The latest version is at http://www.jbc.org/cgi/doi/10.1074/jbc.M117.804922

The small G protein RAS2 is involved in the metabolic compensation of the circadian clock in the circadian model *Neurospora crassa*

Norbert Gyöngyösi[1,2], Anita Szőke[1,2], Krisztina Ella[1], and Krisztina Káldi[1]

[1]Department of Physiology, Semmelweis University, Tűzoltó u. 37-47, Hungary, H-1094
[2]These authors contributed equally to this work.

Running title: Metabolic compensation of the clock by RAS2

Keywords: Circadian rhythm / Entrainment / Glucose compensation / Small GTPase / *Neurospora* / Cyclic AMP (cAMP) / Ras protein

Corresponding author: Krisztina Káldi
Department of Physiology, Semmelweis University, Tűzoltó u. 37-47, Hungary, H-1094 Tel: +36-1-459-1500/60411 Fax: +36-1-266-7480
kaldi.krisztina@med.semmelweis-univ.hu

ABSTRACT

Accumulating evidence from both experimental and clinical investigations indicate a tight interaction between metabolism and circadian timekeeping; however, knowledge of the underlying mechanism is still incomplete. Metabolic compensation allows circadian oscillators to run with a constant speed at different substrate levels and therefore is a substantial criterion of a robust rhythm in a changing environment. Because previous data have suggested a central role of RAS2-mediated signaling in the adaptation of yeast to different nutritional environments, we examined the involvement of RAS2 in the metabolic regulation of the clock in the circadian model organism *Neurospora crassa*. We show that in a *ras2*-deficient strain, the period is longer than in the control. Moreover, unlike in wild type (*wt*), in *Δras2* operation of the circadian clock was affected by glucose: compared with starvation conditions, the period was longer and the oscillation of expression of the *frequency* (*frq*) gene was dampened. In constant darkness the delayed phosphorylation of the FRQ protein and the long-lasting accumulation of FRQ in the nucleus were in accordance with the longer period and the less robust rhythm in the mutant. Whereas glucose did not affect the subcellular distribution of FRQ in *wt*, highly elevated FRQ levels were detected in the nucleus in *Δras2*. RAS2 interacted with the RAS-binding domain of the adenylate cyclase *in vitro*, and the cAMP analogue 8-Br-cAMP partially rescued the circadian phenotype *in vivo*. We propose therefore that RAS2 acts via a cAMP-dependent pathway and exerts significant metabolic control on the *Neurospora* circadian clock.

INTRODUCTION

Circadian rhythms are endogenously generated at the cellular level. Core clock mechanisms control rhythmic expression of a large set of genes, which in turn regulate various biological processes including cell growth, proliferation and metabolism. An important feature of the circadian clock is the ability to display an endogenous rhythm with a constant period length under different environmental conditions. Among these adaptation mechanisms the most intensively investigated process is temperature compensation. Although a tight interaction between metabolism and the circadian clock has been shown at almost all levels of organisms (1-3), it is still poorly understood how molecular timekeeping is compensated against changes in nutrient availability.

Neurospora crassa belongs to the most extensively examined model systems in the field of circadian research and has proved to serve as a

useful tool for the investigation of different aspects of circadian regulation including metabolic compensation of the circadian clock (4). Monitorization of the conidiation rhythm, the time-dependent formation of asexual spores allows easy study of the effect of genetic changes or pharmacologic manipulations on the output of the circadian oscillator (5). The molecular clockwork of *Neurospora* is well characterized (recently reviewed in (6,7)). The transcription factors White Collar-1 (WC-1) and WC-2 and the negative factor Frequency (FRQ) represent the core clock components. Other important regulators of the molecular oscillator include kinases, phosphatases, exosome components and factors controlling the chromatin status (e.g. (8-13)). WC-1 and WC-2 form the WC complex (WCC) that supports expression of *frq*. The FRQ protein interacts with an RNA helicase (FRH) and the casein kinase 1a (9,14) and inhibits the activity of the WCC by facilitating its phosphorylation. FRQ undergoes several steps of phosphorylation that affect distinct functions of the protein (15-17). Shortly after its synthesis, low levels of hypophosphorylated FRQ forms are accumulated in the nucleus and are efficient in the negative feedback. In later phases of the circadian cycle hyperphosphorylation of FRQ interferes with nuclear import of the protein and the cytoplasmic FRQ supports accumulation of the WCC, thereby forming the positive feedback loop. Hyperphosphorylated FRQ interacts with FWD-1 (F-box/WD-40 repeat-containing protein) that facilitates ubiquitination and thus degradation of the protein (18). When FRQ is cleared from the nucleus, the WCC is gradually released from the inhibition and a new cycle starts. Besides, the WCC is the main photoreceptor of *Neurospora* controlling transcription of light-inducible genes and transducing light inputs to the circadian oscillator (19-22). In light/dark cycles, the light-inducible blue-light receptor VIVID inhibits the light-activated WCC by forming an additional negative feedback loop (23-25).

Regarding the interconnections between metabolism and circadian timekeeping, period of the *Neurospora* clock was shown to be independent of the glucose concentration of the medium and CSP1 (conidial separation 1), an important regulator of energy metabolism was characterized as a key component of the adaptation of clock function to different sugar availability (4). According to the model of metabolic compensation proposed by Sancar et al. (4), glucose generally increases the rate of protein synthesis and thus translation of WC-1, the limiting subunit of the WCC. Glucose-dependent suppression of *wc-1* transcription by CSP1, however, compensates for the translation elevation, resulting in glucose-independent expression of the WCC and thus stabilization of the period. Recently, PERIOD-1 (PRD-1), an RNA helicase was also found to control the cycle length in the presence of glucose (26,27).

