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

PhD thesis

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Table of contents

List of abbreviations

APA	Alternative Polyadenylation
ARNT	Aryl hydrocarbon Receptor Nuclear Translocator protein
bd	*band* mutation
C-box	Clock-box
CAMK-1	Calmodulin Kinase-1
cAMP	cyclic AMP
ccg	*clock-controlled gene*
CDC25	Cell Division Cycle 25
CIP	λ-phosphatase
CK-1	Casein Kinase-1
CSP-1	Conidial Separation 1
CT	Circadian Time
DBT	DOUBLETIME
DD	constant darkness
DEXRAS1	Dexamethasone-induced RAS protein 1
D/L transition	dark to light transition, light induction
ECL	Enhanced Chemiluminescence
EDTA	Ethylenediaminetetraacetic Acid
ERK	Extracellular Signal-Regulated Kinase
FAD	Flavin Adenine Dinucleotide
FCD	FRQ-CK1a-interacting Domain
FFC	FRQ-FRH Complex
FC	Fold Change
FDR	False Discovery Rate
FFD	FRH-interacting domain
FGSC	Fungal Genetic Stock Center
FRH	FRQ-interacting RNA Helicase
FRQ	FREQUENCY
FWD1	F-box/WD-40 repeat-containing protein
GAP	GTPase-Activating Proteins

GDP	Guanosine Diphosphate
GEF	Guanine nucleotide Exchange Factors
gna-3	*G protein alpha subunit-3*
GSK	Glycogen Synthase Kinase
GTP	Guanosine Triphosphate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
his3	*histidine biosynthesis gene 3*
H-RAS	Harvey RAS
K-RAS	Kirsten RAS
LB	Luria-Bertani medium
LC	Loading Control
LL	constant light
LOV domain	Light, Oxygen, or Voltage domain
L/D transition	Light to Dark transfer
MAPK	Mitogen-Activated Protein Kinase
MEK	MAPK/ERK Kinase
Mesor	Midline estimating statistic of rhythm
mTOR	mammalian Target Of Rapamycin
NIH3T3	fibroblast cell line
N-RAS	Neuroblastoma RAS
PAS	PER-ARNT-SIM
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PEX	Protein Extraction solution
PER	PERIOD
PI3K	phosphoinositide 3-kinase
PKA	Protein Kinase A
PP1	Phosphatase 1
PP2A	Phosphatase 2A
PP4	Phosphatase 4
PRD-1	PERIOD-1
PRD-4, Chk2	PERIOD-4, Checkpoint kinase 2

QA	Quinic Acid
qRT-PCR	quantitative Real-Time Polymerase Chain Reaction
RAS	Rat Sarcoma virus
RAS2P	Rat Sarcoma 2 Protein
RAS GTPases	RAS Guanosine Triphosphatases
RasGEF	RAS Guanine nucleotide Exchange Factor
RCO-1	Regulation of Conidiation 1
RGB-1	Regulatory subunit of protein phosphatase 2A
ROS	Reactive Oxygen Species
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	SDS-Polyacrylamide Gel Electrophoresis
SET-1/2	Histone-lysine N-methyltransferase, H3 lysine-4 specific 1/2
SIM	Single-minded
SOS1/2	Son of Sevenless 1/2
TTFL	Transcription-Translation Feedback Loop
U2OS	human osteosarcoma cell line
WC-1	White Collar-1
WC-2	White Collar-2
WCC	White Collar Complex
wt	*wild type*
Δrasgef	*rasgef* deficient strain

1. Introduction

1.1. Overview

Due to the Earth's rotation, organisms face daily environmental fluctuations such as the predictable cycle of warmer, brighter days and cooler, darker nights. An internal time-measuring system, the circadian clock, helps prepare for the rhythmic external changes and challenges, enhancing the organism's adaptability (1, 2). This clock regulates almost all physiological processes from the cellular to the organismal level (3-10). Moreover, the circadian clock allows the temporal separation of anabolic and catabolic processes and precisely synchronizes metabolic events with daily environmental fluctuations such as light, temperature, or nutrient (11-14). The circadian rhythm is intrinsically generated and therefore can be maintained even without environmental cues, with the so-called free-run or endogenous period, which is around 24 hours in every eukaryote (15-17).

Time measuring by the circadian clock is based on a transcription-translation feedback loop (TTFL), an evolutionarily conserved mechanism across eukaryotes and some prokaryotes (18). Several model organisms are used to study circadian rhythm, including *Neurospora crassa*, which stands out due to its well-characterized circadian system. In humans, disturbances of the rhythm (e.g., shift work and jet lag) elevate the risk of developing various health problems, including cancer, cardiovascular- and metabolic-diseases (19-21). Several factors and mechanisms ensure the circadian clock's accuracy and stability, maintaining the endogenous rhythm despite the environmental noises. Among these mechanisms, metabolic compensation enables the clock to operate relatively stable even when nutrient availability changes drastically, which can otherwise alter the cell’s basal transcription or translation rates (22-26). Although the nutrient compensation mechanisms have also been described in mammalian cells, most detailed insights come from studies on *Neurospora crassa*.

Nutrient compensation is a complex regulatory network involving numerous kinases, phosphatases, polyadenylation factors, and pathways, including RAS2-mediated signalling (22-25). Since mutations in RAS genes are common in various tumour types (27), and it is known that several components of the RAS pathway are affected by or can influence the circadian regulation (22, 28), detailed exploration of this relationship deserves special attention.

Among the mechanisms controlling metabolic compensation in *Neurospora crassa*, we explored the molecular oscillator function under long-term glucose deprivation and the role of a newly characterized RasGEF protein.

1.2. The circadian rhythm

The circadian rhythm is a self-sustained cycle of biological events that persist even in a constant environment and regulate a wide range of biological functions, including metabolic pathways, the cell cycle, DNA repair, and immune functions, underscoring the vital role of the circadian clock in maintaining homeostasis (29-32). The internal timekeeping system is regularly fine-tuned (entrained) to external time by environmental cues, also called Zeitgebers, such as light or temperature (11, 12, 33). In the absence of Zeitgebers, the circadian clock continues to function with an intrinsic, free-run period of approximately 24 hours - about 25 hours in humans and 22 hours in our model organism *Neurospora crassa* (34-37).

Clock mechanisms are evolutionarily conserved (18) and can be observed in all eukaryotes and some prokaryotes. For this reason, many model organisms can be used for circadian research, such as *Cyanobacteria* (38), fungi (e.g., *Neurospora crassa* (39)), plants (e.g., *Arabidopsis* (40)), insects (e.g., *Drosophila* (41)) and various mammals (42). Although the molecular structure of circadian clock components differs across taxa, the basic principle of how biochemical events can generate circadian oscillation is conserved. Circadian timekeeping likely evolved along three independent pathways during evolution: in prokaryotic cyanobacteria, in plants, and in the eukaryotic clade from which fungi and animals later evolved (18).

Several parameters are used to characterize circadian rhythms. The period refers to the length of a complete cycle of the rhythm, 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. The mesor (midline estimating statistic of rhythm) refers to the average value around which a biological rhythm oscillates over a 24-hour period. The amplitude refers to the magnitude of the rhythm, the difference between the peak and the trough, and reflects the strength of the oscillation (Table 1).

Table 1. Key terms and definitions in circadian research

Amplitude	The extent of circadian oscillation. The difference between the oscillation's highest or lowest points.
Compensation	Maintenance of a constant period (clock speed) despite environmental changes.
Entrainment	An external stimulus (such as the day-night cycle) drives the period and phase of the oscillator function.
Free-run	Circadian oscillation in the absence of environmental cues (e.g., the lack of light, in constant darkness).
Input	A pathway through which an environmental cue entrains or resets the clock.
Mesor	Rhythm-adjusted mean. The average value around which the rhythm oscillates.
Output	A biochemical, physiological, or behavioural process affected or regulated by the clock.
Period	The time of one cycle of the rhythm.
Phase	The current state of the oscillation within a period relative to a reference time point.

Despite the diversity in the molecular structures of the clock, model organisms share many common characteristics in their clock functions. We consider an oscillation as a circadian rhythm if (43):

1) The self-sustaining, endogenous oscillators can maintain a ~24-hour period in a constant environment (in the absence of external cues, free-run rhythm);
2) The clock responds to environmental cues (entrained), ensuring the adjustment of the internal clock to the external time of day;
3) The period does not change significantly due to environmental influence, e.g., temperature and nutrient fluctuations ('temperature and metabolic compensation').

The circadian timekeeping system is based on an evolutionarily conserved transcription-translation feedback loop. In *Neurospora*, *Drosophila*, and mammals, heterodimeric transcription factors that contain the PER-ARNT-SIM (PAS; PER: Period protein; ARNT: Aryl hydrocarbon Receptor Nuclear Translocator protein; SIM: Single-minded)

domain act as positive factors to stimulate the expression of negative factors (36, 44). The negative elements inhibit their own expression by reducing the activity of the positive factors (43-45). The cyclic activation and repression of clock components generate a self-sustaining and robust ~24-hour rhythm. This mechanism regulates the expression of numerous genes (*clock-controlled genes*, *ccgs*), which mediate the effect of the clock on various cellular functions (Figure 1). Significance of the rhythm is indicated by the fact that approximately 25-40% of the mammalian genes are under circadian control (46-50).

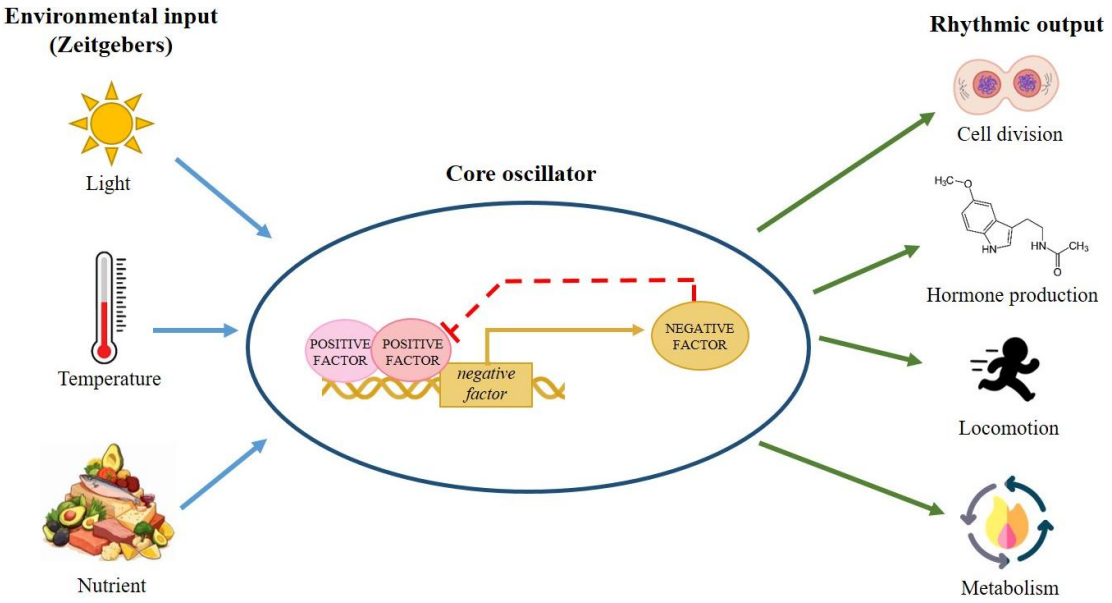

Figure 1. General structure of the transcription-translation feedback loop

Positive regulatory factors assemble into a transcription complex that drives expression of the negative factor(s). The negative factor, upon accumulation inhibits the positive transcription factors, repressing its own expression. Zeitgebers serve as inputs that synchronize the internal clock with the external environment. Numerous molecular processes are governed by the circadian clock.

Disruption of the circadian rhythm (e.g., nocturnal light and irregular eating in shift workers) increases the risk of various metabolic, neurodegenerative, and immunological diseases or cancer incidence (19-21).

1.3. *Neurospora crassa* as a model organism

The filamentous fungus *Neurospora crassa* was first described in a French bakery as an invasive red bread mold. It took over 100 years to begin its journey to become one of the

best-studied model organisms in cell biology research, including the cellular and molecular aspects of chronobiology (51, 52).

Neurospora possesses several qualities that make it an ideal model organism, including short generation time, rapid growth (~3mm/h), endurance, cheap culturing, easy genetic modification, and an already well-characterized circadian clock (52).

Since *Neurospora crassa* was first used as a model organism for genetic studies in the 1920s (53), numerous single- and multiple-mutant strains have been isolated or generated. As a mostly haploid organism in the life cycle, the phenotype of *Neurospora* is immediately visible, thereby simplifying genetic studies. Different *Neurospora* strains have been collected and stored by specialized organizations, such as the Fungal Genetic Stock Center (FGSC), which makes the strains available to the scientific community.

Based on the conserved features of endogenous time measuring, research on the *Neurospora* circadian clock has provided an opportunity to better understand the functioning of molecular oscillators in higher eukaryotic organisms as well (43).

1.4. Circadian rhythm detection in *Neurospora crassa*

Neurospora crassa's asexual spore development (conidiation) is under circadian control, and this rhythmic output can be easily assayed in the laboratory. Regulation of conidiation by the circadian clock provides a fitness advantage to the organism; in *Neurospora*, sporulation typically occurs at night, when spores are less likely to be damaged by harmful UV light (54).

As vegetative hyphae grow across the surface of the agar medium, the clock supports the production of aerial hyphae in the late evening. These aerial structures mature over approximately 12 hours and eventually differentiate into fluffy orange macroconidia. Following conidiation, undifferentiated hyphal cells continue vegetative growth, initiating a new cycle. The pattern of the conidiation band creates a „fossil record" of clock function (54). In *Neurospora*, the period of the conidiation rhythm is ~22-hours in constant darkness (DD) at 25°C and is effectively compensated between 18-30°C. The rhythmic spore formation can be entrained by various light/dark (L/D) and temperature cycles and reset by light or temperature pulses (43).

In *Neurospora*, the conidiation rhythm is more pronounced and therefore, often monitored in strains carrying the *band* (*bd*) mutation, a T79I point mutation in the *ras-1*

gene (28). The *bd* mutation does not alter the core circadian oscillator but affects its output by making the cells insensitive to CO_2 accumulation in race tubes, thereby enhancing the visibility of the rhythmic conidiation pattern.

Conidiation rhythm can be explored in the so-called race tube assays. The race tubes are 30 cm long glass tubes bent 45° upward at both ends and contain a glucose-free agar medium (54) (Figure 2). Conidia are inoculated at one end of the race tube, and the tubes are incubated in constant light (LL) usually at 25°C for ~24 hours to synchronize the circadian clock, then placed into constant darkness. The growth front is marked daily under a red safe light, which does not entrain the clock. We use a software (55) to densitometrically analyse the race tube images. Sine curves are fitted by the program and the period and phase are determined accordingly. Conidiation rhythm can also be explored under entrained conditions, such as light/dark or temperature cycles.

