ADAPTATION OF THE CIRCADIAN CLOCK TO LOW NUTRIENT SUPPLY IN *NEUROSPORA CRASSA*

PhD thesis

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1. Introduction

Circadian clocks are internal timekeeping systems that help organisms anticipate and adapt to daily environmental changes, such as light intensity, temperature fluctuations, and nutrient availability. They regulate almost all physiological processes from the cellular to the organismal level. Circadian rhythms persist even under constant conditions and are maintained by highly conserved transcription-translation feedback loops. In humans, disruption of circadian timing through shift work or jet lag has been linked to an increased risk of metabolic disorders, cardiovascular diseases, and cancer.

The period of the circadian rhythm refers to the length of a complete cycle, typically around 24 hours under constant conditions. The phase defines the time of a given point of the cycle (usually a peak or trough) relative to a reference point, which is in most systems, the time point of light on. Key features of the circadian clock are temperature and metabolic compensations, which maintain the period stable even under changing temperature or nutrient conditions.

Several model organisms are used to study circadian rhythm, including the filamentous fungus *Neurospora crassa*, which stands out due to its well-characterized molecular oscillator. Its daily conidiation rhythm provides an easily measurable clock

output in the race tube assay, allowing for the easy detection of period and phase changes. The core clock of *Neurospora* is built around the White Collar Complex (WCC) transcription factor, composed of WC-1 (White Collar-1) and WC-2 (White Collar-2), the positive components of the *Neurospora* clock. The WCC activates transcription of the negative regulator *frq* (*frequency*). FRQ forms a complex with other components, like CK-1a (Casein Kinase-1a), which can phosphorylate and inactivate the WCC, closing the negative feedback loop. This inhibition reduces the transcription of *frq*. FRQ is gradually phosphorylated throughout the day, leading to its degradation, allowing a new cycle to begin approximately every 24 hours.

As a blue light receptor, WC-1 is also involved in light detection, which is necessary for synchronizing the clock to external light conditions.

Post-translational regulation of FRQ and the WCC, especially by phosphorylation through CK-1a, CKII (Casein Kinase II), PKA (Protein Kinase A), and other kinases, and dephosphorylation by PP1 (Protein Phosphatase 1), PP2A (Protein Phosphatase 2A), and PP4 (Protein Phosphatase 4), plays a critical role in maintaining robust oscillations under different environmental conditions.

RAS (Rat Sarcoma) proteins are small GTPases (Guanosine Triphosphatases) that regulate growth, proliferation, and metabolism. Their activity is controlled by guanine nucleotide exchange factors (GEFs), which activate RAS by promoting GDP-GTP exchange, and GTPase-activating proteins (GAPs), which accelerate inactivation.

In *Neurospora*, mutations in *ras-1* and *ras-2* alter circadian behaviour. *ras-2* deletion specifically impairs metabolic compensation of the circadian clock.

RAS-MAPK signalling influences clock function in other organisms by affecting transcription factors and kinases such as ERK (Extracellular Signal-Regulated Kinase) and GSK3β (Glycogen Synthase Kinase 3β), pointing to a conserved interaction between RAS pathways and circadian regulation.

In *Neurospora*, several factors and mechanisms ensure the accuracy and stability of the circadian clock, maintaining the endogenous rhythm despite environmental noise. Transcriptional regulators such as CSP-1 (Conidial Separation 1) and RCO-1 (Regulation of Conidiation 1) adjust the expression of *wc-1* and *frq* in response to nutrient limitations, while RNA-binding proteins, alternative polyadenylation, and nonsense-mediated decay fine-tune mRNA stability and processing. Chromatin modifiers like SET-1 and SET-2

(Histone-lysine N-methyltransferase, H3 lysine-4 specific 1/2), as well as signalling pathways involving PKA and GSK, adjust clock protein stability and phosphorylation. RAS2-cAMP signalling also plays a crucial role in maintaining stable rhythms across nutrient conditions.

My research work focused on how long-term glucose deprivation reorganizes the core clock components in *Neurospora* and the role of a previously uncharacterized RasGEF protein in the metabolic compensation. Given the evolutionary conservation of RAS signalling, we also tested whether similar mechanisms exist in mammalian cells by using the U2OS (human osteosarcoma) cell line. Our results provide new insights into how nutrient status influences circadian robustness across species.

2. Objectives

Our goal was to investigate the relationship between the circadian rhythm and metabolism of *Neurospora crassa*:

2.1. Mechanism of adaptation of the circadian oscillator to long-term glucose deprivation:

- Comparison of clock function under starvation and control conditions
- Identification of regulatory pathways involved in the adaptation of the clock to nutrient withdrawal

2.2. Investigation of the role of a RasGEF protein in the metabolic compensation of the circadian oscillator:

- Characterization of the circadian oscillator and the rhythmic output in the *rasgef* deletion strain
- Studying the possible role of RasGEF in the nutrient compensation of the clock
- Characterization of the regulation of *rasgef* expression
- Searching for signalling pathways and interaction partners of RasGEF
- Characterization of parallel mechanisms in the metabolic compensation of the mammalian circadian clock

3. Methods

Neurospora cultures were incubated in a medium containing either high (2%) or low (0.01%) glucose for at least 40 hours. Gene expression was analyzed to assess clock function. Protein expression and phosphorylation were examined with western blot.