In yeast, the RAS-mediated pathway is central in the signal transduction of glucose sensing. This pathway controls the transcription of at least one hundred genes and thus contributes to the adaptation of cellular functions to changes in the metabolic environment (28,29). Since yeast cells and *Neurospora crassa* express similar RAS proteins (30) and the *Neurospora* clock is thoroughly characterized at the molecular level, *Neurospora* may represent a good tool for the investigation of the interactions between RAS-signaling and metabolic regulation of the circadian clock.

RAS small G-proteins are universal eukaryotic signal transducers that belong to the superfamily of small monomeric GTPases. In most organisms several RAS isoforms are expressed. Although they share common sets of upstream regulators and downstream effectors, various experimental data support the functional specificity of the different RAS isoforms (31). RAS proteins play a critical role in the regulation of metabolism, cell growth, proliferation and oncogenic transformation (32,33), all representing processes that are interlinked with the circadian rhythm. Indeed, increasing body of evidence suggest interconnections between RAS-mediated pathways and the circadian clock in different organisms. Expression of RAS family members was found to be regulated by the circadian clock in some mammalian tissues (34-36), and functional analysis also revealed that the RAS/MAPK pathway may serve as output and/or is able to modulate outputs of the circadian clock (37-45). In *Neurospora*, activating mutation of RAS-1 (present in the *band* (*bd*) strain) enhances the conidiation rhythm and the light-induced transcription of the positive factor WC-1 (46).

Moreover, we recently showed that the phase of conidiation is more sensitive to temperature changes in the *bd* strain than in *wt* (47). Although in most cases the molecular basis is not well understood, RAS GTPases seem to be involved in clock control mechanisms in higher organisms as well. In *Drosophila*, RAS regulates the activity of the positive clock factor CLOCK/CYCLE (48). In mice, the dexamethasone-induced RAS protein 1 (DEXRAS1) is suggested to be an input factor of the circadian clock (49-52). Both experimental data and bioinformatic analysis suggest that oncogenic RAS proteins are involved in the deregulation of the circadian clock in cancer cells (53). Finally, it was recently shown that RAS-signaling affects the circadian clock in the suprachiasmatic nucleus of mice acting, at least partially, via ERK and glycogen synthase kinase (GSK), and thereby fine-tunes the circadian period (54).

Our goal in this study was to investigate whether RAS2 is involved in the metabolic compensation of the circadian clock of *Neurospora crassa*. Our results indicate that stability of the molecular clock function against changes in glucose availability is dependent on RAS2. We show that both phosphorylation and subcellular localization of FRQ are dependent on RAS2. We propose that RAS2 acting via a cAMP-dependent pathway links the molecular oscillator to the glucose sensing pathway and thereby is an important component of the nutritional compensation of the circadian period.

RESULTS

The *ras2* mutation affects conidiation rhythm

To study the possible interplay between the RAS2-mediated signal transduction pathway and the circadian clock, we aimed to examine the circadian behavior of the *ras2* deletion strain. *Δras2* (FGSC #12467) was generated during the Neurospora Genome Project (55) and had a phenotype similar to that of the *smco7* mutant described earlier (56). On agar slants, *Δras2* formed less aerial hyphae than *wt* and accordingly, produced relatively few conidia located primarily in a crescent form at the upper top of the medium (Fig 1A). When inoculated in liquid medium, *Δras2* grew slowly compared to *wt* and produced mycelia dominantly in colonial form (small balls) instead of mycelial mats (Fig S1A).

To confirm that deletion of *ras2* is responsible for the morphological defects of the mutant, we generated a strain expressing a tagged form of RAS2 under the control of the *cpc-1* promoter in the *Δras2* background. Comparable *ras2* transcript levels were detected in *Δras2, cpc-1-ras2* and *wt* and an anti-FLAG antibody recognized a protein band with the expected molecular weight (29.5 kDa) in the total cell lysates of *Δras2, cpc-1-ras2,* but detected no signal in the lysates prepared from either *Δras2* or *wt* cells (Fig 1B). Expression of $RAS2_{FLAG}$ rescued the morphological defects observed in the mutant, indicating that the fusion protein is functionally active and the altered morphology was a consequence of *ras2* deficiency (Fig 1A, Fig S1A).

To analyze the conidiation rhythm of *Δras2*, race tube assays were performed under constant conditions (Fig 1C). Similarly to the *smco7* mutant (56), *Δras2* had a reduced growth rate compared to *wt*. In accordance with literature data (7,46), *wt Neurospora* did not display conidiation rhythm on minimal medium but showed sustained banding when the reactive oxygen species generator menadione was present. In *Δras2*, however, no conidiation rhythm was detected even when high concentrations (100 μM) of menadione were applied, suggesting that deletion of *ras2* affects the rhythmic output.

Since entrained conditions generally support conidiation rhythm (47), we incubated race tube cultures in 12/12-hours light/dark cycles. Under these conditions, also *Δras2* displayed sustained banding for several days (Fig S1B). However, when the phase of conidiation was thoroughly analyzed and compared with that of *wt*, a significant delay was observed in *Δras2*, suggesting that RAS2 activity affects the clock function also under entrained conditions (Fig 1D). In *Δras2, cpc-1-ras2* the phase of banding was similar to the phase detected in *wt*, indicating that expression of $RAS2_{FLAG}$ in the mutant background results in rescue of the clock function.