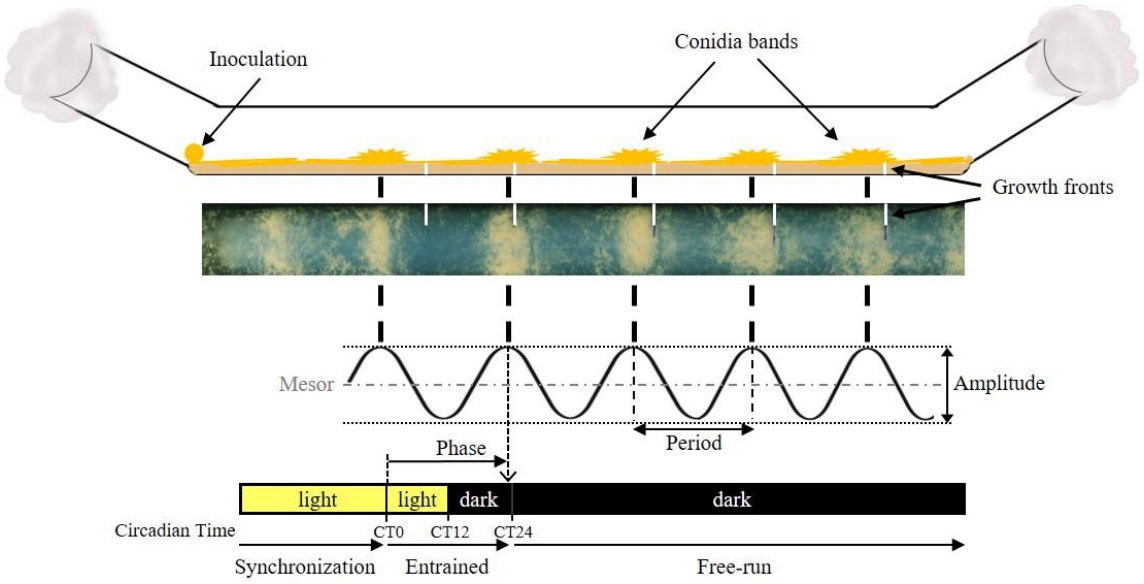

Figure 2. Conidia formation of *Neurospora* is controlled by the circadian clock

The most upper panel shows a schematic race tube indicating the inoculation site, the growth fronts and the conidial bands. Black dashed lines indicate the positions of the corresponding conidial bands on the image of a representative race tube. Middle panel: sine curve fitted on the densitometric data. Period, phase, and amplitude marked. Lower panel: Light-dark phases are indicated: Constant light, followed by 12/12-hour light/dark cycles (entrainment), then free-run rhythm in constant darkness.

Circadian time (CT) enables the comparison of rhythm properties (e.g., phase) across strains and organisms with different endogenous periods. For this purpose, the period is

divided into 24 equal parts, each defined as one circadian hour. CT0 corresponds to the subjective dawn and CT12 to the subjective dusk in free-run conditions, reflecting the organism's internal clock. In contrast, Zeitgeber time (ZT) is defined by external cues, most commonly the light-dark cycle, where ZT0 and ZT12 mark lights on and off, respectively.

1.5. Transcription-translation feedback loop in *Neurospora crassa*

The molecular mechanism of circadian time measuring is evolutionarily highly conserved. The core components of the clock constitute a transcription-translation negative feedback loop, interlocked with additional stabilizing feedback loops.

In *Neurospora,* the positive components, WC-1 (White Collar-1) and WC-2 (White Collar-2) form the heterodimeric White Collar Complex (WCC) through the interaction of their PAS domains (43, 44, 56). PAS domains regulate various physiological and developmental processes in eubacteria, archaebacteria, and eukaryotes, mostly in response to environmental cues (57). In the *Neurospora* oscillator, FRQ (FREQUENCY) is a main component of the negative clock factor. FRQ acts as a scaffold protein in the formation of the FRQ–FRH–CK-1a complex, where FRH (FRQ-interacting RNA helicase) stabilizes and governs the nuclear localization of FRQ, while CK-1a (casein kinase-1a) mediates the progressive phosphorylation of FRQ and other core clock components (58-60). FRQ contains a nuclear localization signal (NLS) enabling its nuclear translocation, as well as two PEST-like motifs (PEST-1 and PEST-2) (61-63). These motifs contribute in different ways to clock regulation: PEST-1-dependent interactions promote FRQ degradation, whereas PEST-2 is involved in the control of WC-1 accumulation (63, 64).

In the transcription-translation feedback loop, the WCC binds to the Clock-box (C-box) on the *frq* promoter, thereby supporting *frq*'s transcription (65). A significant delay is built into the process, causing FRQ protein levels to peak 4-8 hours after the RNA levels (66). FRQ binds to the FRH protein via the FRH-interacting domain (FFD) (67), forming the FRQ-FRH complex (FFC) (68). FFC associates with CK-1a through the FRQ-CK-1a-interacting domains (FCD1-2) of FRQ, and within a few hours enters the nucleus. This complex promotes the phosphorylation of the WCC, thereby inactivating it as a transcription factor (69). The inactivated WCC leaves the nucleus and accumulates in the

cytosol (70, 71) (Figure 3A). FRQ is progressively phosphorylated throughout the day, primarily mediated by CK-1a. After a critical phosphorylation threshold, FRQ's interaction with CK-1a ceases (64, 72, 73), and it associates with F-box/WD-40 repeat-containing protein (FWD1), causing FRQ to be degraded within 14-18 hours via the ubiquitin-proteasome pathway (74). When FRQ levels in the nucleus sufficiently decrease, the WCC is released from the inhibition, and its dephosphorylated active forms enter the nucleus to start a new cycle. In *Neurospora*, WCC activity, *frq* RNA, and FRQ protein levels oscillate over a cycle of approximately 22 hours (64, 72, 73).

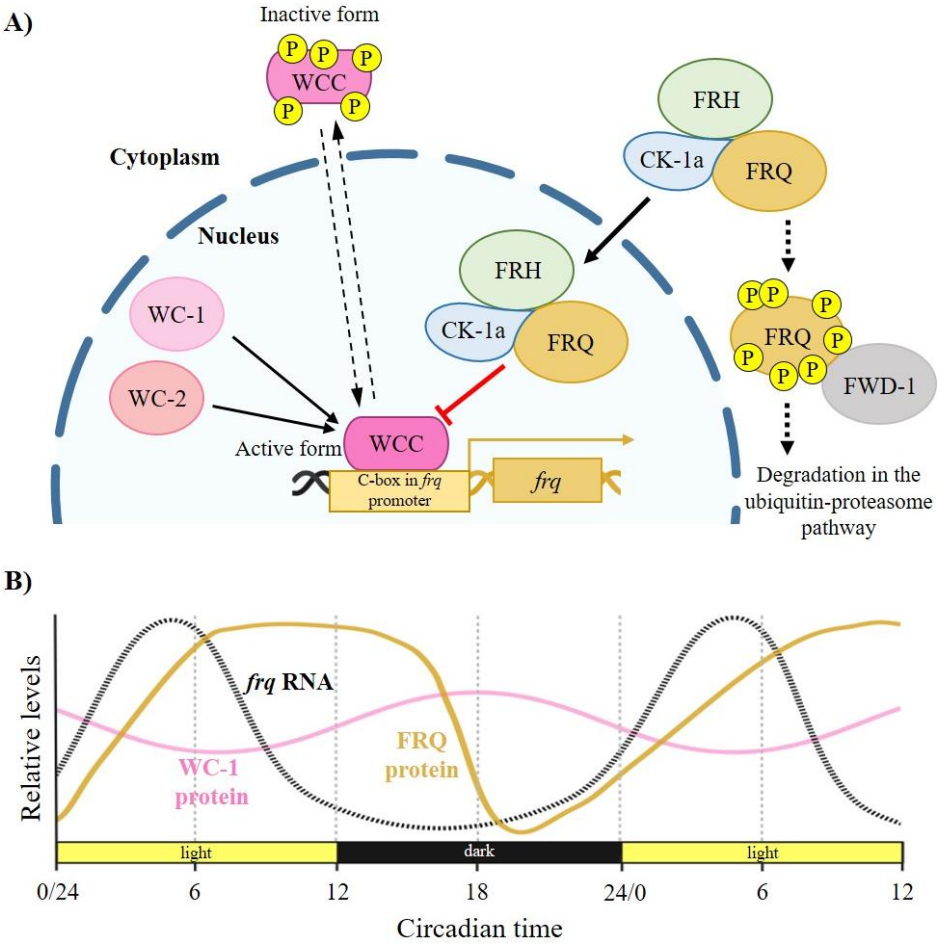

Figure 3. Schematic presentation of the transcription-translation feedback loop and circadian oscillation in *Neurospora crassa*

A) In the cytoplasm, newly synthesized FRQ rapidly binds to FRH, forming the FRQ-FRH complex. This complex interacts with CK-1a and translocates into the nucleus, where promotes phosphorylation of the WCC, leading to its inactivation and dissociation from the *frq* promoter. Phosphorylated WCC accumulates in the cytoplasm, while FRQ undergoes progressive phosphorylation which leads to its inactivation and degradation.

B) *frq* RNA peaks early in the cycle, followed by delayed accumulation of FRQ protein. WC-1 peaks during the subjective night.

In contrast to *frq*, *wc-1* and *wc-2* transcript levels do not exhibit a circadian rhythm. However, WC-1 protein accumulates rhythmically, peaking at subjective night (Figure 3B) (75). The rhythm of WC-1 levels is dependent on post-translational regulatory mechanisms, which, in part, are controlled by FRQ. Thus, FRQ plays a dual role; on the one hand, it represses its own expression (negative feedback), on the other hand, it promotes WC-1 accumulation (positive feedback) (76).

As a blue light receptor, WC-1 is also involved in light detection, which is necessary to entrain the clock to the external light conditions (77, 78). WC-1 has a LOV (Light, Oxygen, and Voltage) domain, which binds a FAD (Flavin Adenine Dinucleotide) molecule as chromophore (78, 79). Upon light exposure, WC-1 undergoes a conformational change that increases its interaction with WC-2. The light-activated WCC binds to consensus GATA sequences in the promoters of blue light-regulated genes, initiating transcriptional responses to environmental light cues (80), for instance in *al-1* and *al-2*, which are crucial for carotenoid biosynthesis (81-83).

The transcription-translation loop can regulate the expression of many *clock-controlled genes (ccgs)* that in turn control various physiological processes. The best-characterized *ccgs* in *Neurospora* are involved in asexual spore formation, such as *ccg-1* and *ccg-2* (84-86). The clock also modulates the expression of genes involved in metabolism, DNA repair and various stress responses (87).

1.5.1. Post-translational regulation of the *Neurospora* clock components

Post-translational modifications, such as phosphorylation and dephosphorylation, are involved in stabilizing the core clock proteins and regulating their activity and subcellular localization.

1.5.1.1. FRQ modification by phosphorylation

FRQ undergoes multiple rounds of phosphorylation, a conserved mechanism also observed in PERIODs (PERs), the negative clock components of mammals and *Drosophila*. Early phosphorylation events promote FRQ nuclear export and ultimately initiate its degradation, while specific phosphorylations, particularly at the C-terminal

region, enhance its stability (64, 88). Generally, hypophosphorylated FRQ is more stable, whereas extensive phosphorylation targets it for efficient degradation (89, 90).

Several kinases are involved in the phosphorylation of FRQ:

- CK-1a and CKII (casein kinase-2) are the central kinases in FRQ phosphorylation. CK-1a is functionally identical to DOUBLETIME (DBT) in *Drosophila* (91-93) and CKIδ/CKIε in mammals (94, 95), supporting the conserved nature of the circadian clock (96);
- PRD-4 (checkpoint kinase 2, ortholog of the mammalian Chk2) phosphorylates FRQ in response to DNA damage (97, 98);
- CAMK-1 (calmodulin kinase-1) phosphorylates FRQ to regulate light entrainment (99-102);
- PKA (protein kinase A) stabilizes FRQ by phosphorylation, possibly by inhibiting ubiquitination (103) and is involved in the regulation of WCC-independent *frq* transcription (104).

1.5.1.2. Phosphorylation-dependent control of the White Collar Complex

- CK-1a in complex with FFC phosphorylates the WCC, thereby closing the negative feedback loop (60, 105);
- PKA is also able to phosphorylate the WCC, further inhibiting its DNA-binding ability and preventing its nuclear translocation (106);
- PKC phosphorylates WC-1, presumably reducing its stability and activity (107, 108);
- GSK (glycogen synthase kinase) binds to the WCC and phosphorylates both subunits, thereby promoting their degradation (109).

The phosphorylation state of the WCC is different under light and dark conditions. In darkness, a significant fraction of the WCC is in a hypophosphorylated, transcriptionally active form that promotes the expression of *frq* and other *ccgs*. The active form is relatively unstable and is degraded more rapidly (71, 110). Exposure to light leads to the formation a larger and transcriptionally very active WCC which becomes phosphorylated at additional sites (111).

1.5.1.3. The role of phosphatases in the circadian regulation

Phosphatases counterbalance the activity of kinases, modulating the phosphorylation states of both FRQ and WCC, thereby influencing the dynamics of the circadian clock. They also show conserved functions across species, e.g., in *Drosophila* and mammals (37, 112-119). In *Neurospora crassa*, the following phosphatases are involved in clock regulation:

- PP1 (phosphatase 1) stabilizes FRQ (120);
- PP2A (phosphatase 2A) is the main phosphatase dephosphorylating the WCC, and thereby reactivating it for a new circadian cycle (37); it also opposes the action of casein kinase II (CKII) by dephosphorylating FRQ, thereby delaying the completion of the negative feedback loop (120);
- PP4 (phosphatase 4) dephosphorylates both FRQ and WCC, allowing nuclear WCC entry (106).

1.6. Interactions between RAS-mediated signalling and the circadian clock

In humans, the RAS (Rat Sarcoma virus) superfamily of small GTPases (small G-proteins) contains about 160 members, divided into five families (RAS, RHO, RAB, ARF, and RAN). Within the RAS family, the RAS guanosine triphosphatases (RAS GTPases) subfamily contains three classical isoforms in humans: H-RAS, K-RAS (variants 4A and 4B), and N-RAS (121-123). These RAS proteins act as molecular switches, cycling between an inactive Guanosine Diphosphate- (GDP) and an active Guanosine Triphosphate (GTP)-bound state and regulate key processes such as proliferation, survival, and metabolism (124).

Their activity is tightly controlled by guanine nucleotide exchange factors (GEFs), which activate RAS by promoting GDP-GTP exchange, and GTPase-activating proteins (GAPs), which accelerate inactivation. Among GEFs, SOS1 (Son of Sevenless 1) and SOS2 are the best studied and are essential for cellular homeostasis (125). Unfortunately, mutations in RAS genes or their regulators are among the most frequent drivers of tumorigenesis, highlighting their significance (27).