Strains with elevated PKA activity (*mcb*) and altered FRQ-CK-1a interaction (*frq*Δ*FCD1-2*) were used. GSK function was tested using the *qa-gsk* strain, in which its expression is regulated by a quinic acid-inducible promoter.

The interaction between RasGEF-RAS2P was confirmed by an *in vitro* interaction assay.

U2OS cells carrying a luciferase reporter under the control of the *Bmal1* (mammalian positive factor) promoter were used to monitor circadian rhythmicity under varying glucose concentrations. SOS1 and ERK inhibitors were applied to assess their effects at different glucose levels.

3.1. Race tube analysis

Neurospora strains were grown in bent glass tubes containing glucose-free solid medium. Vegetative hyphae are grown linearly, while conidial band formation is under circadian control. Growth fronts were marked daily, and band spacing was analysed with the ChronOSX 2.0.3.4 software.

3.2. Protein extraction and subcellular fractionation

Mycelium was ground in liquid nitrogen and protein was extracted using a buffer supplemented with protease and phosphatase inhibitors. Protein concentration was measured at 280 nm (NanoDrop One). Nuclear and cytosolic fractions were separated using a modified method described by Luo et al. (1998).

3.3. Western blot

Proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane. Membranes were stained with Ponceau S to verify equal loading, blocked in TBS containing 5% milk, and incubated with the appropriate primary and secondary antibodies. Detection was performed using the enhanced chemiluminescence method.

3.4. RNA analysis

RNA was extracted using TriReagent. The RNA concentration and quality were determined using the Nanodrop One device, and cDNA was synthesized using the RevertAid First Strand cDNA Synthesis Kit.

qRT-PCR was performed using a LightCycler 480 with TaqMan probes. Ct values were calculated using the second derivative method and normalised to the *gna-3* housekeeping gene.

3.5. *In vitro* interaction partner assay

pGEX4T1 and *pGEX4T1-ras2p* plasmids were expressed in Rosetta™ (DE3) cells. GST or GST-RAS2P proteins were bound to glutathione-agarose beads and incubated with Flag-tagged RasGEF protein lysates. After washing, bound proteins were eluted and interactions were analysed using western blot with anti-FLAG antibodies.

3.6. Statistical analysis

Data were analysed in Statistica 13; cosinor analyses in R 4.3.3; graphs in GraphPad Prism 8.0. Significance was set at $p < 0.05$.

4. Results

4.1 Long-term glucose deprivation affects the circadian clock

My colleagues showed that *Neurospora* maintained rhythmic expression of *frq* and other *clock-controlled genes* despite long-term glucose deprivation. While the cytoplasmic levels of the WCC decreased, nuclear levels remained stable, supporting continued transcriptional activity of clock genes.

4.1.1. frq RNA stability is not dependent on glucose supply

As *frq* transcription stops after a light-to-dark transfer of the culture, following *frq* transcript levels under these conditions allows one to assess degradation of the RNA. However, the decay rate of *frq* RNA did not differ significantly between high- and low-glucose conditions, indicating that RNA stability was unchanged and the transcriptionally active WCC pool was maintained even under starvation conditions.

4.1.2. Reduced light response in starvation

WCC also acts as a photoreceptor. Upon light exposure, a large fraction of WCC rapidly becomes activated, triggering a transient but pronounced induction of light-sensitive genes, followed by an adaptation. We measured the light-induced transcription of *frq*, *wc-1*, and *al-2*, where the initial induction was reduced in starved cultures, suggesting that the light-

activatable pool of the WCC was reduced in long-term glucose deprivation.

4.1.3. Role of protein kinases

Enhanced PKA activity in the *mcb* strain abolished starvation-induced molecular changes in the circadian oscillator, highlighting PKA as a central mediator of nutrient-dependent clock control. GSK, on the other hand, was essential for starvation-induced hyperphosphorylation of FRQ. Mutation of the FRQ FCD1-2 domains, which disrupt CK-1a binding, did not prevent starvation-induced FRQ hyperphosphorylation but caused moderate alterations in WC levels under glucose limitation.

4.2 The role of a RasGEF in the metabolic compensation of the circadian rhythm

Based on the relationship between RAS-mediated signalling and circadian rhythms in different organisms, and our previous findings regarding the tight link between RAS signalling and the metabolic regulation of the clock in *Neurospora*, we aimed to examine the role of RasGEF activity in the starvation response.

4.2.1. RasGEF deletion alters circadian period and phase and participates in temperature compensation

The *rasgef* (NCU 09758) knock-out strain has delayed conidiation phase under both light/dark and temperature cycles,

revealing that RasGEF can affect the clock function. The period is longer in the *rasgef* mutant in constant darkness with a more pronounced difference at low temperatures, indicating also the RasGEF role in adaptation to cold stress.