Involvement of RAS2 in glucose compensation of the circadian clock

To further analyze the effect of *ras2* deficiency on clock function, we generated a *Δras2* strain that expressed luciferase under the control of the *frq* promoter. In the first experiments, we used a

medium containing no glucose and followed the rhythm of *frq*-promoter activity in constant darkness (Fig 2A, upper panel). Although a robust rhythm was detected in both the *Δras2* and the *wt* background, the circadian period was significantly longer in the mutant than in the control strain suggesting that the RAS2-mediated signaling interacts with the molecular clock (Fig 2B). As it was expected from earlier data (4), addition of glucose to the medium did not affect either the robustness or period of the rhythm in *wt* (Fig 2A, lower panel and Fig 2B). In the *ras2* deficient strain, however, a dampening in the amplitude was observed and the period was more than two hours longer compared to *wt*. These data indicate that compensation of the oscillator function against glucose requires the action of RAS2.

Next, we examined whether expression of *ras2* is controlled by the circadian clock in *wt*. As shown in Fig 3, whereas *frq* levels displayed rhythmic changes in our samples, *ras2* mRNA did not oscillate indicating that *ras2* is not a clock-controlled gene in *Neurospora*.

Expression and phosphorylation of the molecular clock components is affected by the *ras2* mutation

In the next experiments we compared expression and phosphorylation of the main clock components in *wt* and *Δras2*. Electrophoretic mobility of the core clock proteins FRQ, WC-1 and WC-2 is dependent on their phosphorylation status, *i.e.* phosphorylation is reflected by the presence of protein forms with slower electrophoretic mobility (57,58). On the other hand, activity of the clock components correlates with their phosphorylation status; *i.e.* hypophosphorylated nuclear FRQ is active in the negative feedback by supporting phosphorylation and thus inactivation of the WCC (16).

As the results of the *in vivo* luciferase measurements suggested that impact of the *ras2* mutation on the clock function depends on glucose, *Neurospora* was inoculated in glucose containing standard medium. In constant light (LL), only a slight difference in FRQ protein level was detected and this was represented by a higher accumulation of hypophosphorylated FRQ forms in *Δras2* (Fig 4A, left panel). WC-1 levels were higher and the hyperphosphorylated fraction of the protein dominated in *Δras2* compared to *wt*. Although WC-2 expression levels were similar in both strains, a slightly higher fraction of the hyperphosphorylated forms was detected in *Δras2*. To further examine the effect of the *ras2* mutation on the molecular clock, we performed subcellular fractionation and analyzed the expression of clock proteins in cytosol and nucleus (Fig 4A, right panel). A higher fraction of the hypophosphorylated FRQ forms was detected in the nucleus of *Δras2* compared to *wt*. In accordance with the higher activity of hypophosphorylated FRQ in promoting phosphorylation of the WCC, hyperphosphorylated forms of both WC proteins dominated in *Δras2* nuclei. Moreover, a substantial fraction of WC-1 was detected in the cytosol of the mutant, indicating that the excess of WC-1 in *Δras2* is mainly present in an inactive cytosolic form.

To further investigate the molecular oscillator, we analyzed the expression of FRQ in cultures grown in constant darkness (DD). Although both strains displayed time-dependent changes in FRQ levels on the first day (Fig 4B, Fig S2A), FRQ signals peaked later in *Δras2* than in *wt*. This difference was also well represented by the reduced *frq* RNA expression and the very low level of newly synthesized hypophosphorylated FRQ forms in *Δras2* at DD12 (Fig 4C). During the second circadian cycle both the levels and the phosphorylation of FRQ displayed oscillation in the *wt*, whereas less pronounced changes in the phosphorylation and expression of the protein were detected in *Δras2* (Fig 4B, Fig S2A). In *wt* newly synthesized FRQ becomes progressively phosphorylated from DD16, therefore we investigated both expression levels and phosphorylation of FRQ between DD16 and DD26 with a more detailed resolution on the same gel (Fig 4D). Overall FRQ levels were lower in *Δras2* than in *wt*. To asses FRQ levels in *Δras2* relative to *wt*, we constructed a calibration line by loading the gel with increasing quantities (25-125 %) of the *wt* protein extracts and determined relative FRQ levels at DD16 when newly synthesized FRQ is already well detectable in both strains (Fig S2B, Fig 4E left panel). We found significantly lower FRQ expression in *Δras2* lysates compared with *wt* samples. In addition, whereas in *wt* FRQ was gradually and continuously shifted towards the

hyperphosphorylated forms, in *Δras2* this shift was slow and the highly phosphorylated forms of the protein were absent even at DD26 (Fig 4D). When the ratio of hypo- and hyperphosphorylated FRQ forms was determined, the results indicated that distribution of these FRQ forms displayed a marked difference over time in the two strains (significant time x strain interaction of Repeated Measures ANOVA) (Fig 4E right panel). Thus, absence of RAS2 affects phosphorylation of FRQ also under constant conditions.

According to data in the literature, rhythm of WC-1 expression is — in most cases — not as robust as that of FRQ (59,60). While WC-1 showed time-dependent changes in *wt* in our hands with the first trough and peak phases similar to those reported by others (59,61), no rhythm of WC-1 expression could be detected in *Δras2* (Fig S2C and D). This finding also suggests that the molecular clock function is less robust in the mutant than in *wt*. We quantified and compared WC-1-specific signals in *wt* and *Δras2* at DD24 and found no significant difference (Fig S2E).