Once activated by GTP, RAS can initiate multiple downstream cascades that are highly conserved across eukaryotes. The RAF-MEK-ERK pathway is a major effector branch:

once phosphorylated, ERK (Extracellular signal-Regulated Kinase) translocates to the nucleus to regulate transcription factors and thereby cell cycle and growth (126). A second branch is the PI3K (phosphoinositide 3-kinase)-AKT-mTOR (mammalian Target Of Rapamycin) pathway, which links RAS activity to protein synthesis and metabolism. Beyond these canonical pathways, RAS interacts with a variety of other effectors, adding further layers of regulatory potential (126, 127).

Increasing evidence suggests that circadian rhythms and the RAS signalling pathway are closely linked. In *Drosophila*, RAS signalling pathway regulates the activity of the positive clock factors, the CLOCK/CYCLE transcriptional complex (128). Moreover, mutations in the *neurofibromatosis-1* affect a downstream member of the RAS pathway, the MAPK (Mitogen-Activated Protein Kinase), which alters the oscillation of clock genes, and the circadian rhythm of locomotor activity (129). In *Neurospora crassa*, a T79I point mutation in the *ras-1* gene (*bd* mutation) enhances one of the circadian rhythmic outputs, leading to a robust conidiation rhythm (28). This strain is therefore a widely used tool in chronobiology research. Additionally, RAS2 affects the phosphorylation state of FRQ and is involved in nutrient compensation (22). In vertebrates, circadian regulation of RAS signalling has also been reported. In the chicken pineal gland, the MAPK has been shown to exhibit diurnal oscillations in its tyrosine phosphorylation and enzymatic activity (130). Similarly, in the murine suprachiasmatic nucleus, RAS can affect the period of the clock via influencing the activity of ERK and glycogen synthase kinase 3β (131). Furthermore, the dexamethasone-induced RAS protein 1 (DEXRAS1) is likely an input signal of the clock (132-134). RAS activation, MEK (MAPK/ERK kinase) and ERK phosphorylation in the mouse liver have also been shown to be under circadian control (135).

1.7. Compensation mechanisms stabilize the circadian period against changes in the environmental temperature and nutrient levels

Organisms constantly interact with their environment and change their physiology to adapt to changes in their surroundings efficiently. A key feature of the circadian clock is, however, its ability to buffer or maintain the nearly stable period against external changes such as temperature fluctuation and nutrient availability. This ability of circadian

oscillators is called compensation is critical for precise timekeeping in a natural environment.

Temperature compensation has been a long-researched area in chronobiology, as temperature generally increases the speed of biochemical processes (26, 136), which could speed up the operation of the molecular oscillator, and thereby shorten the circadian period. In various model organisms, such as *Neurospora crassa*, *Drosophila melanogaster* and mammalian cell lines, temperature compensation is suggested to be based on a series of phosphorylations, with the enzyme casein kinase-1 playing a prominent role (137-139).

As mentioned in the Introduction, nutrient supply is a crucial Zeitgeber of the circadian rhythm, allowing the clock to synchronize its operation with the environment (140-144), while, on the other hand, the clock regulates many metabolic pathways (145-149). Nutrient supply can also affect the speed of biochemical processes. This effect can be buffered by metabolic (or nutrient) compensation. Metabolic compensation has been demonstrated from *cyanobacteria* to eukaryotes (150). It was studied at multiple levels in *Neurospora*, its entire mechanism however is still not understood. Metabolic compensation in mammalian cells has been hardly characterized. Matsumura et al. showed that different fetal bovine serum concentrations in the culture media did not significantly affect the expression, amplitude, phase, or period of clock genes in mouse NIH3T3 mouse fibroblasts (151). Similarly, in human U2OS osteosarcoma cells, only minimal period changes were observed at various physiological glucose levels (23). These findings suggest that mammalian circadian clocks are also relatively robust against nutrient fluctuations.

Most information of nutrient compensation comes from *Neurospora crassa* (23), where the circadian period remains robust across a wide range of glucose concentrations. This stability is achieved through regulation at multiple molecular levels, such as transcriptional, post-transcriptional, and post-translational, and involves several key factors, including CSP-1 (Conidial Separation 1), RCO-1 (Regulation of Conidiation 1), PRD-1 (Period-1), SET-1/2 (Histone-lysine N-methyltransferase, H3 lysine-4 specific 1/2), polyadenylation factors, PKA, and the small GTP-binding protein RAS2 (Table 2). CSP-1 is a glucose-induced transcriptional repressor whose expression is directly activated by the WCC. Under high-glucose conditions, *csp-1* transcription rate increases,

and the elevated CSP-1 limits the transcription of *wc-1*, preventing excessive accumulation of the limiting positive clock component which could shorten the period. CSP-1 acts in complex with the corepressors RCO-1 and RCM-1, via binding to the promoters of *ccgs*, including *wc-1*, in a glucose-dependent manner (25, 26, 152, 153).

Although its role partially overlaps with that of CSP-1, RCO-1 also functions as a global transcriptional repressor that, besides regulating other cellular processes, modulates the amplitude and period of *frq* RNA and FRQ protein levels by repressing WCC-independent *frq* transcription (154). When RCO-1 function is compromised, *frq* can also be expressed even via WCC-independent mechanisms, mainly by relieving of repression by chromatin regulators (155, 156) or activation by alternative pathways such as PKA (104), which enable *frq* expression even when WCC activity is low.

PRD-1 encodes a highly conserved ATP-dependent RNA helicase that indirectly influences the *Neurospora* circadian clock. This post-transcriptional modifier and its orthologs regulate mRNA processing and stability in many organisms (157, 158). While *wild type* strains maintain stable circadian periods across varying glucose levels, *prd-1* mutants have longer periods as carbon availability increases. Further analysis revealed that PRD-1 localization is sensitive to glucose; it is mainly in the nucleus under high-glucose conditions, and becomes degraded under starvation, linking its function to nutrient availability (159). This suggests that PRD-1 acts during nutrient-rich conditions to adjust processing and stability of clock transcripts as a post-transcriptional „brake" (159).

Polyadenylation factors emerged as key regulators in a genetic screen for mutants with impaired metabolic compensation, where mutations led to period shortening under high nutrient levels, indicating that proper poly(A) tail processing is essential for stable rhythmicity. Beyond polyadenylation, nonsense-mediated mRNA decay (NMD) regulates period length primarily through controlling *ck-1a* expression, which contains an unusually long 3′ UTR. Loss of NMD components (*upf1* or *upf2*) elevates *ck-1a* levels, resulting in period shortening under nutrient-rich conditions. NMD mutants also show increased *wc-2* and reduced *frh* expression, while *frh* itself is strongly downregulated during glucose starvation, suggesting direct nutrient control of both arms of the clock (23, 25).

Alternative polyadenylation (APA) adds another layer of post-transcriptional regulation that may support metabolic compensation. Core clock genes such as *ck-1a*, *frq*, *wc-2*, *rco-1*, and *csp-1* affect APA events, potentially influencing mRNA stability and circadian period. Intact APA regulation helps stabilize the expression of clock components under nutrient stress, and thus maintains circadian period despite metabolic fluctuations.

Chromatin modifiers also play crucial roles. Two SET-domain methyltransferases, SET-1 and SET-2, directly regulate *frq* expression. SET-1 represses *frq* via H3K4 methylation through the COMPASS complex, while SET-2 prevents improper WCC-independent *frq* transcription by maintaining H3K36 methylation (23, 24, 154, 155). Deletion of SET-2 results in *frh* downregulation and a short period under starvation, linking chromatin regulation to helicase-mediated feedback control (23, 154, 155).

Loss of *pkac-1*, which encodes the catalytic subunit of PKA, subtly alters compensation through nutrient-dependent phosphorylation, while deletion of *rbg-28*, a ribosome biogenesis factor, causes complete arrhythmicity at high nutrient concentrations, implicating translation in nutritional compensation.

The RAS2 pathway is also crucial in coupling nutrient availability to circadian clock function in *Neurospora crassa*. Deletion of *ras2* prolongs the circadian period and dampens rhythm amplitude, particularly under glucose-rich conditions, indicating impaired metabolic compensation. In *Δras2*, FRQ protein levels are reduced, phosphorylation is delayed, and nuclear accumulation of hypophosphorylated FRQ increases, disrupting proper negative feedback regulation. These defects are glucose-dependent, suggesting a specific role of RAS2 in nutrient sensing. While RAS2 does not influence MAPK activation, it interacts with adenylate cyclase, and pharmacological activation of the cAMP pathway partially restores clock function in the mutant. These findings demonstrate that RAS2, via cAMP signalling, is essential for maintaining the circadian period across metabolic states (22).

Table 2. Factors involved in metabolic compensation of the *Neurospora* circadian clock

Level of regulation	**Factor(s)**	**Main function in metabolic compensation**
Transcriptional regulators	CSP-1, RCO-1, RCM-1	Glucose-induced repressors, it prevents the overaccumulation of WC-1 and maintains rhythmic *frq* expression
Chromatin modifiers	SET-1, SET-2,	Histone methyltransferases, repress improper *frq* transcription
Translational regulators	RGB-28	Ribosome biogenesis, required for rhythmicity under high nutrient
Post-transcriptional regulators	PRD-1, polyadenylation factors, NMD, APA	RNA stability and processing, regulation of *ck-1a*, *frh* and *wc-2*
Signalling regulators	PKA, RAS2	Glucose sensing, maintains period across metabolic states

Mechanisms of metabolic compensation characterized in *Neurospora* have functional parallels in mammalian systems, therefore the results from *Neurospora* may provide a basis for understanding this process in higher eukaryotes (25).

2. Objectives

Our aim was to study the mechanism of metabolic compensation of the circadian clock in the model organism *Neurospora crassa*, with a particular focus on clock's adaptation to starvation and the role of RAS signalling. Accordingly, the two main lines of investigation are outlined below:

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. 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

3.1. *Neurospora* strains

The following *Neurospora crassa* strains were obtained from FGSC (160): *wt* (FGSC #2489), *wt,bd* (FGSC #1858) used as *wild type* strains, *mcb* (FGSC #7094), and *Δrasgef* (FGSC #11866). FGSC #11866 and FGSC #7094 were created during the *Neurospora* Genome Project (161). Prof. Michael Brunner provided the *qa-gsk* (109) strain.

My colleagues generated *histidine biosynthesis gene 3 (his3), Δrasgef* and *his3, Δrasgef, bd* by crossing *Δrasgef* with *wt, his3* (FGSC #6103) or *wt, bd his3*. To generate $rasgef_{Flag}$ and $rasgef_{Flag}$*, bd*, the *pMF-ccg1-2xFlag-rasgef* plasmid was used for transformation of *his3, Δrasgef* and *his3, Δrasgef, bd*, respectively (162). All crosses were performed according to the protocol on the FGSC website.

3.2. Plasmid construction

Wild type (FGSC #2489) cDNA served as template in the Polymerase Chain Reactions (PCR). PCR was performed with a Bio-Rad T100 Thermal Cycler. The Phusion High-Fidelity PCR Kit (Thermo Fisher Scientific #F553L) was used according to the manufacturer's instructions. The PCR reaction mixture was purified using the Promega Wizard SV Gel and PCR Clean-Up System kit (Promega #A9281) according to the manufacturer's instructions. Digestions with restriction endonucleases were performed according to the recommendations (Thermo Fisher Scientific).

To create the *pMF-ccg1-2xFlag-rasgef* plasmid, where two *flag-tags* are linked at the N-terminus, we amplified the *rasgef* coding sequence from the cDNA using the following primers (*rasgef*-specific regions are underlined):

Forward primer: 5'- AAAAAGGCGCGCCGCATCGCAGAGTAGCCGAC-3'

Reverse primer: 5'-AAAAACCCGGGCTAGACTTGAGCAGAGGTAGGCA-3'

The resulting *rasgef* PCR product and the *pMF-ccg1-2xFlag-frh* (163) vector were digested with *SgsI* (#ER1891) and *CfrI* (#ER0172) enzymes, and the *frh* sequence was replaced with *rasgef*.

To generate *pGEX4-ras2p*, where *ras2p* is N-terminally tagged with a *gst-tag*, we amplified the *ras2p* coding sequence using the following primers:

Forward primer: 5'-AAAAGGATCCGACCTACCTACCTCCAATATC-3'

Reverse primer: 5'-AAAAGCGGCCGCGGCTGTCGGATGCGAGAAG-3'

The generated *ras2p* PCR product and the *pGEX4T1* (Cytiva #27-1542-01) vector were digested with *EcoRI* (#ER0271) and *NotI* (#ER0592) enzymes.

The *pBM60-ClaI-ΔFCD1-2* plasmid (73) was a gift of Prof. Michael Brunner.

For ligation, T4 DNA Ligase (Thermo Fisher Scientific #EL0011) was used as described in the manufacturer's instructions. The ligates were heat-shock transformed into competent *Escherichia coli ⊿H5α* cells (#EC0112) according to the ThermoFisher Scientific protocol and selected using Luria-Bertani (LB) (164) solid medium containing 50 mg/mL ampicillin. Picked colonies were incubated in LB medium with ampicillin overnight at 37°C. Plasmid DNAs were isolated using the Thermo Scientific GeneJET Plasmid Miniprep Kit (#K0503) as described in the instructions. The constructs were mapped by restriction enzyme digestions and agarose gel electrophoresis. Finally, the plasmids were sent to Microsynth AG for Sanger sequencing.

The selected plasmids were introduced into the corresponding *his3* deletion strain of *Neurospora* by electroporation using the Bio-Rad Gene Pulser, following the FGSC protocol (160). If the sequence was inserted into the locus, the strains were grown in the absence of histidine, enabling the selection of successfully transformed strains. Expression of the corresponding proteins was later confirmed by western blot and race tube analysis.

To generate the *frqΔfcd1-2* strain, the *pBM60-ClaI-ΔFCD1-2* plasmid (73) from Prof. Michael Brunner was used to transform *bd, frq10 his-3* (162).

3.3. *Neurospora* culture conditions

Strains were maintained by growing on solid slant medium containing Vogel's medium (165) supplemented with 50 ng/ml biotin, 2% agar, and 2% glucose.

The standard liquid medium contained Vogel's medium with 10 ng/ml biotin, 0.5% L-arginine, and 2% glucose. Under low-glucose condition ("starvation"), the glucose concentration was reduced to 0.01%.

A mycelium mat was grown in Petri dishes in standard liquid medium in constant darkness at room temperature. For further culturing, equal-sized mycelial mats were punched and grown in standard liquid medium for 24 hours at 25°C, constant light, and under continuous shaking at 90 rpm. Cultures were grown in at least of 150 ml of liquid medium per mycelium ball to keep the incubation conditions as constant as possible. The

mycelium balls were washed with distilled water before changing the medium. Mycelia were harvested by filtering, frozen in liquid nitrogen and stored in a -80°C freezer until use.