4.2.2. RasGEF supports metabolic compensation in *Neurospora*

Under low glucose condition, in the *rasgef* deletion strain the amplitude of the *frq* RNA oscillations was reduced, starvation-induced reduction of WC-1 and WC-2 was less pronounced, and the rhythm of the expression of the *clock-controlled gene*, *ccg-2* was dampened compared to the wild type (*wt*) strain. These results suggest that RasGEF affects the core clock and helps maintain the expression of circadian output genes during nutrient limitation.

4.2.3. RasGEF is clock-controlled and regulated by light

rasgef RNA showed circadian oscillations in constant darkness. Its RNA peaked in antiphase to *frq*. Expression was modestly repressed by light. CDC25, the yeast homolog of RasGEF, is known to be regulated by phosphorylation. To determine whether the *Neurospora* RasGEF is similarly modified, we analysed the protein by western blots in high and low glucose media. We found that RasGEF was a phosphoprotein that shifted

to a hypophosphorylated, nuclear-localized state upon starvation.

4.2.4. RasGEF-RAS2P-PKA signalling

In fungi, RAS signalling is functionally linked to the cAMP/PKA pathway, which is responsive to changes in glucose availability. The glucose-induced cAMP production was absent in the *rasgef* knock-out strain, indicating defective PKA pathway activation. Affinity purification and mass spectrometry analysis suggested that RAS2P is a RASGEF interaction partner. This interaction was confirmed using an *in vitro* interaction assay.

4.2.5. Conservation of RasGEF signalling in mammalian cells

In U2OS cells, *Bmal1*-luciferase rhythms were maintained between 0.5 to 25 mM glucose concentrations. Inhibition of SOS1 (the mammalian homolog of RasGEF) or MEK1/2 (MAPK/ERK Kinase 1/2) impaired rhythmicity under low glucose conditions, demonstrating that RAS-MAPK signalling contributes to circadian robustness under nutrient limitation in mammalian cells.

5. Conclusions

Our results show that the circadian clock of *Neurospora crassa* remains functional under long-term glucose starvation, demonstrating robust metabolic compensation. Starvation reduced the cytosolic pool of the WCC, weakening light responses, but preserved enough nuclear WCC to sustain transcription of *frq* and other *clock-controlled genes*. The stability of *frq* RNA remained unchanged, indicating that its turnover is independent of the glucose content of the medium. Manipulating signalling pathways revealed that reduced PKA activity supports transcriptional compensation, while increased GSK activity contributes to the starvation-induced hyperphosphorylation of FRQ, which likely weakens its inhibition of the WCC. Although CK-1a is an essential FRQ kinase, starvation-induced phosphorylation occurred largely independently of FRQ-CK-1a interaction.

We characterized a RasGEF as a key factor in nutrient compensation. Deletion of rasgef impaired both metabolic and temperature compensation, delayed conidiation phase, increased WCC levels under starvation, and disrupted output gene regulation. RasGEF interacts with RAS2P, a previously uncharacterised small GTPase, and influences cAMP/PKA signalling, positioning it upstream of several known clock

regulators. The RasGEF shows circadian-regulated expression, glucose-dependent phosphorylation, and subcellular relocalisation.

Extending the work to U2OS cells demonstrated that glucose-responsive RasGEF signalling is conserved. Inhibition of SOS1, the mammalian homolog of *Neurospora*'s RasGEF, or its downstream ERK pathway, impaired rhythmicity only under low-glucose conditions, mirroring the phenotype of *Neurospora*.

In conclusion, this study offers a detailed insight into how the circadian system detects and responds to fluctuations in nutrient availability. The coordinated action of PKA, GSK, CK-1a, PP2A, and RasGEF signalling forms a flexible regulatory network capable of preserving clock robustness under challenging conditions.

These findings enhance our understanding of metabolic compensation in a model fungal system and highlight evolutionarily conserved pathways that may be relevant to circadian regulation in higher organisms, including humans.

6. Bibliography of the candidate's publications

6.1. Publications related to the thesis:

Sárkány, Orsolya; Szőke, Anita; Pettkó-Szandtner, Aladár; Kálmán, Eszter Éva; Brunner, Michael; Gyöngyösi, Norbert; Káldi, Krisztina. Conserved function of a RasGEF-mediated pathway in the metabolic compensation of the circadian clock. FEBS JOURNAL, 20 p. (2025). IF (2025): 4.2

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6.2. Publications not related to the thesis:

Révész, Fruzsina; Tóth, Erika M; Kriszt, Balázs; Bóka, Károly; Benedek, Tibor; Sárkány, Orsolya; Nagy, Zsuzsa; Táncsics, András. *Sphingobium aquiterrae sp.* nov., a toluene, meta- and para-xylene-degrading bacterium isolated from petroleum hydrocarbon-contaminated groundwater. INTERNATIONAL JOURNAL OF SYSTEMATIC AND EVOLUTIONARY MICROBIOLOGY 68: 9pp. 2807-2812., 6 p.(2018). IF (2018): 2.166

∑IF: 12.766