Since phosphorylation of FRQ may influence nuclear accumulation of the protein, we analyzed cytosolic and nuclear FRQ fractions during a circadian cycle in *wt* and the *ras2* deficient strain (Fig 5). Following the LD transition, in the first 8 hours in both strains a similar decrease of FRQ levels was observed in both cellular compartments. In *wt* FRQ levels peaked at DD16 and then became rapidly reduced with a kinetics similar to that described earlier (62). In accordance with the delayed synthesis of FRQ, cytosolic protein levels increased more slowly in *Δras2* than in the *wt*. Increase of the nuclear amount of the protein was, however, similar to that in the *wt*, and instead of a rapid reduction of nuclear FRQ levels seen in *wt*, a substantial fraction of FRQ was detected in the nucleus even at DD24. To analyze this difference, we determined nuclear/cytosolic FRQ-specific signal ratios. At both DD16 and DD20 significantly higher ratios were obtained for *Δras2* than for *wt*. (Fig 5). In addition, Repeated Measures ANOVA with two factors (time and strain) showed significant strain effect for the whole period analyzed (Fig S3). In summary, above data suggest that RAS2 is involved in the control of subcellular localization of FRQ under constant conditions.

Response of the molecular clock to glucose is dependent on RAS2

Based on our results showing that the circadian phenotype of *Δras2* is dependent on glucose, we aimed to examine how FRQ expression and subcellular distribution respond to glucose in *Δras2* and *wt*. We cultured mycelia in starvation medium overnight in LL and then treated them with different levels of glucose for four hours (Fig 6). When a relatively low concentration (0.5%) of glucose was applied, high levels of FRQ accumulated in the nucleus of *Δras2*, whereas nuclear FRQ levels remained low in *wt*. Addition of glucose in higher concentration (4%) effectively increased the accumulation of hypophosphorylated and thus total levels of FRQ in *wt* as well. However, when protein signals were quantified, significantly higher fraction of FRQ was detected in the nuclei of *Δras2* than in the nuclei of *wt*. To exclude the possibility that osmotic changes contributed to the observed effect of high glucose levels in *Δras2*, we repeated the experiment by adding NaCl instead of glucose in the same osmotic concentration. NaCl did not affect the nuclear levels of FRQ in *Δras2* (Fig S4A). Moreover, FRQ was also shifted towards the nucleus in *Δras2* when cultures were incubated overnight in a glucose containing medium (Fig S4B). Our observations indicate that RAS2 plays an important role in the control of nuclear levels of the oscillator protein in response to glucose.

Analysis of the signaling pathway between RAS2 and the circadian clock

RAS2 was found to control both a MAP kinase cascade and the adenylate cyclase-dependent pathway in *Saccharomyces cerevisiae* (63-65). The MAPK component of the cascade is represented by Kss1. Therefore, we examined the levels of the active forms of MAK2, the *Neurospora* ortholog of Kss1 (66,67). Based on the sequence homology (67), we used a phospho-specific commercial antibody that recognizes the phosphorylated and thus active form of the mammalian MAPK (ERK). According to data in the literature (67,68), a protein band with the approximate molecular weight of 43 kDa could be detected in both strains (Fig S5). Although phospho-MAK2 levels showed relative large variations when independent cultures were

compared, we found no consistent strain-specific differences.

Based on the data showing that RAS2 activates adenylate cyclase in yeast (28), in the next experiments we addressed whether *Neurospora* RAS2 similarly interacts with the adenylate cyclase. We generated an *E. coli* strain expressing a fusion protein containing the RAS-binding domain of adenylate cyclase and an N-terminal GST-tag. The fusion protein was coupled to glutathione agarose and the resin was incubated with the lysate of *Δras2, cpc-1-ras2* containing $RAS2_{FLAG}$. $RAS2_{FLAG}$ was specifically bound to the resin, suggesting that RAS2 may be an interaction partner of the adenylate cyclase (Fig S6A). To further examine the possible involvement of the RAS/adenylate cyclase-mediated pathway in the glucose response of the clock, we tested the effect of the cell-permeable and relatively stable cAMP analogue 8-Br-cAMP in the presence of glucose. 8-Br-cAMP had no effect on the rhythm in *wt, frq-luc* (Fig 7A), whereas it slightly but significantly shortened the period in *Δras2, frq-luc* (Fig 7B). Thus, presence of the cAMP analogue partially rescued the impaired function of the circadian clock of *Δras2*. Moreover, addition of 8-Br-cAMP reduced the glucose-induced nuclear accumulation of FRQ in the mutant (Fig S6B), also suggesting that a cAMP-mediated pathway may link RAS2 to the molecular clock.

DISCUSSION

From fungi to mammals RAS family GTPases play important roles in signaling systems controlling cellular metabolism, cell growth and differentiation (32,33). In mammals, importance of RAS proteins as regulators of cell growth is emphasized by the prevalence of upregulation of RAS-mediated signaling in many tumor types (69). Expression of the mammalian RAS protein can rescue the loss of endogenous RAS in yeast, suggesting the functional conservation of RAS proteins among eukaryotic organisms (70). We found that in *Neurospora* the circadian period is significantly longer in the *Δras2* mutant than in *wt*, suggesting that RAS2 activity is crucial for normal function of the circadian clock. Moreover, in contrast to *wt* both the period and robustness of the rhythm were sensitive to glucose in *Δras2*; the period was significantly increased and the oscillation became dampened in glucose-containing medium. These findings indicate that activation of RAS2-mediated signaling is part of the compensatory mechanisms needed for robust clock function under different metabolic conditions. Both expression and phosphorylation of molecular clock components are characteristically affected by the deletion of *ras2*. Increased abundance in *Δras2* of the hypophosphorylated forms of FRQ — which are therefore more active in the negative feedback — is in accordance with the enhanced accumulation of the hyperphosphorylated and thus inactive forms of the WCC. In a recent work the speed of the phosphorylation cascade affecting FRQ activity in the negative feedback was proposed as the key factor determining circadian period (15). Our data showing delayed phosphorylation of FRQ in DD in *Δras2* together with a longer period are in good accordance with this model. Based on the data presented in this study we propose that RAS2 may fine-tune the circadian clock by controlling a cAMP-dependent pathway. In our model, elevation of glucose levels could lead to both increased translation rate and thus accumulation of newly synthesized hypophosphorylated FRQ forms and enhanced activity of the RAS2-dependent pathway. As a distal component of this pathway, PKA could directly or indirectly support phosphorylation of FRQ and thereby counteract the nuclear accumulation of FRQ forms active in the negative feedback. Conversely, in the *ras2* deletion strain in the presence of glucose, the tardy clearance of FRQ from the nucleus may result in prolonged inhibition of the WCC and thus an increase of the period and a dampening of the oscillation.