3.4. Race tube assay

The minimal race tube medium contained Vogel's medium supplemented with 0.17% arginine, 3.2% agar, and 205 nM biotin. Glucose was omitted. Race tubes were inoculated with conidia and incubated at 25°C under constant light for 24 hours to synchronize the cells. Additional experimental settings will be indicated later. The circadian parameters were analysed using the ChronOSX 2.0.3.4 software (55, 166). To check clock function under entrained conditions, the first 1-2 days of the experiment were excluded from the analysis.

3.5. RNA analysis

The samples were homogenized under liquid nitrogen in a mortar, and RNA was isolated using the TriReagent (Sigma Aldrich #93289) isolation reagent according to Chomczynski's protocols (167, 168). The RNA concentration and quality of the samples were determined using the Nanodrop One device, and cDNA was synthesized using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific #K1622) according to the manufacturer's instructions.

Quantitative real-time PCR (qRT-PCR) was performed using a LightCycler480 instrument and TaqMan probes. Roche LightCycler® 480 Probes Master was used and supplemented with 1.2 µM forward and reverse primers, 0.33 µM probe, and 20 ng cDNA. The primers and probes used during the experiments are listed in Table 3.

Table 3. Oligonucleotide sequences used in qRT-PCR measurements (5'-3')

Gene name	Forward primer	Reverse primer	Probe
al-2	ACCTGGCCAATT CGCTCTTT	GACAGAAGGAGTAC AGCAGGATCA	[6-FAM] CTGGTCGACTCCGCATT [TAMRA]

ccg-2	GCTGCGTTGTCG GTGTCAT	GGAGTTGCCGGTGTT GGTAA	[6-FAM] AATGTGGTGCCAGCGTCAA GTGCTG [TAMRA]
frq	TTGTAATGAAAG GTGTCCGAAGGT	GGAGGAAGAAGCGG AAAACA	[FAM] ACCTCCCAATCTCCGAACT CGCCTG [TAMRA]
gna-3	ATATCCTCACTTG ACACAAGCC	CGGAGTCTTTAAGGG CGTTATT	[6-FAM] TCCAACATCCGTCTCGTGT TTGCT [TAMRA]
rasgef	GCCGACGTCATC AGAAACAA	GTTGGTAGGCGGGAG ACAAA	[6-FAM] CAAGGCCCCCACAGACGGC C [TAMRA]
wc-1	ACCTCGCTGTCCT CGATTTG	TGCTGGGCCTCTTTC AACTC	[6-FAM] CCGTCCGACATCGTGCCGG [TAMRA]

Using the LightCycler Relative Quantification Software, we determined the C_t (threshold cycles) values by calculating the maximum of the second derivative. During the relative quantification of the expression, we compared the examined gene expression to the level of the housekeeping gene, *G protein alpha subunit-3* (*gna-3*).

3.6. Protein analysis

The protein extraction (PEX) solution contained: 50 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 137 mM NaCl, 5 mM ethylenediaminetetraacetic acid (EDTA), 1.4 mM glycerol, 1 mM Ser-protease inhibitor (PMSF), 12 nM Cys-, Ser-, Thr-protease inhibitor (Leupeptin), 15 nM aspartyl-protease inhibitor (Pepstatin) and phosphatase inhibitor cocktail (PhosStop, Roche #4906845001) used at 1x final concentration according to the manufacturer's instructions. The PEX solution was added to the homogenized mycelium, incubated, and removed by multiple centrifugations of cell debris as described earlier (55, 64). The concentration and quality of the protein extract were determined using a NanoDrop One spectrophotometer at 280 nm.

For subcellular fractionation, the density gradient method described by Luo et al. was used with some modifications (169). The buffer volumes were reduced to 1/10, and the

centrifugation parameters for nucleus and cytosol separation were modified to 8800 g, 2 min, and 4°C.

For λ-phosphatase (CIP) treatment (2 U/mg protein), none of the phosphatase inhibitors was added to the PEX solution, and the samples were incubated with the enzyme for 60 minutes at 30°C.

The protein extracts were heated with SDS-containing reducing buffer (146 mM sodium dodecyl sulfate (SDS), 2.88 M glycerol, 1.88 M β-mercaptoethanol, 105 mM Tris (pH 6.8), and 3.14 μM bromophenol blue) at 95°C for 10 min and proteins were separated based on their molecular weight using SDS-PAGE (SDS-polyacrylamide gel electrophoresis). For immunodetection of the proteins, a semi-dry blot technique was used. Ponceau S staining served as a loading control. The list of primary and secondary antibodies is shown in Table 4. Incubations with any of the listed antibodies was carried out either for 2 hours at room temperature or overnight at 4°C. Detection was performed using the enhanced chemiluminescence (ECL) method (170) and X-ray film.

Table 4. Primary and secondary antibodies for western blot

Protein	Primary antibody	Secondary antibody
FRQ	anti-FRQ (mouse, monoclonal) 1:10	Goat Anti-Mouse IgG (H + L)-HRP Conjugate (Bio-Rad #1706516) 1:4000
WC-1	anti-WC-1 (rabbit, polyclonal) 1:500	Goat Anti-Rabbit IgG (H + L)-HRP Conjugate (Bio-Rad #1706515) 1:4000
WC-2	anti-WC-2 (rabbit, polyclonal) 1:500	Goat Anti-Rabbit IgG (H + L)-HRP Conjugate (Bio-Rad #1706515) 1:4000
FLAG	anti-FLAG (mouse, monoclonal, Sigma Aldrich #F3165) 1:5000	Goat Anti-Mouse IgG (H + L)-HRP Conjugate (Bio-Rad #1706516) 1:4000
GST	anti-Glutathione-S-Transferase (GST) antibody (rabbit, polyclonal, Sigma Aldrich #G7781)	Goat Anti-Rabbit IgG (H + L)-HRP Conjugate (Bio-Rad #1706515) 1:4000
RGB-1	anti-RGB-1 (rabbit, polyclonal) 1:5000	Goat Anti-Rabbit IgG (H + L)-HRP Conjugate (Bio-Rad #1706515) 1:4000

3.7. cAMP measurement

Neurospora crassa cell lysate was also used for cAMP measurement, following the manufacturer's instructions (cAMP-Screen Direct™ Cyclic AMP Immunoassay System, Applied Biosystems, Waltham, Massachusetts, United States).

3.8. Investigation of the interaction between RasGEF and GST-RAS2P

To study the interaction between Rat sarcoma 2 protein (RAS2P) and RasGEF, *pGEX4* and *pGEX4-ras2p* plasmids were transformed into Rosetta™(DE3) competent cells (Sigma-Aldrich #70954) according to the manufacturer's instructions. Expression of the fusion protein was induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside for 2 h at 37°C with shaking, followed by lowering the temperature to 15°C for overnight incubation. The cells were centrifuged at 4500g for 15 min at 4°C, and the supernatant was removed. The cell pellet was resuspended in lysis buffer (Sigma-Aldrich #20-188) and sonicated on ice at 25% power for 45 s three times (Bandelin Sonoplus HD 2070 homogeniser). The samples were centrifuged at 6000g for 15 min at 4°C, and the supernatants were used. GST or GST-RAS2P proteins were bound to glutathione-agarose beads (Sigma-Aldrich #G4510). The beads were washed with Phosphate Buffered Saline (PBS) (Sigma-Aldrich #D8537) and incubated with 20 mg of $rasgef_{Flag}$ cell lysate in 1.5 mL at 4°C for 1.5 hours with continuous agitation, then washed 3x with PBS and eluted with SDS-containing reducing buffer. The interaction was detected using an anti-FLAG antibody with western blot.

3.9. Analysis

The ChronOSX 2.0.3.4 software (developed by T. Roenneberg) was used to evaluate the race tube experiments. Statistical evaluations were performed using Statistica 13 (Statsoft Inc., Tulsa, Oklahoma, United States), and cosinor analyses were performed using R 4.3.3 and R Studio (version 2024.04.2+764). Data were plotted using GraphPad Prism 8.0 software. During qRT-PCR, Ct values were calculated using LightCycler Relative Quantification Software.

Effects were considered significant if the p-value was less than 0.05. Significance levels are indicated as follows: *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$.) Error bars indicate ±SEM.

4. Results

In line with the aims presented in section 2, my research work focused on two main questions. Accordingly, the Results chapter is divided into two main sections. First, we examined how long-term glucose deprivation affects clock function (Chapter 4.1.), then we studied the role of the RAS signalling pathway in the nutrient compensation of the circadian clock (Chapter 4.2.).

4.1. Long-term glucose deprivation affects the circadian clock

Previous studies of our workgroup showed that long-term glucose withdrawal leads to molecular rearrangements in the *Neurospora* circadian clock (171). The levels of the positive clock components, WC-1 and WC-2, decreased to 15-20% of baseline within a few hours following glucose deprivation. In contrast, levels of the negative clock component, FRQ, remained nearly constant, but the protein became hyperphosphorylated (171).

Despite these changes, robust circadian oscillations of *frq* RNA and FRQ protein were observed under different carbon-supply conditions. Furthermore, no significant difference in *frq* and *wc-1* transcript levels was detected between standard and starvation conditions. This result pointed to a compensatory mechanism that either stabilizes *frq* RNA or maintains the active pool of the WCC at a "normal" level under nutrient-limited conditions (171).

We addressed *frq* RNA stability. Importantly, *frq* transcription stops after light to dark (L/D) transition, thus, changes in *frq* levels are proportional to the rate of RNA decay (63, 172). We measured *frq* levels following a L/D transfer in both media (Figure 4). We found that the kinetics of *frq* RNA changes after the transfer were similar under both conditions, indicating that *frq* RNA stability is not dependent on glucose content of the medium.

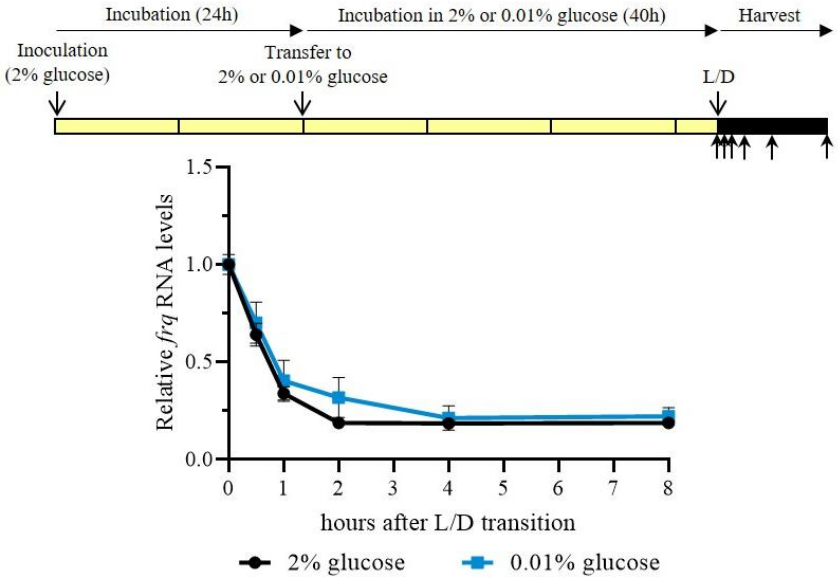

Figure 4. Stability of *frq* RNA is not affected by glucose availability

The upper panel shows the schematic outline of the experiment design. After incubating the *wt* strain in standard liquid medium for 24 h, mycelia were transferred to standard (black line) or starvation medium (blue line) in LL. Then 40 hours of incubation, cultures were transferred to DD, marking time point 0. Samples were collected at the specified time points thereafter. RNA levels were normalized to time point 0 for each condition (n = 6, mean ± SEM, one-phase decay analysis, n.s.).

WCC also acts as a photoreceptor, and its light-responsive activity is known to differ between an adapted steady-state and in the initial phase of the light response. Upon light exposure, a large fraction of WCC rapidly becomes activated, triggering a transient but pronounced induction of light-sensitive genes. To assess whether this light-activatable pool of WCC is affected by nutrient availability, we examined the light response of *frq*, *wc-1*, and *al-2* (70, 173) under both standard and starvation conditions (Figure 5).

For all three genes, the initial increase in RNA levels was lower in starved than non-starved cultures, while the "steady state" levels after light adaptation were similar under both conditions (Figure 5). The difference in the kinetics of light induction suggests that the light-inducible pool or the photoreceptor function of WCC is reduced upon glucose deprivation.

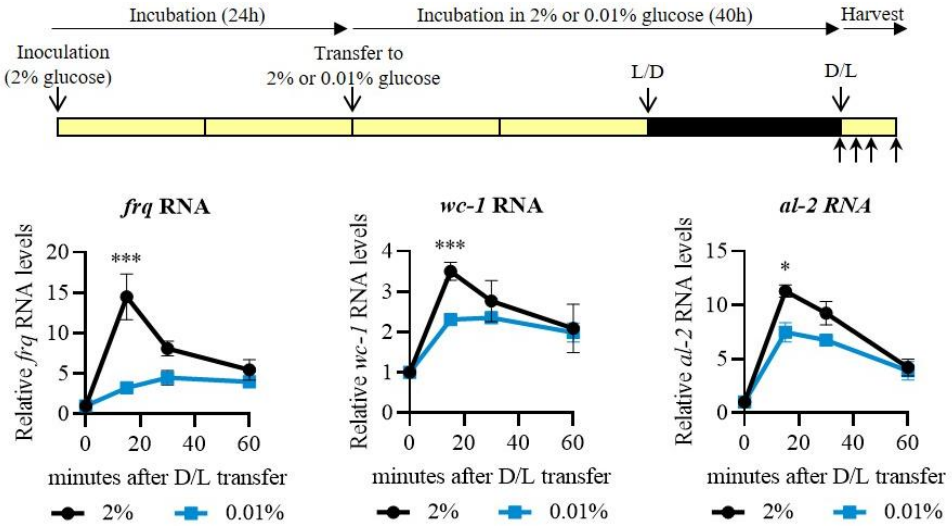

Figure 5. The light-induced activity of the WCC is reduced upon glucose deprivation

The upper panel shows the schematic outline of the experiment design. After incubating the *wt* strain in standard liquid medium for 24 h, mycelia were transferred to standard or starvation medium in LL. Following a 24-hour incubation, the cultures were transferred to constant darkness for 16 h and light-induced. Samples were collected at the indicated time points after the dark/light transfer. Relative RNA levels were normalized to those measured at time point 0 (n = 5-11, mean ± SEM, repeated measurement ANOVA with significant time*treatment interaction, followed by Tukey's HSD test).

