffer (PEB): |        |
|----------------------------------|--------|
| Reagent                          | Amount |
| 0.5 M Tris pH 6.8                | 1.0 mL |
| 10% SDS                          | 2.0 mL |
| Glycerol                         | 1.0 mL |
| ddH <sub>2</sub> O               | 6.0 mL |

| Separating gel (12.5% acrylamide): |              |
|------------------------------------|--------------|
| Reagent                            | Amount       |
| Water                              | 2.03 mL      |
| 1.5M Tris pH 8.8                   | 1.25 mL      |
| 10% SDS                            | 50 $\mu$ L   |
| 40% acrylamide                     | 1.56 mL      |
| PhosTag                            | 50 $\mu$ L   |
| 10 mM MnCl <sub>2</sub>            | 50 $\mu$ L   |
| 10% Ammonium Persulfate (APS)      | 25.0 $\mu$ L |
| TEMED                              | 5.0 $\mu$ L  |

| Stacking Gel:         |              |
|-----------------------|--------------|
| Reagent               | Amount       |
| Water                 | 1.56 mL      |
| 0.5 M Tris pH 6.8     | 0.645 mL     |
| 10% SDS               | 25.9 $\mu$ L |
| 40% acrylamide        | 259 $\mu$ L  |
| 0.4% Bromophenol blue | 10 $\mu$ L   |
| 10% APS               | 13 $\mu$ L   |
| TEMED                 | 2.59 $\mu$ L |

| Running buffer: |        |
|-----------------|--------|
| Reagent         | Amount |
| Water           | 1 L    |
| Glycine         | 14.4 g |
| Tris base       | 3.0 g  |
| SDS             | 1.0 g  |

| Anode buffer I: |        |
|-----------------|--------|
| Reagent         | Amount |
| Water           | 1 L    |
| Tris base       | 34.3 g |
| Methanol        | 100 mL |
| pH              | 10.4   |

| Anode buffer II: |        |
|------------------|--------|
| Reagent          | Amount |
| Water            | 1 L    |
| Tris base        | 3.03 g |
| Methanol         | 100 mL |
| pH               | 10.4   |

| Cathode buffer:    |        |
|--------------------|--------|
| Reagent            | Amount |
| Glycine            | 3.0 g  |
| Tris base          | 3.03 g |
| ddH <sub>2</sub> O | 900mL  |
| Methanol           | 100 mL |
| pH                 | 9.4    |

| 10X Blocking Buffer: |        |
|----------------------|--------|
| Reagent              | Amount |
| BSA                  | 5.0 g  |
| TBST                 | 50 mL  |

| 1X TBST:           |        |
|--------------------|--------|
| Reagent            | Amount |
| Tris               | 1.21 g |
| NaCl               | 8.5 g  |
| ddH <sub>2</sub> O | 1L     |
| pH                 | 7.5    |
| Tween-20           | 1 mL   |

| TTBST:   |        |
|----------|--------|
| Reagent  | Amount |
| Tris     | 20 mM  |
| NaCl     | 500 mM |
| Tween 20 | 0.3%   |
| pH       | 7.5    |