FRQ can be phosphorylated by PKA *in vitro* (71), suggesting that FRQ may be a direct substrate of the kinase. Moreover, the *pkac-1*^{ko} strain lacking the major catalytic subunit of PKA partially resembles the phenotype of the *Δras2* strain. Reduced growth rate and dampened rhythm of FRQ oscillation, although to different extents, were detected in both strains. However, expression of the WCC was differentially affected by the lack of PKAC-1 and RAS2. Whereas in *pkac-1*^{ko} expression of the WCs was low, WC-1 levels in *Δras2* were elevated in LL and were similar to the

wt levels in DD, suggesting that RAS2-independent PKA activity is sufficient to support expression of the WC proteins in the mutant. Expression of WC-1 is controlled by both light-dependent and light-independent mechanisms and is posttranslationally supported by FRQ (16,19,61,72,73). How the absence of RAS2 influences this complex regulation is still unclear.

Beside the cAMP-mediated signaling, a MAPK kinase module homologous to the mammalian Raf/Mek/Erk pathway can also be activated by the RAS system in different fungi (66,74). However, we did not find a clear difference in MAK2 activity between *Δras2* and *wt* in our samples. As MAP kinases other than Erk (Jun amino-terminal kinase (JNK) and p38) were also described as downstream effectors of RAS GTPases in higher eukaryotes and their homologous are present also in *Neurosposra* (5,75), a role of these pathways in the metabolic regulation of the clock of *Neurospora* cannot be excluded. Moreover, as MAP kinases are usually sensitive to the metabolic state of the cell (e.g. (76)), their contribution to the control of the circadian clock may be dependent on culturing conditions.

While data from the literature already implied that RAS-mediated pathways affect circadian time-keeping in different organisms, our study indicates for the first time that RAS GTPases may constitute a link between metabolism and the molecular clock. Our data suggest that in *Neurospora* RAS2 is required to maintain a robust rhythm with constant circadian period independent of changes in glucose availability in the environment. We propose that in addition to CSP1 and PRD-1 (4,26,27), RAS2 is a mediator of metabolic compensation of the *Neurospora* clock. While loss of CSP1 shortens the period in response to glucose, *prd-1* and *ras2* mutants show period lengthening under these conditions. This suggests that operation of counteracting systems may be required for stabilization of the circadian period in changing metabolic environment. The molecular mechanisms underlying this metabolic control are only partially understood and a plausible model has only been provided for the action of CSP1. Whereas CSP1 controls WC-1 levels, the RAS2-mediated pathway seems to influence primarily the action of FRQ in a glucose-dependent manner.

EXPERIMENTAL PROCEDURES

Plasmid construction

cpc-1-Flag-Strep-ras2 was constructed in two steps. By using *Neurospora* genomic DNA as template, a PCR product including the coding region of *ras2* and nucleotides coding for an N-terminal Strep-tag was amplified and inserted between the XbaI and HindIII sites of a p3XFLAG-CMV™-7.1 vector (Sigma Aldrich). This construct served as a template for the PCR product containing the coding region of *ras2* and nucleotides coding for two N-terminal FLAG-tags and a STREP-tag. This product was cloned as an AscI-SpeI fragment into *cpc-1-vvd-Strep* (25) resulting in replacement of the *vvd-Strep* region with the *Flag(2x)-Strep-ras2* fragment.

Neurospora Strains and Culture Conditions

The strains used in this study were the *wt* strain (FGSC #2489), *wt, his3* (FGSC #6103), *wt, frq-luc* (47), the *Δras2* (FGSC #12467) mutant deriving from the Neurospora Genome Project (55), the *Δras2, frq-luc* and the *Δras2, cpc-1*-ras2 strains. For generation of *Δras2, frq-luc*, a *Δras2, his3* strain was first generated by crossing *wt, his3* and *Δras2* according to the protocol published on the home page of the Fungal Genetics Stock Center (URL: http://www.fgsc.net/neurospora/NeurosporaProtocolGuide.htm) and pBM60-P*frq-luc-trpC* (generous gift of M. Brunner, (77)) was integrated into the *his-3* locus of *Δras2, his3* by electroporation (78). *Δras2, cpc-1-ras2* was generated by the integration of *cpc-1-Flag-Strep-ras2* into the *his-3* locus of the *Δras2, his3* strain.