4.1.1. Modulators involved in metabolic compensation of the molecular clock in *Neurospora crassa*

Our particular aim was to uncover modulators and signalling routes contributing to glucose-dependent modifications of the circadian clock and the regulation of nutrient compensation.

As mentioned in the Introduction section, CK-1a is a key kinase affecting the function of the core clock proteins. CK-1a is associated with the FRQ-FRH complex through the FCD1 and FCD2 (73, 105) domains of FRQ. This complex can phosphorylate WCC and thereby inhibit its activity. Moreover, CK-1a phosphorylates FRQ, leading to changes in both FRQ's function and stability. To investigate the glucose-dependent effect of CK-1a on clock components, we used the *frqΔFCD1-2* strain (73), in which a mutation in the FCD1-2 interaction domain of FRQ prevents its binding to CK-1a (73) (Figure 6).

frqΔFCD1-2 displayed an electrophoretic mobility shift upon glucose withdrawal, suggesting that starvation-induced phosphorylation of FRQ is not dependent on its interaction with CK-1a. The difference in WC levels between starved and non-starved cultures was moderate compared to *wt* (Figure 6A). *frqΔFCD1-2* RNA levels were low under standard conditions and remained compensated upon glucose deprivation (Figure 6B). Our data suggest that stable recruitment of CK-1a to FRQ is not essential for the starvation-dependent hyperphosphorylation of FRQ and the compensation of *frq* RNA, but it slightly affects glucose-dependent changes of the WC levels.

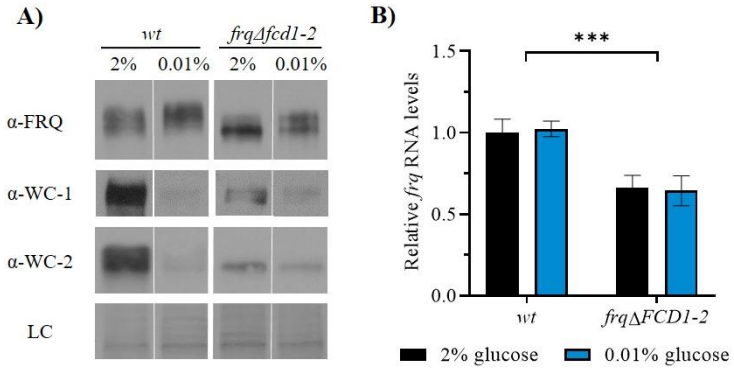

Figure 6. Impaired FRQ-CK-1a interaction affects the WCC level in starvation

A) Mycelial discs of the indicated strains were incubated in standard liquid medium for 24 h in LL, then transferred to standard or starvation medium for 40 h. Ponceau staining is shown as loading controls (LC).

B) RNA levels were normalized to those of the *wt* grown in standard medium (n = 4-5, mean ± SEM, factorial ANOVA with significant strain effect, followed by Tukey's HSD test).

As PKA can modify both FRQ and WC-1 (103, 106) and its activity is dependent on glucose in both *Neurospora* and yeast (174-176), in the following experiments we focused on its possible role in the nutrient compensation of the clock.

In the *mcb* strain (176), one of the regulatory subunits of PKA is mutated, resulting in constitutively high enzymatic activity. Although FRQ remained hyperphosphorylated in the mutant, *frq* RNA increased upon glucose deprivation (Figure 7A and B). In addition, WC-1 and WC-2 levels did not decrease during starvation, also indicating a dominant role for PKA in the metabolic compensation of the clock.

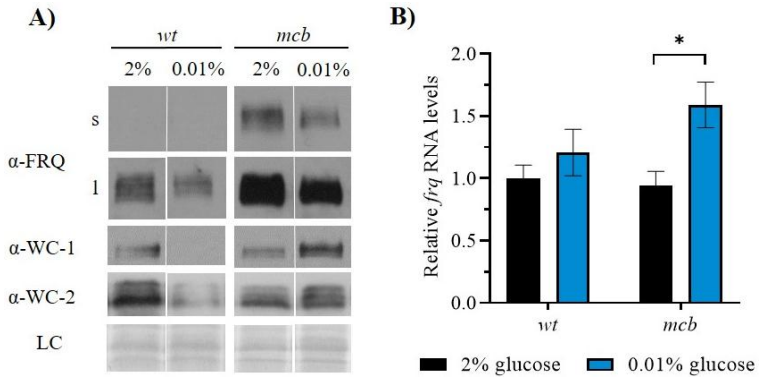

Figure 7. PKA is a key component of glucose adaptation of the clock

A) Experiments, with the indicated strains, were performed as described in Figure 6. s: short exposure, l: long exposure. Ponceau staining is shown as loading controls (LC).
B) RNA levels were normalized to those of the *wt* grown in standard medium (n = 8-9, mean ± SEM, factorial ANOVA with significant treatment effect, followed by Tukey's HSD test).

Glycogen synthase kinase is an important factor of the starvation response in many organisms, including yeast (177), and was shown to fine-tune the circadian period in *Neurospora crassa* (109). As GSK is an essential protein, to reduce GSK expression, we used a *qa-gsk* strain in which the gene expression is driven by the quinic acid (QA)-inducible *qa-2* promoter (109). Experiments were performed without QA, resulting in very low GSK expression. According to previous findings (109), in the presence of 2% glucose, WC-1 and FRQ levels were elevated in *qa-gsk*. The starvation response of the clock was partially affected by GSK depletion (Figure 8A). While WC levels were reduced, even to a higher extent than in *wt*, FRQ did not become hyperphosphorylated and *frq* RNA levels moderately decreased upon starvation in the mutant (Figure 8B). These data suggest that GSK supports the phosphorylation of FRQ during starvation, but this modulation of FRQ is not sufficient to impact WCC levels. Reduced phosphorylation of FRQ in the *qa-gsk* strain may enhance the negative feedback effect at low glucose levels and lead to the slight reduction of *frq* RNA.

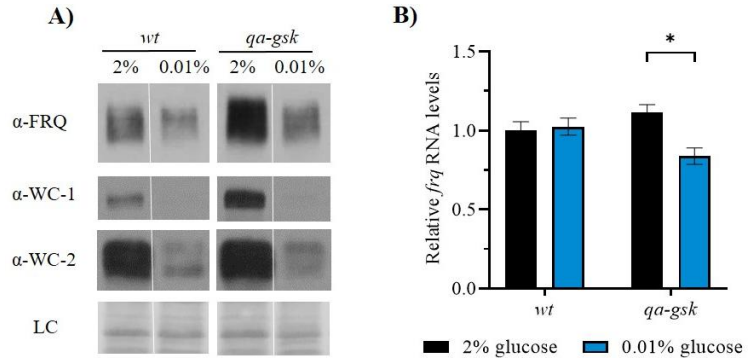

Figure 8. GSK affects both the glucose-dependent phosphorylation of FRQ protein and *frq* RNA level changes upon starvation

A) Experiments, with the indicated strains, were performed as described in Figure 6. The medium was supplemented with 15µM QA during the first 24 h of incubation, then fresh medium was added without QA. Ponceau staining is shown as loading control (LC).
B) RNA levels were normalized to those of the *wt* grown in standard medium (n = 6, mean ± SEM, factorial ANOVA with significant strain*treatment interaction, followed by Tukey's HSD test).

The possible role of PP2A as mediator acting between nutrient level and the circadian clock was also investigated by my colleagues. The results indicated a role for PP2A in the glucose-dependent modifications of the positive clock components (171).
Overall, we found that multiple signalling routes are involved in the glucose-dependent adaptation of the circadian clock function, probably also interacting with each other. A summary of these pathways including a scheme is presented in the Discussion of the thesis (Figure 24).

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 the tight link between RAS signalling and metabolic regulation, we aimed to further examine the possible activity of RAS-pathways in the metabolic compensation of the clock. In this project, we focused on the role of RasGEF activity. *Neurospora* contains four RasGEF domain-encoding genes, two of which (NCU 06500 and NCU 09758) include all conserved domains found in CDC25 (Cell Division Cycle 25) (178), the thoroughly characterized RasGEF of *Saccharomyces cerevisiae* (179),

which plays a central role in glucose sensing. While a deletion strain for NCU 6500 is available only as a heterokaryon, suggesting an essential function of the protein product, a knock-out strain homozygous for the deletion of NCU 09758 (FGSC #11866) was generated during the *Neurospora* Genome project (161). Hence, we focused our investigation on the RasGEF encoded by NCU 09758.

4.2.1. Deletion of RasGEF affects the circadian clock function

First, we examined the conidiation phenotype of the *rasgef* gene-deficient strain (FGSC #11866) and compared it with that of the *wt* (FGSC #2489).

In accordance with literature data, *wt* (FGSC #2489) did not exhibit rhythmic conidiation on minimal medium; however, rhythmicity could be induced by the addition of the ROS (Reactive Oxygen Species) generator menadione (28). *Δrasgef* (FGSC #11866 background) was less sensitive to menadione than *wt* (FGSC #2489 background), indicating a potential influence of RasGEF on the clock output (Figure 9).

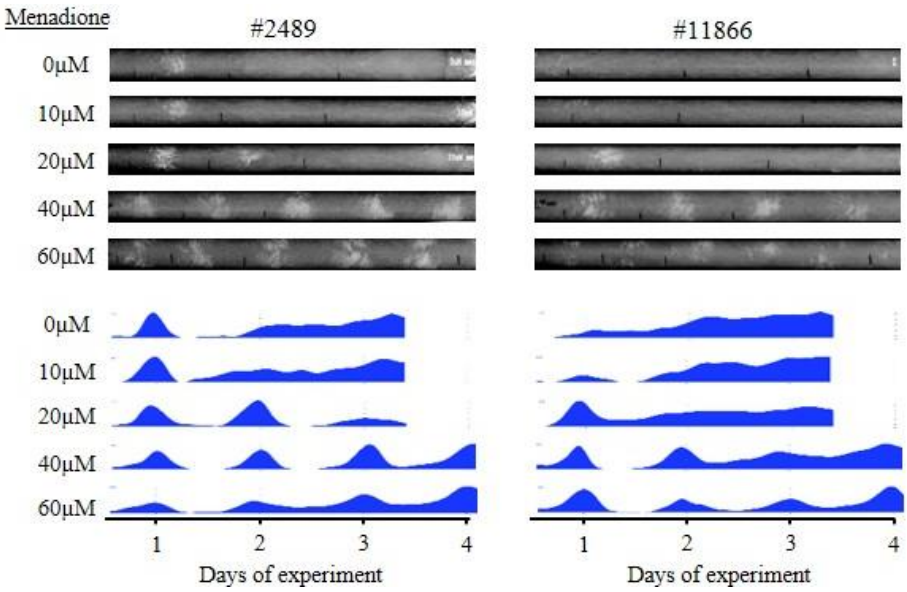

Figure 9. Conidiation is less sensitive to menadione in *Δrasgef* (FGSC #11866) than in *wt* (FGSC #2489)

Race tubes containing different concentrations of menadione were inoculated with the indicated strains. After 2 days of incubation in LL, the race tubes were transferred to DD. Upper panels: Images of representative race tubes. Lower panels: Averaged densitometric curves of race tube duplicates from a representative experiment are shown (n = 4). As

menadione affects growth rate, different numbers of days could be displayed on the densitometric plots.

For better visualization of the conidiation rhythm, the *ras-1* strain (*band*) showing robust rhythm of spore formation even on minimal medium was crossed with *Δrasgef* (FGSC #11866). For convenient reading, in the following sections of the thesis the crossed and the control *bd* strain will be referred to as *Δrasgef* and *wt*, respectively.

To further examine how the absence of RasGEF affects circadian parameters, we followed the conidiation rhythm of *Δrasgef* and *wt* under 12 h light/dark and 22°C/30°C temperature cycles (Figure 10A and B). The gene-deficient strain showed a phase delay under both conditions.

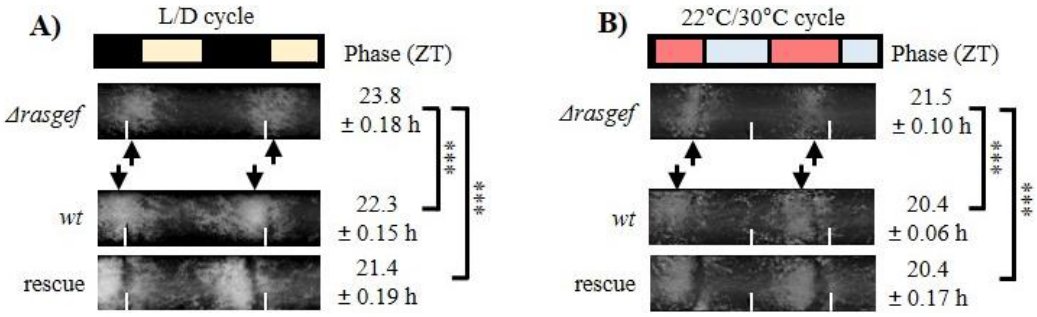

Figure 10. The conidiation phase is delayed in *Δrasgef* compared to *wt* under both 12/12 h L/D and temperature cycles. The phenotype is restored by RasGEF$_{Flag}$ (rescue) expression

A) Race tubes were incubated under 12/12 h L/D cycles without glucose. The white lines refer to the same time points within the panel. For easier comparison of the conidial bands' positions, images were slightly adjusted to ensure similar daily growth distances in the parallel samples. Conidiation peaks are indicated by arrows and given in Zeitgeber time (ZT) (n = 23 (rescue), 66 (*Δrasgef*) and 59 (*wt*); mean ± SEM, one-way ANOVA with significant strain effect for phase).

B) Race tubes were incubated under 22/30 °C temperature cycles without glucose. Conidiation peaks are indicated by arrows and given in Zeitgeber time (ZT) (n = 8; mean ± SEM, one-way ANOVA with significant strain effect for phase).

When examined in constant darkness at different temperatures, *Δrasgef* was more sensitive to low temperatures compared to *wt,* showing a dampened rhythm at 19°C (Figure 11A).

Most biochemical reactions have a temperature coefficient (Q_{10}) of ~2-3, meaning their rates double or triple when the temperature is increased by 10°C (180). Circadian clocks, however, show temperature compensation: their period length changes only minimally across physiological temperature ranges, usually less than ~10% over 10-12°C, corresponding to a Q_{10} of about 1.05-1.10 (137, 181, 182).

In our data, the *wild type* strain behaved as expected, with only minor period changes (Q_{10} ~ 1.08). *Δrasgef*, however, displayed a longer period and a larger change in the period than *wt* between 22 and 30°C, with the most pronounced deviation at lower temperatures, corresponding to a higher Q_{10} (~1.13) (Figure 11B). These findings suggest that RasGEF contributes to stabilization of the circadian period at lower temperatures.

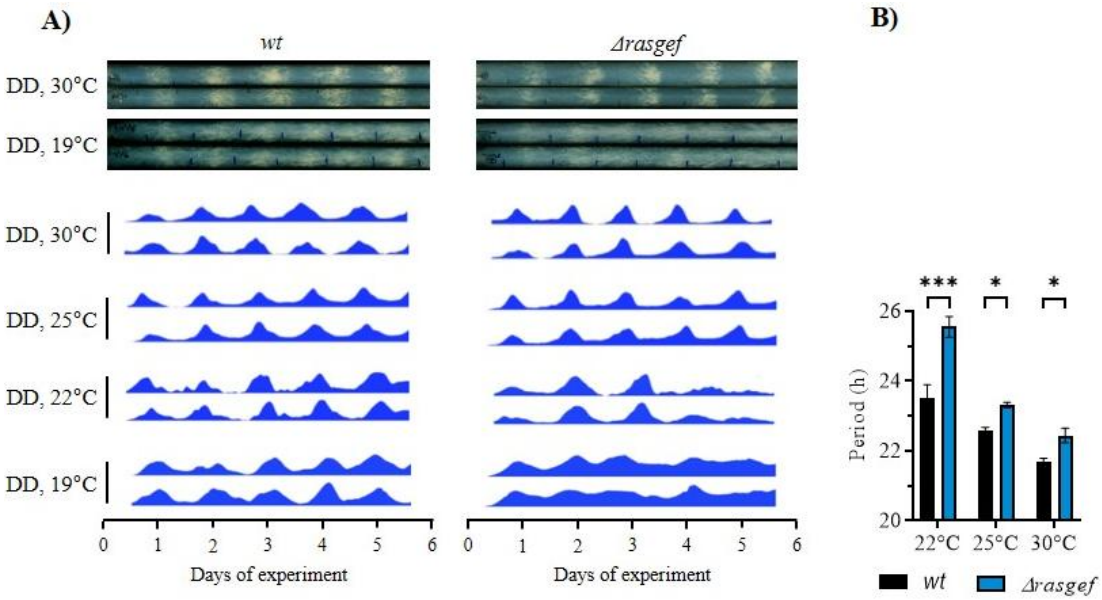

Figure 11. The conidiation rhythm of *Δrasgef* shows impaired temperature compensation and dampening at low temperature

A) Race tubes without glucose were incubated in DD at the indicated temperatures. Upper panels: Images of representative tubes are shown. Lower panels: Densitometric analysis of the race tubes incubated at the indicated temperatures.

B) Race tubes were analysed with the ChronOSX program and the periods at different temperatures were determined (n = 13-23, mean ± SEM, factorial ANOVA with significant strain*treatment interaction, followed by Fisher's LSD test).

To confirm that the absence of the *rasgef* gene was responsible for the phase and period effects in *Δrasgef*, we generated a strain expressing a FLAG-tagged version of RasGEF under the control of the *ccg-1* promoter in the *Δrasgef,bd* background (*rasgef*$_{Flag}$, *bd*) (183). Expression of RasGEF$_{Flag}$ abolished the phase delay and period difference observed in the gene-deficient strain relative to the *wt* (Figure 10A and B) (183). This indicates that the fusion protein was functionally active and the altered rhythm in the mutant strain was a consequence of the absence of RasGEF.

4.2.2. Investigation of RasGEF's role in the metabolic compensation of the circadian rhythm

As our group previously showed that the RAS-mediated pathway affects the nutrient compensation of the clock in *Neurospora crassa* (22), we examined whether the effect of *rasgef* deletion on the conidiation rhythm depends on glucose. Interestingly, in the presence of glucose, a delayed conidiation phase was observed in both *wt* and *Δrasgef* compared to the glucose-free medium. Nevertheless, the period difference between the strains in glucose-free medium was not detected in the presence of glucose (Figure 12). This suggests that RasGEF may play a role in adapting the circadian clock to low carbohydrate availability.

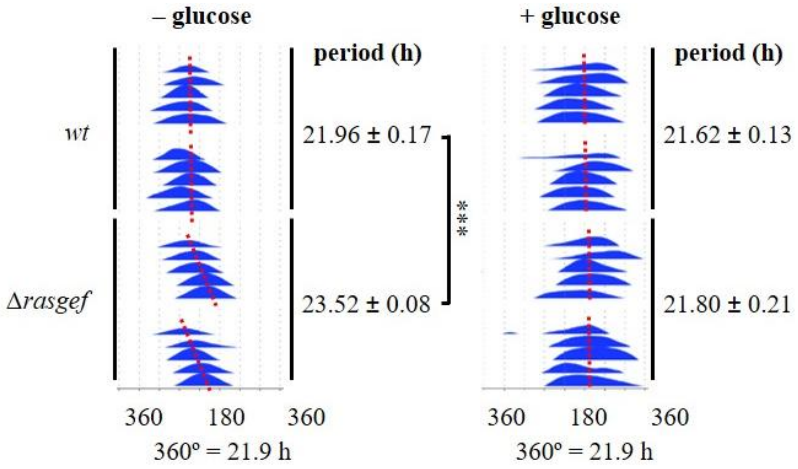

Figure 12. The period is dependent on glucose in *Δrasgef*

Race tubes containing no (- glucose) or 0.3% glucose (+ glucose) were inoculated with the indicated strains. After 24 h incubation in constant light (LL), the tubes were transferred to DD (25°C). Conidial densities of consecutive cycles are plotted below each other, with one cycle normalized to 360° (corresponding to the wild type free-run period of 21.9 h). This normalization allows direct comparison of rhythms between strains. A

phase delay is seen when the peaks shift to the right in the presence of glucose, while a period difference is visible when successive cycles fail to align across rows without glucose in the *Δrasgef* strain. The plots show representative individual race tubes (n = 4, mean ± SEM, factorial ANOVA with significant strain*treatment interaction, followed by Tukey HSD test).

Next, we measured the oscillation of *frq* RNA in liquid cultures at low (0.01%) and standard (2%) glucose levels. As previously shown, the *frq* RNA rhythm was similar under both conditions in *wt* (171) (Figure 13A). Under standard glucose conditions, *frq* oscillation in *Δrasgef* resembled that in the *wt*. However, *frq* levels were upshifted and showed a reduced amplitude under glucose deprivation, suggesting a role of RasGEF in the adaptation of the circadian oscillator to starvation (Figure 13B).

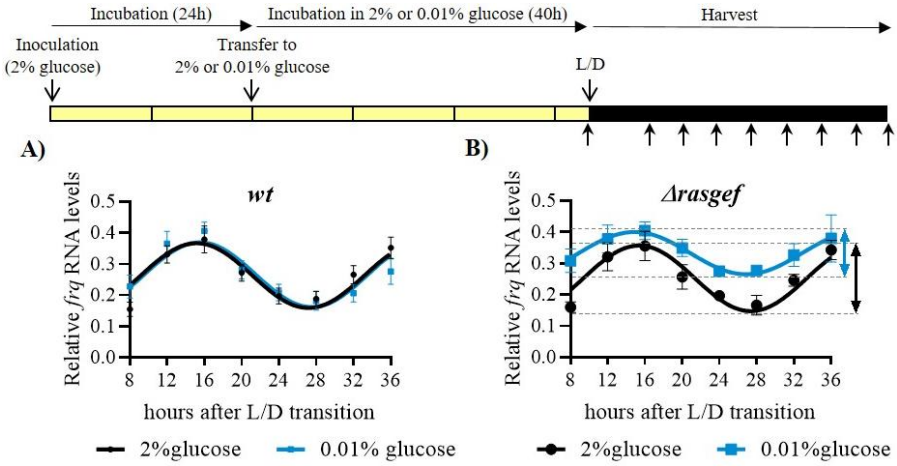

Figure 13. *frq* oscillation alters in *Δrasgef* upon glucose deprivation

The upper panel shows the schematic outline of the experimental design. Following a 24 h incubation of the indicated strains in standard liquid medium, mycelia were transferred to standard (black) or starvation medium (blue) in LL. After 40 hours, the cultures were transferred to DD. RNA was extracted from samples collected at the indicated time points.
A) *frq* RNA rhythm in *wt* under standard (black) and starvation (blue) conditions. Sine curves were fitted to the time series (n = 6-8, mean ± SEM, cosinor analysis: solid line indicates significant fitting).
B) *frq* RNA rhythm in *Δrasgef* under standard (black) and starvation (blue) conditions. Sine curves were fitted to the time series (n = 6-8, mean ± SEM, $p_{mesor} = 0.015$ in *Δrasgef*, cosinor analysis: solid line indicates significant fitting).

We also examined expression of the main clock components at the protein level. Our research group previously described that under long-term glucose deprivation, FRQ became hyperphosphorylated, while the levels of WC components were significantly reduced compared to standard conditions (171).

FRQ expression was similar in *Δrasgef* and *wt* under both growth conditions. WC-1 and WC-2 were slightly overexpressed under standard conditions, and the reduction in WCC caused by starvation was less significant in the mutant compared to the *wt* (Figure 14).

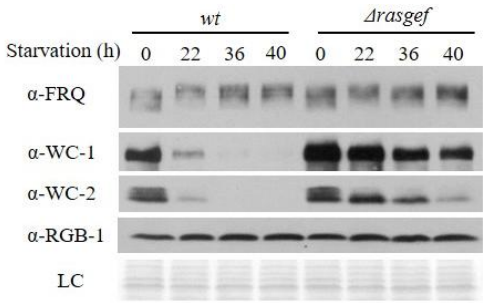

Figure 14. In *Δrasgef*, glucose-dependent changes in the WCC are less pronounced compared to *wt*

Mycelial discs were incubated in LL for 24 hours in standard liquid medium and then transferred to starvation medium (time point 0). Samples were harvested at the indicated time points. Cell extracts were analysed by western blotting. RGB-1 (Regulatory subunit of protein phosphatase 2A) detection and Ponceau staining are shown as loading controls (LC).

To further investigate how RasGEF impacts the circadian clock function, we examined the time-dependent expression of *ccg-2* encoding a conidial hydrophobin essential for spore dispersal. Importantly, *ccg-2* is a glucose-repressible output gene. In standard medium, *ccg-2* expression showed similar phase and amplitude in *wt* and Δ*rasgef* (Figure 15). Under glucose starvation, the transcript levels increased in both strains, although to a lesser extent in the mutant. Moreover, while the rhythm was preserved in *wt,* it was markedly dampened in Δ*rasgef.* These results suggest that the absence of RasGEF compromises the circadian system's ability to adapt gene expression to low-glucose environments.

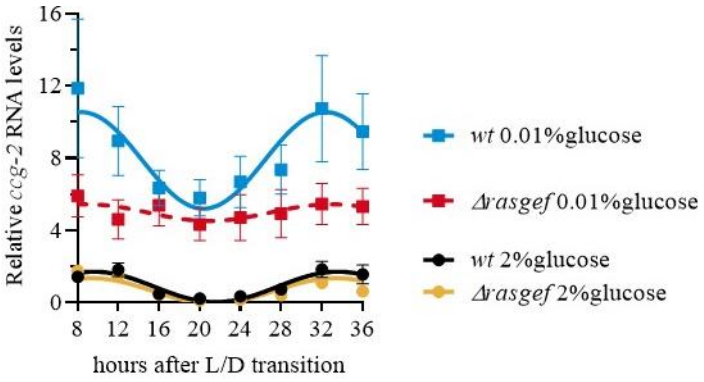

Figure 15. *ccg-2* does not display rhythmic expression in starved *Δrasgef*

ccg-2 RNA levels in samples from the experiment described in Figure 13. were quantified and analysed (n = 6-8, mean ± SEM, cosinor analysis: solid line indicates significant fitting, whereas dashed line indicates no significant fitting to a 24-hour sinusoidal function).

4.2.3. *rasgef* is a clock-controlled gene that can be influenced by light and glucose availability

We monitored *rasgef* transcript levels in constant darkness to assess whether it is under circadian regulation. In standard medium, *rasgef* RNA showed clear rhythmicity and was approximately twice as abundant as under glucose-starved conditions, where only minor fluctuations were observed (Figure 16).

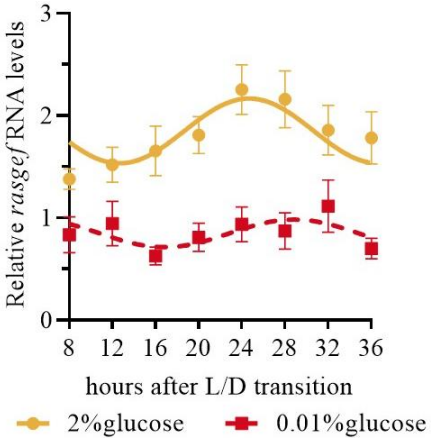

Figure 16. *rasgef* expression is under circadian control

rasgef RNA levels were quantified in the *wt* samples from the experiment described in Figure 13 (n = 6-8, solid line indicates significant fitting, dashed line indicates no significant fitting to a 24-hour sinusoidal function).

The *rasgef* promoter activity was investigated by my colleagues using a luciferase reporter strain. *rasgef* displayed robust oscillation that was in antiphase with that of *frq* (55, 183), suggesting that *rasgef* is an evening-specific gene (78, 184).

To examine whether light affects *rasgef* expression, dark-grown cultures were exposed to light, and transcript levels of *rasgef* and *frq* were measured. As expected, *frq* showed strong light induction with glucose-dependent kinetics (Figure 17A) (171). In contrast, *rasgef* expression modestly declined under both nutrient conditions (Figure 17B), suggesting that *rasgef* is repressed by light.

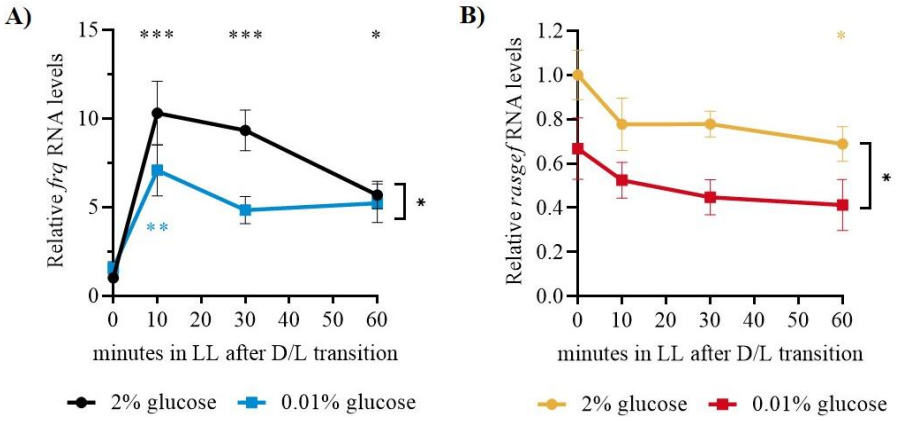

Figure 17. *rasgef* expression is slightly repressed by light

A) *frq* RNA levels were quantified in the *wt* samples from the experiment described in Figure 5.