Liquid cultures were incubated at 90 rpm and 30°C if not indicated otherwise. Vogel's media were supplemented with 0.5% L-arginine, 10 ng/ml biotin and the indicated amount of glucose. The standard liquid medium contained 2% glucose. Race tubes were prepared as described (72). When indicated, the race tube medium was supplemented with menadione. Race tubes were incubated at 30°C. Analysis of race tubes was performed by densitometry using the ChronOSX 1.0.7 β software (T. Roenneberg, LMU Munich) and according to the protocol described (47). The first day of the entrainment was excluded from the analysis of race tubes. In each series of

experiments data deriving from the same days were analyzed for all race tubes.
8-Br-cAMP was dissolved in NH_4OH and pH of the medium was restored by the addition of HCl.

Protein analysis
Extraction of *Neurospora* protein and Western blots were performed as described (13,47). For the subcellular fractionation the method described by Luo et al. (79) was used with slight modification. Briefly, buffer volumes were reduced to 1/10 and the centrifugation step to separate cytosol and nuclei was modified to 8800 g for 2 minutes at 4°C. 450 and 300 µg protein from cytosolic and nuclear fractions were analysed by SDS-PAGE, respectively. Detection of total protein levels (TP) by Ponceau staining was used as loading control. Detection of RGB-1 as a cytosolic marker was used to control the subcellular fractionation.
For the analysis of protein levels optical density of protein bands was quantified using the ImageJ software. Density values were corrected for loading differences using a well-defined region of the total protein stain as described (80). When all replicates could not be loaded onto the same gel, values obtained in *Δras2* samples were normalized to the control values determined in the corresponding *wt* or untreated samples on the same Western blot.

RNA analysis
Total RNA was extracted with the TriPure Isolation Reagent (Roche) and transcript levels were quantified as described earlier (47) and in the Appendix Supplementary Methods.

In vivo luciferase assay
96-well white plates with solid media containing Vogel´s salt solution, 0.1% L-sorbose, 1% agarose, 10 ng/ml biotin and 150 µM beetle luciferin (Promega) were inoculated with the luciferase-expressing strains. When indicated, 0.05% glucose and 1 mM 8-bromo-cAMP were added to the medium. Cultures were incubated for one day in constant darkness and subsequently for one day in constant light at 30ºC. Following a light-dark transfer, luminescence was detected for 4-5 days and data were analyzed as described (47).

Statistical analysis
For statistical analysis, the STATISTICA 7.0 (Statsoft Inc., Tulsa, OK, USA) software was used. Data in all figures are presented as mean ± SEM. Results were considered to be statistically significant if p value was < 0.05.

ACKNOWLEDGMENTS
We thank Krisztina Makara for excellent technical assistance and Ágnes Südy for critical reading of the manuscript. We are especially grateful to Michael Brunner for providing antibodies against WC-1, WC-2 and FRQ. We gratefully acknowledge the Fungal Genetics Stock Center and the *Neurospora* Genome Project for providing *Neurospora* strains. This work was supported by an EMBO-HHMI startup grant to K.K. and the National Research, Development and Innovation Office - NKFIH (K115953). K.K. is a Merit Scholar of the Semmelweis University.

CONFLICT OF INTEREST
The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS
N.G. and K.K. designed the experiments and wrote the manuscript. N.G., A.S. and K.E. performed the experiments.

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FIGURE LEGENDS

FIGURE 1.
RAS2 controls conidiation rhythm in *Neurospora crassa*. (A) The growth phenotype of *Δras2* is characteristically different from that of *wt* and is rescued by the expression of $RAS2_{FLAG}$ in the *Δras2, cpc-1-ras2* strain. Representative slants of the indicated strains are shown. (B) Expression of the tagged version of RAS2 in the *Δras2* background. Left panel: *ras2* RNA levels in the indicated strains grown on slants were determined relative to *actin* RNA by qPCR (n=4, SEM). Right panel: Indicated strains were grown in liquid cultures in LL. Whole cell extracts were analyzed by Western blotting using an anti-FLAG antibody. *: unspecific band, TP: total protein. (C) Menadione does not induce conidiation rhythm in *Δras2*. Race tubes were inoculated with *wt* and *Δras2* and contained menadione, as indicated. Following incubation in constant light for two days, race tubes were transferred to constant darkness. Representative race tubes are shown. The first black line on each race tube marked the growth front at the time point of the LD transition. (D) Phase of banding is delayed in *Δras2* under entrained conditions. Race tubes inoculated with conidia of *Δras2*, *wt* or *Δras2, cpc-1-ras2* were incubated in 12/12-hours light/dark cycles. For better comparison of the position of conidial bands, images were fitted so that the daily growth distances were similar in the parallel samples. Representative race tubes are shown. Average phases of the peak of conidiation were calculated and statistically analyzed (n=7 (*wt*), 18 (*Δras2*), 14 (*Δras2, cpc-1-ras2*), SEM, ***$p < 0.005$, two sample t-test).

FIGURE 2.
RAS2 controls the circadian clock in a glucose-dependent manner. (A) The *ras2* mutation affects the period and robustness of the oscillation of *frq* promoter activity. *In vivo* luciferase assay was performed on the indicated strains with (lower panel) or without (upper panel) glucose in the medium. Time-dependent changes in luciferase activity as averaged curves are shown from parallel measurements (10-14 samples) of a representative experiment. Black and red arrows indicate peak phases of luciferase activity in *wt, frq-luc* and *Δras2, frq-luc*, respectively. (B) The circadian period is increased and shows glucose sensitivity in *Δras2*. Time-dependent changes of bioluminescence in the indicated strains were detected as described in (A) and periods were analyzed with the Chrono program (n=22-28, SEM, **$p < 0.01$, two-way ANOVA, Tukey HSD test).