B) *rasgef* RNA levels were quantified in the *wt* samples from the experiment described in Figure 5.

Relative *frq* and *rasgef* RNA levels were normalized to those measured at time point 0 under standard conditions (n = 8, mean ± SEM, repeated measures ANOVA with significant treatment*time interaction, followed by Fisher's LSD test).

CDC25, the yeast homolog of RasGEF, is known to be regulated by phosphorylation (185-191). To determine whether the *Neurospora* RasGEF is similarly modified, we analysed $RasGEF_{Flag}$-expressing strains by western blots.

Two distinct bands of RasGEF were detected on western blots, and treatment of the protein lysates with an alkaline phosphatase (CIP) resulted in a shift of RasGEF to a faster-migrating form, indicating that it was phosphorylated (Figure 18A). In nutrient-rich medium, the hyperphosphorylated form was predominant, while glucose starvation

led to enrichment of the hypophosphorylated species. Subcellular fractionation revealed that $RasGEF_{Flag}$ localizes mainly to the cytoplasm under high glucose, but shifts to the nucleus in starved cultures (Figure 18B). These results suggest that glucose regulates both the phosphorylation status and the nuclear localization of RasGEF, resembling the nutrient-dependent control of CDC25 in yeast (185-191).

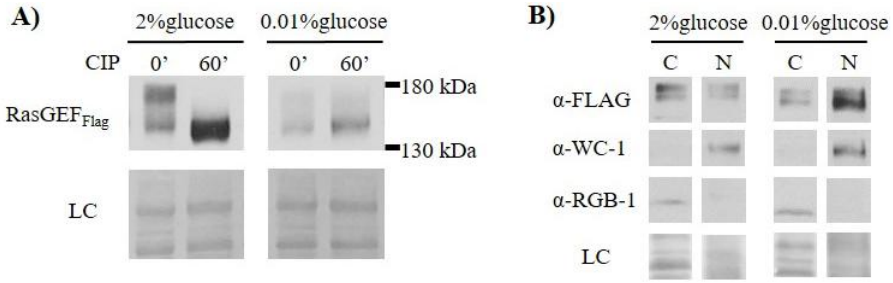

Figure 18. RasGEF is a phosphoprotein and its subcellular localization depends on its phosphorylation state

A) *rasgef$_{Flag}$* cultures were grown in standard liquid medium for 24 h in LL, and then transferred to either standard or starvation medium for 40 h in LL. Protein lysates were prepared, analysed by western blotting using an anti-FLAG antibody. When indicated, the protein lysates were treated with CIP for 60 minutes. Ponceau staining is shown as loading control (LC).

B) Experiments were performed with the *rasgef$_{Flag}$*. Nuclear (N) and cytosolic (C) fractions were analysed by western blotting. RGB-1 is shown as a cytosol marker, WC-1 is dominantly localized to the nucleus. Ponceau staining was used as loading control (LC).

4.2.4. Characterization of the signalling pathway linked to RasGEF

In fungi, RAS signalling is functionally linked to the cAMP/PKA pathway, which is responsive to glucose availability (192-195). To test this regulation in *Neurospora*, we measured cAMP levels in *wt* and *Δrasgef* upon increasing the glucose concentration.

While glucose significantly increased cAMP concentrations in *wt*, no significant induction was observed in the mutant (Figure 19), indicating that RasGEF is essential for glucose-triggered elevation of cAMP production.

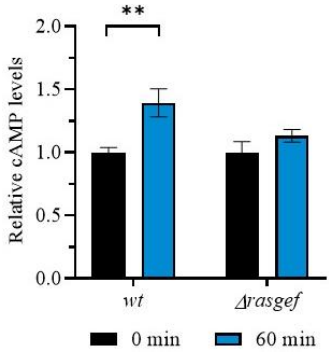

Figure 19. Glucose-induced cAMP elevation is impaired in *Δrasgef*

Indicated strains were grown as described in Figure 18A. Following the 40 h incubation in the starvation medium, 2% glucose was added to the cultures. Samples were harvested at the indicated time points and cAMP levels were determined in the whole cell lysates and normalized to those measured at time point 0 (n = 8, mean ± SEM, repeated measurement ANOVA followed by Tukey's HSD test).

To search for interaction partners of RasGEF, we performed a FLAG-based affinity purification followed by mass spectrometry using lysates from the *rasgef*$_{Flag}$ (FGSC #11866 background) and *wt* (FGSC #2489 background) as a control. Among monomeric G proteins, Rat sarcoma 2 protein (RAS2P) showed the highest fold change (FC) and lowest false discovery rate (FDR), suggesting a strong association (Table 5) (183). Gene ontology analysis highlighted significant enrichment in GTPase activator functions, supporting a role of RasGEF in monomeric G-protein signalling (183).

Table 5. Top 10 significantly enriched proteins identified by FLAG-based affinity purification and mass spectrometry (p value <0.01, FDR < 0.05).

Protein ID	Protein Name	logFC	p value	FDR
Q7S5W4	Centrin 3	11.304646	1.43E-05	9.67E-04
U9W4J8	DUF1941-domain-containing protein	10.563087	1.66E-11	3.40E-08
A0A0B0DTI2	Acyltransferase-domain-containing protein	9.9806461	9.01E-10	1.23E-06
Q7S8G8	Pyridoxamine phosphate oxidase	9.7252724	1.09E-07	3.42E-05
U9W2W2	DUF1421 multi-domain protein	9.6381016	3.75E-05	1.99E-03
P23622	Sulfate permease 2	9.581044	1.40E-09	1.43E-06

Q7S5H7	GTPase Ras2p	9.5007514	1.09E-06	1.55E-04
Q7RXZ8	Acetoacetyl-CoA synthase	9.3297794	5.92E-09	4.04E-06
V5IMI3	Heme peroxidase	9.1929231	7.70E-07	1.17E-04
Q7S2C8	Late endosomal/lysosomal adaptor and MAPK and MTOR activator 1	9.1529501	0.0033638	0.0476747

To validate the interaction between RAS2P and RasGEF, a GST-RAS2P fusion protein was expressed in *E. coli* and immobilized on glutathione-agarose beads, allowing selective binding of GST-tagged proteins. These beads were incubated with protein lysate from *N. crassa* expressing $RasGEF_{Flag}$ (FGSC #11866 background). After washing to remove nonspecific binding partners, the retained proteins were eluted and subjected to SDS-PAGE followed by western blotting using an anti-FLAG antibody. This approach specifically recovered $RasGEF_{Flag}$ (Figure 20), confirming that RAS2P directly interacts with RasGEF *in vitro*.

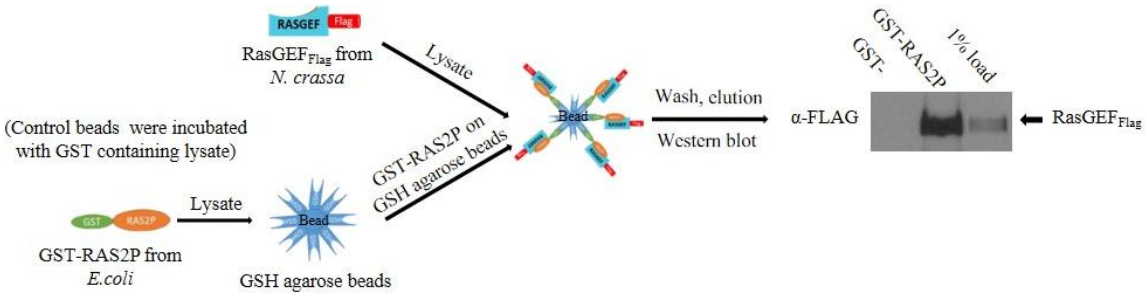

Figure 20. $RasGEF_{Flag}$ interacts with RAS2P *in vitro*

Schematic outline of the experiment. GST-tag expressed in *E. coli* was used as a negative control. 1% load shows a small fraction of the FLAG-tagged RasGEF used in the *in vitro* interaction assay, as input reference.

4.2.5. The adaptation of the circadian clock in mammalian cells is also affected by RasGEF signalling

We next aimed to investigate glucose compensation in the mammalian circadian clock and to evaluate the potential involvement of RAS-mediated signalling.

Time-dependent luminescence changes were followed in U2OS cells expressing *Bmal1-luciferase* under varying glucose concentrations. As *Bmal1* encodes one of the core positive components of the mammalian circadian clock, its rhythmic promoter activity

allows monitoring of clock oscillations. The cell culture work was performed by one of my colleagues, while my task was the subsequent analysis of the phase and period. Rhythmicity was maintained across a wide range of glucose levels and only a slight phase advance was observed at low glucose levels (0.5-1 mM) (Figure 21A). Importantly, the period length remained stable regardless of glucose levels (Figure 21B), indicating that, similar to *Neurospora*, the mammalian clock also exhibits metabolic compensation (23, 196).

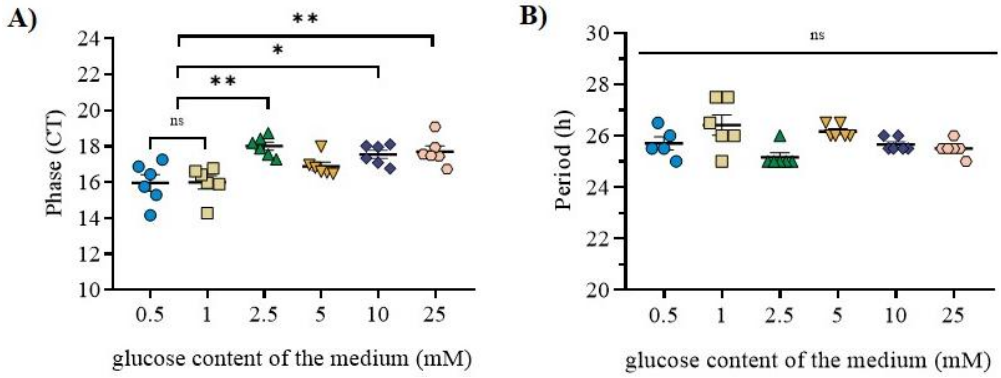

Figure 21. Rhythmic *Bmal1* promoter activity can be detected over a wide range of glucose levels with stable period and a slight phase advance at low glucose levels

A) Phase analysis shows a slight advance under low glucose. Following the 2 h synchronisation, *Bmal1-luc* cells were cultured in the presence of the indicated glucose concentration. Luminescence was detected for 5 days following synchronization (n = 5-6, mean ± SEM, one-way ANOVA with significant treatment effect for phase analysis, followed by Tukey's HSD test).

B) Averaged periods based on data from the experiment described in A) remains stable across all glucose conditions.

To determine whether SOS1 - the mammalian homolog of *Neurospora* RasGEF - plays a role in maintaining clock function under low-glucose conditions, U2OS cells were treated with BAY-293, the specific SOS1 inhibitor (197) (Figure 22).

At 0.5 mM glucose, inhibition of SOS1 resulted in a faster dampening of the *Bmal1-luc* signal compared to untreated controls (Figure 22A). In contrast, at 10 mM glucose, the oscillation remained unaffected by the inhibitor (183) (Figure 22B). The rapid damping limited the period analysis at low glucose levels to 2-3 days, but during this time no significant difference in the period was observed (Figure 22C).

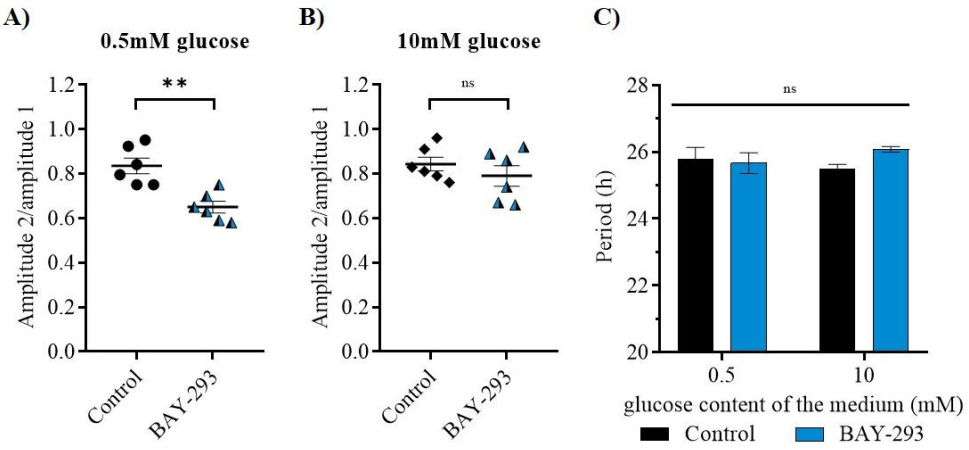

Figure 22. Inhibition of SOS1 results in rhythm dampening at low glucose level

A-B) Following the 2 h synchronization, the cell culture medium was supplemented with either the SOS1 inhibitor BAY-293 (600 nM) or vehicle (DMSO), as indicated. Amplitude changes from the first to the second day in the presence or absence of BAY-293 at the indicated glucose levels. Amplitude ratios (difference in the relative bioluminescence between the second peak and nadir/difference in the relative bioluminescence between the first peak and nadir) were calculated and normalized to the changes of the absolute bioluminescence (indicator of cell number changes) (n = 8, mean ± SEM, factorial ANOVA followed by Fisher's LSD test).

C) Averaged periods based on data from the experiment described in (A-B) are indicated (n = 6, mean ± SEM, factorial ANOVA followed by Tukey's HSD test).

Since SOS1 activates the RAS/MAPK/ERK signalling pathway, which has previously been implicated in circadian regulation (198), we next tested whether this pathway also contributes to the control of the clock in a glucose-dependent manner.

Cells were treated with an inhibitor of MEK1/2, U0126 that prevents ERK activation (199). Under low-glucose conditions, U0126 treatment significantly dampened the rhythm and resulted in a longer period (183) (Figure 23). At high glucose levels, the inhibitor had, however, no effect on these rhythm parameters. Following 90 h of treatment, the inhibitor was washed out and replaced with fresh medium containing the original glucose levels. Rhythmicity promptly recovered, indicating that the reduced amplitude was due to clock inhibition rather than loss of cell viability (183).

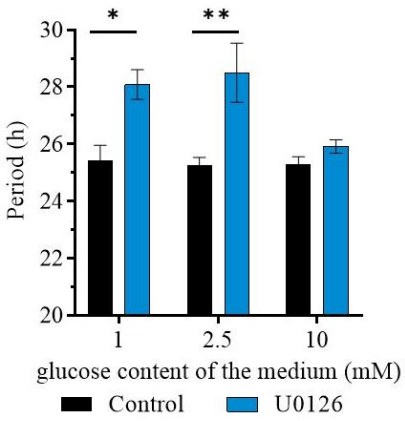

Figure 23. The ERK inhibitor affects the period at low glucose levels

Experiment was performed as described in Figure 23A-B. Following synchronization, the cell culture medium was supplemented with either the ERK inhibitor U0126 (2 μM) or vehicle (DMSO), as indicated (n = 6, mean ± SEM, factorial ANOVA followed by Tukey's HSD test).