FIGURE 3.
Expression of *ras2* is not rhythmic. Liquid cultures of *wt* were synchronized by a light-dark transfer and harvested at the indicated time points in DD. Levels of *frq* (black line) and *ras2* (gray line) RNA were determined relative to *actin* RNA by qPCR (n=3, SEM).

FIGURE 4.
Expression and oscillation of the molecular clock components are altered in *Δras2*. (A) RAS2 affects both expression and phosphorylation of FRQ and the WC proteins in LL. Liquid cultures were inoculated with the indicated strains and incubated in constant light for 3 days. Whole cell extracts (left panel) or cytosolic (C) and nuclear (N) fractions (right panel) were analyzed by Western blotting. Solid and dashed arrows indicate hyper- and hypophosphorylated forms of the proteins, respectively. RGB-1 was detected as a cytosolic marker (81). In the right panel for WC-2 both long (l) and short (s) exposures are shown. (B) FRQ oscillation is different in *wt* and *Δras2*. Cultures of *wt* and *Δras2* were harvested at the indicated time points in DD and protein expression was analyzed by Western blotting. Representative Western blots are shown. (TP: total protein) (C) Reduced expression of *frq* RNA and hypophosphorylated FRQ forms in *Δras2* at DD12. RNA (upper panel) and protein extracts (lower panel) were prepared from cultures harvested at DD12. RNA levels were determined relative to *actin* (n=4, SEM, $*p < 0.05$, two sample t-test). Protein levels were analyzed by Western blotting. Representative Western blot is shown. The dashed arrow indicates newly synthesized hypophosphorylated forms of FRQ. (D) Phosphorylation of FRQ is affected by RAS2 in constant darkness. Experimental details as described for (B). Protein expression was analyzed by Western blotting. The dashed line indicates the midpoint of the electrophoretic mobility of FRQ at DD16 in *wt*. Protein signals above and below the dashed line at all time points were considered as hyper- and hypophosphorylated protein forms, respectively. (E) Quantification of FRQ expression and phosphorylation. Left panel: FRQ levels at DD16 in *Δras2* relative to *wt* were determined by densitometry. A calibration curve (Fig S2B) was constructed by loading the gel with different quantities (25-125 %) of *wt* protein extract and FRQ levels in *Δras2* relative to *wt* were calculated. 100 % corresponded to mean FRQ levels in the *wt* samples (n=4, SEM, $***p < 0.005$, paired t-test). Right panel: Hyper- and hypophosphorylated FRQ forms (see Fig 4D) were quantified by densitometry and the ratio of hyper- and hypophosphorylated protein forms in *wt* and *Δras2* is shown at the indicated time points (n=3, SEM, $***p < 0.005$, significant time x strain interaction (ANOVA with two factors, time: repeated, strain: non-repeated), Tukey HSD test).

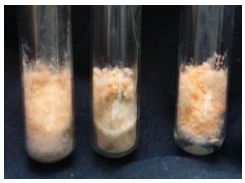
FIGURE 5.
RAS2 is involved in regulation of nuclear FRQ levels in DD. Cultures of *wt* and *Δras2* were harvested at the indicated time points in DD. Cytosolic (C) and nuclear (N) fractions were prepared and FRQ levels were analyzed by Western blotting. Left panel: Representative Western blots are shown. RGB-1 was detected as a cytosolic marker. (TP: total protein) Right panel: Nuclear/cytosolic signal ratios were calculated, and values of *Δras2* samples were normalized to the control ratio determined for the corresponding *wt* sample on the same Western blot (n=3-4, SEM, $*p <0.05$,$**p < 0.01$, one sample t-test).

FIGURE 6.
RAS2 prevents glucose-dependent accumulation of FRQ in the nucleus. *wt* and *Δras2* mycelia grown in standard liquid medium were transferred into a medium containing no glucose. Following an incubation for 16 hours, cultures were treated with either 0.5% or 4% glucose for four hours, as indicated. The experiment was performed in LL. FRQ expression in whole cell extracts (T) or cytosolic (C) and nuclear (N) fractions were analyzed by Western blotting. Left panel: Representative Western blots are shown. RGB-1 was detected as a cytosolic marker. (TP: total protein) Right panel: Nuclear/total signal ratios were calculated for the samples incubated without glucose or with 4 % glucose, as indicated. Values of *Δras2* samples were normalized to the control ratio determined for the corresponding *wt* sample on the same Western blot (n=4, SEM, $*p < 0.05$, one sample t-test).

FIGURE 7.
A cAMP analogue affects the circadian rhythm of the *ras2*-deficient strain. (A) 8-Br-cAMP does not influence the rhythm of *frq*-promoter activity in *wt*. 1 mM 8-Br-cAMP was added to the glucose-containing medium. Left panel: Time-dependent changes in luciferase activity as averaged curves are

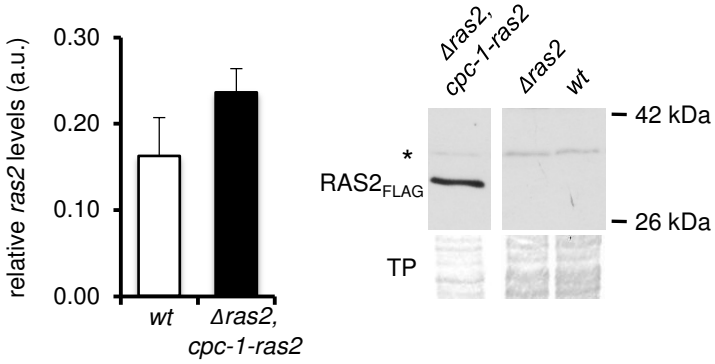
shown from a representative experiment (n=6). Right panel: Periods were analyzed with the Chrono program (n=24, SEM). (B) Shorter circadian period of *Δras2* in the presence of 8-Br-cAMP. For experimental details see (A). (Left panel: n=4; right panel: n=19-20, SEM, ***p < 0.005, two sample t-test)