Above findings suggest that both SOS1 and ERK contribute to the maintenance of robust circadian oscillations under nutrient-limited conditions in mammalian cells.

5. Discussion

Organisms continuously adapt to fluctuations in nutrient availability, which can profoundly impact core cellular processes, including circadian regulation. In *Neurospora crassa*, metabolic compensation ensures that the endogenous oscillator remains functional and works with a stable period even at very different levels of carbon sources. Our findings extend prior observations by demonstrating that this compensation is preserved under severe reduction of nutrient levels, and is accompanied by drastic changes in the levels and stoichiometry of clock components. We observed that long-term glucose deprivation led to a marked reduction in WC-1 and WC-2 protein levels, while FRQ remained stable but became hyperphosphorylated. Notably, the nuclear pool of the WCC was retained, suggesting that a minimum level of nuclear WCC suffices to sustain a stable TTFL. In parallel, the cytosolic WCC pool, potentially serving as a light-responsive reservoir, was strongly depleted (171). In line with this, we found that the initial, not-adapted phase of the light induction of the clock components, *frq* and *wc-1*, and that of a carotenoid synthesis regulator, *al-2* were significantly attenuated under starvation (Figure 5). These results indicate that the photoreceptor function of the WCC is particularly sensitive to nutrient availability, whereas its role in clock regulation is preserved.

To determine whether *frq* RNA stability contributes to compensation, we monitored decay kinetics following an L/D transition in both standard and starvation conditions. The similarity in *frq* RNA degradation profiles across conditions indicated that glucose depletion does not influence transcript stability, pointing instead to upstream regulatory mechanisms as compensatory drivers (Figure 4).

There is also strong evidence that nutrient limitation can trigger ROS production and mitochondrial stress. ROS can influence circadian regulation by modulating FRQ phosphorylation, WCC stability, and downstream outputs, similar to the effects we observed during glucose starvation (28, 55, 200). However, unlike direct ROS elevation, where *frq* expression typically shows a phase advance, we did not detect such a shift under starvation. This suggests that the cellular response to glucose deprivation can not be attributed to ROS level changes.

To further investigate the possible control mechanisms, we focused on key kinases and phosphatases involved in clock regulation. Using the *mcb* mutant strain, which displays

enhanced PKA activity, we found that PKA signalling is a central modulator of nutrient-induced clock remodelling. Elevated PKA activity prevented FRQ hyperphosphorylation, preserved WCC levels, and preceded starvation-induced transcriptional changes (Figure 7).

We also demonstrated that glucose starvation-induced GSK activation contributes to FRQ hyperphosphorylation. This modification may reduce FRQ-mediated repression of WCC, thereby allowing adequate transcription of *frq* and downstream genes even at lower levels of the transcription factors (Figure 8). CK-1a is a central regulator of the feedback activity of FRQ. We used the *frqΔFCD1-2* mutant, in which the binding site for CK-1a is disrupted. Starvation-induced FRQ hyperphosphorylation was largely retained in this strain, suggesting that kinases other than CK-1a, such as GSK, mediate these changes. The absence of substantial *frq* RNA level differences between *frqΔFCD1-2* and *wt* under starvation further supported the notion that altered FRQ-CK-1a interaction did not fully account for the metabolic compensation of the circadian clock (Figure 6).

Figure 24 provides a summary of the potential mechanism by which the *Neurospora* circadian clock might adjust to changes in nutrient availability, as suggested by literature data and our findings. In summary, the coordinated action of PKA, GSK, and PP2A forms a regulatory network that fine-tunes the phosphorylation and expression dynamics of FRQ and WCC, ensuring that the clock can properly operate even when resources are strongly limited.

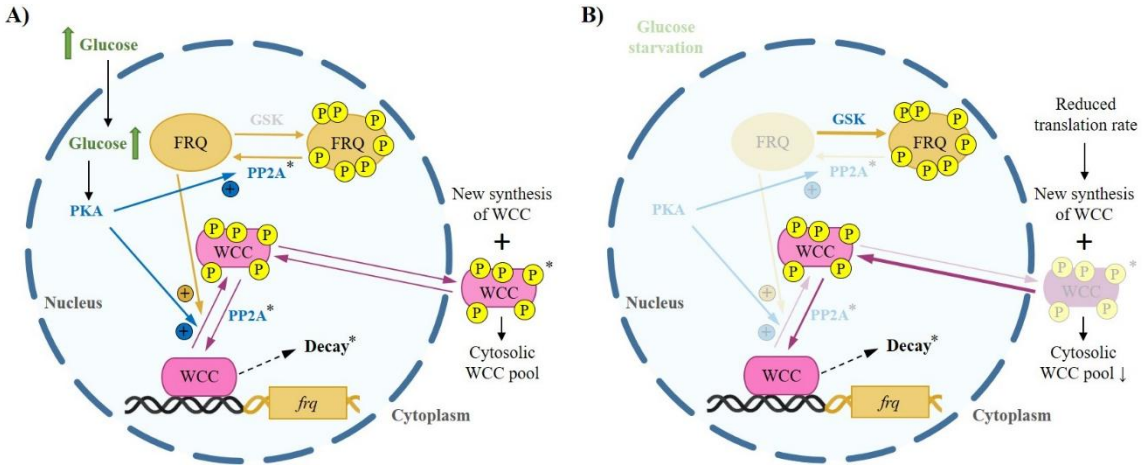

Figure 24: Schematic overview of how negative feedback and signalling pathways involving PKA, PP2A, and GSK regulate the circadian clock under high glucose conditions (A) and during glucose starvation (B)

Glucose deprivation leads to reduced activity of both PKA and PP2A, while GSK activity increases. PKA likely plays a central role in mediating glucose-dependent changes to the clock. When PKA activity drops during starvation, the WCC becomes more susceptible to destabilization, yet *frq* transcription remains compensated. PKA can also influence PP2A activity. Under starvation, the combined effect of decreased PP2A and increased GSK activity enhance FRQ phosphorylation, weakening its negative feedback on the WCC. This helps maintain an appropriate active pool of the WCC, despite a reduction in its overall level. In the figure, more intense colors indicate higher activity of the components or processes, while lighter shades represent reduced activity. Asterisks (*) indicate mechanisms which were investigated by my colleagues, and therefore were not presented in detail in the Results. They are, however, included in this model to provide a comprehensive overview of the system.

To further dissect the mechanism of nutrient compensation, we investigated the role of the RAS pathway, which was known to respond to glucose. Our second study identified RasGEF as a mediator of nutrient-responsive circadian regulation in both *Neurospora* and mammalian U2OS cells. In *Δrasgef*, conidiation was phase-delayed (Figure 10), metabolic and temperature compensation were impaired (Figure 11), and *frq* levels increased under glucose starvation (Figure 13), correlating with elevated WCC abundance. Expression of the clock-controlled gene *ccg-2* was also dysregulated, suggesting a broader disruption of output pathways (Figure 15).

Interestingly, *rasgef* expression exhibited circadian rhythmicity (Figure 16), peaking in the subjective night and being mildly suppressed by light (Figure 17). Although this rhythm was dispensable for clock function - as demonstrated by phenotypic rescue with a constitutively expressed *rasgef* - it underscores the intricate coupling between circadian and metabolic regulatory circuits. We also found that RasGEF undergoes glucose-dependent phosphorylation and translocates between cytosol and nucleus (Figure 18). Under starvation, RasGEF became hypophosphorylated and enriched in the nucleus, a pattern resembling its yeast homolog CDC25. These modifications likely influence RasGEF's ability to activate RAS proteins and downstream effectors.

We identified RAS2P as a primary RasGEF interactor in *Neurospora* (Figure 20). RAS2P had not been previously characterized. Our data suggest that it operates in a pathway distinct from RAS2, which controls circadian period primarily under nutrient-rich

conditions. Together, these findings propose a functional divergence between RAS paralogs in the regulation of clock robustness depending on nutrient availability.

In *Δrasgef,* cAMP levels failed to respond to glucose, linking RasGEF activity to cAMP/PKA signalling (Figure 19). Given that PKA modulates FRQ stability, WCC inactivation, translation, and PP2A activity, RasGEF might be in a central position within the nutrient-responsive signalling network supporting metabolic compensation. It is important to note that in fungi such as *Neurospora*, cAMP signalling drives rapid adaptation to available glucose, whereas in mammals it integrates hunger signals to maintain systemic energy balance. To assess how nutrient availability impacts circadian regulation in mammals, we analysed circadian oscillations of *Bmal1-luciferase* in U2OS cells cultured at varying glucose concentrations. Rhythmic *Bmal1-luc* expression was preserved across a wide range of glucose concentrations (0.5 - 25 mM). A slight phase advance was observed at low glucose, yet the period remained largely unchanged (Figure 21). Inhibiting SOS1 - RasGEF's mammalian homolog - dampened the amplitude and stability of rhythms under glucose-limited conditions, while the rhythm at higher glucose levels remained unaffected (Figure 22). Similarly, MEK1/2 inhibition flattened oscillations and lengthened the period only in low-glucose media (Figure 23). These data confirm that SOS1/ERK signalling supports clock function under energy stress, mirroring the role of RasGEF in *Neurospora*. SOS1 promotes RAS/ERK signalling required for cellular adaptation and survival under metabolic stress. Loss of SOS1 or ERK activity in mammalian cells impairs autophagy, delays stress-responsive signalling, and increases susceptibility to apoptosis during glucose deprivation (201). The understanding of how glucose supply can affect RasGEF function is still incomplete. Recent studies have shown that fructose-1,6-bisphosphate can bind and regulate SOS1, suggesting a direct metabolic input into RAS/ERK signalling (202).

In summary, our findings reveal a conserved signalling axis linking RasGEF to nutrient-dependent circadian regulation in fungi and mammalian cells. The RasGEF-PKA/ERK pathway acts as a sensor of glucose status, controlling circadian robustness under adverse metabolic conditions. Our work contributes to the understanding of how endogenous clocks adapt to environmental and physiological fluctuations, and may offer new perspectives for studying metabolic disorders and circadian misalignment also in disease contexts.

6. Conclusions

The presented findings contribute to our understanding of how the circadian clock adapts to changes in nutrient availability, focusing primarily on the model organism *Neurospora crassa* and extending key findings to the mammalian system. Using long-term glucose starvation as a tool to perturb metabolic homeostasis, we demonstrated that the circadian clock remains functionally intact despite significant changes in the abundance and stoichiometry of its core components. This flexibility is known as metabolic compensation, that preserves period of the circadian clock under challenging conditions. Our data indicate that the WCC is particularly sensitive to glucose availability, especially in its cytoplasmic form, while nuclear WCC levels remain sufficient to sustain transcription of *frq* and other output genes even under severe limitation of the carbon sources. Moreover, we found that metabolic compensation is maintained across a broad range of glucose concentrations even in mammalian cells.

Targeted manipulation of signalling pathways revealed the importance of PKA and GSK in modulating the phosphorylation state and stability of clock components. Reduced PKA activity under starvation facilitated transcriptional compensation, while GSK activation contributed to FRQ hyperphosphorylation. These changes modulate feedback strength and likely permit the clock to function at lower level of the WCC.

We identified RasGEF as a regulator of nutrient compensation. Deletion of *rasgef* disrupted multiple aspects of clock function, including output gene expression and compensation to metabolic and temperature changes. RasGEF operates through the RAS2P monomeric GTPase and modulates the PKA pathway, suggesting a hierarchical signalling framework linking nutrient sensing to clock regulation.

Inhibition of SOS1 or ERK, mammalian homologs of the components of the *Neurospora* RasGEF signalling pathway, impaired rhythmic clock function under low-glucose conditions, demonstrating functional conservation of the mechanism.

Altogether, this work identifies RasGEF signalling as a central and conserved regulator of circadian robustness under nutrient stress. The findings provide a framework for understanding how metabolic status influences timekeeping and may inform studies of circadian disruption in diseases characterized by altered nutrient signalling.

7. Summary

The circadian clock enables organisms to anticipate and adapt to daily environmental cycles. A key property of the clock is its robustness against metabolic fluctuations, allowing it to maintain a stable period across varying nutrient levels - known as metabolic compensation. This dissertation investigates the molecular mechanisms underlying nutrient-dependent modulation of the circadian clock, using *Neurospora crassa* as a model organism, and explores the conservation of these mechanisms in mammalian cells.

In *Neurospora*, we found that the circadian clock remains functional under glucose starvation, despite a marked decrease in cytoplasmic WCC levels and hyperphosphorylation of FRQ. Nuclear WCC levels were preserved, allowing stable *frq* expression and circadian oscillation. However, light-induced transcriptional activation was attenuated under starvation, suggesting weakened photoreceptor function. Our results also showed that the *frq* mRNA decay rate did not differ between nutrient conditions, indicating that *frq* RNA stability is unaffected by glucose availability. We further identified key signalling components that mediate these compensatory responses. Using mutant strains, we showed that PKA activity modulates starvation-induced changes in clock components. GSK was necessary for FRQ hyperphosphorylation, and partial disruption of FRQ-CK-1a interaction affected WCC levels.

Furthermore, we identified RasGEF, a guanine nucleotide exchange factor, as an essential regulator of clock robustness under nutrient limitation. Loss of *rasgef* impairs metabolic and temperature compensation, disrupts rhythmic gene expression, and alters the timely pattern of conidiation. Importantly, RasGEF signals through RAS2P and affects the cAMP/PKA pathway, reinforcing the integration of nutrient sensing with circadian regulation.

Finally, we extended our findings to mammalian cells using U2OS cell lines expressing *Bmal1-luciferase*. Luminescence rhythms remained stable across a wide glucose concentration range (0.5-25 mM), and pharmacological inhibition of SOS1 or ERK signalling revealed their importance for circadian robustness under low glucose conditions. These results suggest that RasGEF/SOS1-mediated pathways are conserved regulators of nutrient compensation in eukaryotic clocks.

Together, these results identify molecular pathways that maintain clock function during nutrient limitation and provide evidence for their evolutionary conservation.

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9. Bibliography of the candidate's publications

9.1. Publications relevant to the dissertation

I. 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

II. Szőke, Anita; Sárkány, Orsolya; Schermann, Géza; Kapuy, Orsolya; Diernfellner, Axel C R; Brunner, Michael; Gyöngyösi, Norbert**; Káldi, Krisztina. Adaptation to glucose starvation is associated with molecular reorganization of the circadian clock in *Neurospora crassa*. ELIFE 12 Paper: e79765, 26 p. (2023). IF (2023): 6.4

Cumulative impact factor: 10.6

9.2. Publications unrelated to the dissertation

I. 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

Cumulative impact factor of all publications: 12.766

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