A



wt *Δras2* *Δras2, cpc-1-ras2*

B



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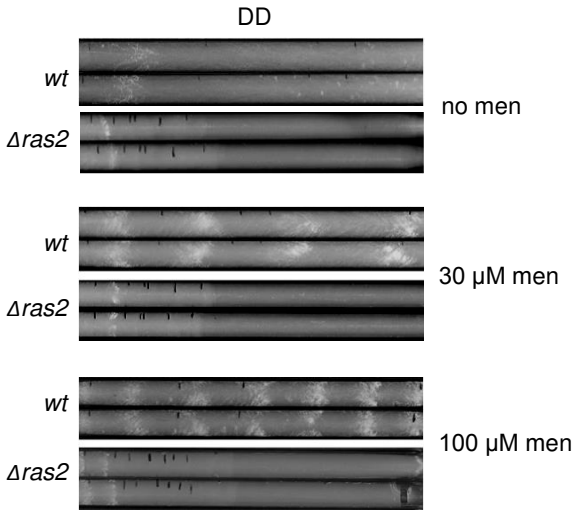
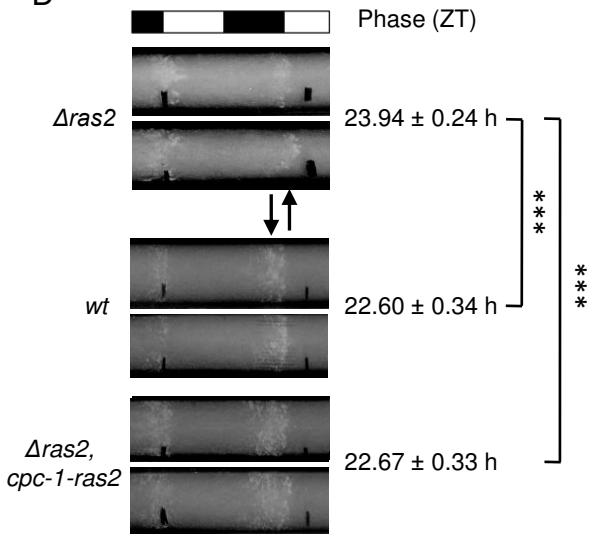
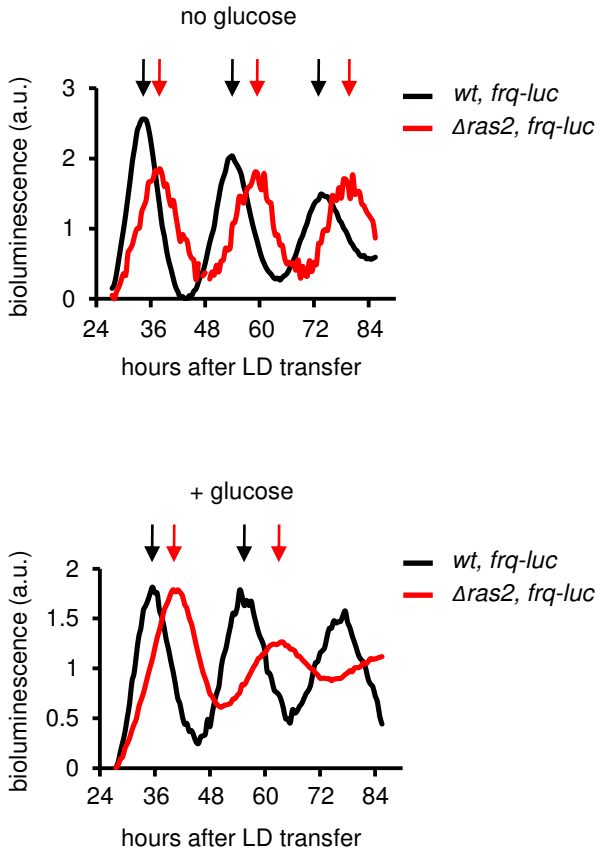


D



A


no glucose
bioluminescence (a.u.)
3
2
1
0
24
36
48
60
72
84
hours after LD transfer
wt, frq-luc
Δras2, frq-luc
+ glucose
bioluminescence (a.u.)
2
1.5
1
0.5
0
24
36
48
60
72
84
hours after LD transfer
wt, frq-luc
Δras2, frq-luc

B

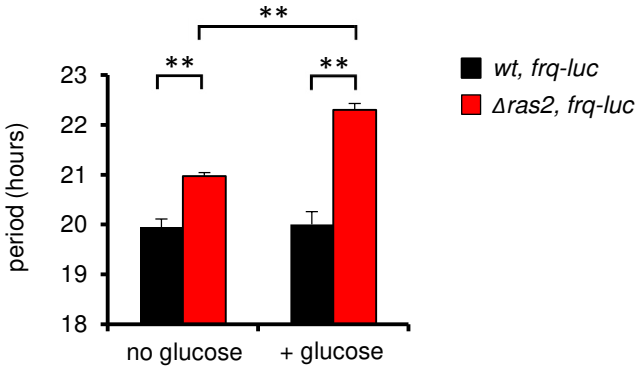

**
**
**
period (hours)
23
22
21
20
19
18
no glucose
+ glucose
wt, frq-luc
Δras2, frq-luc

Fig 3

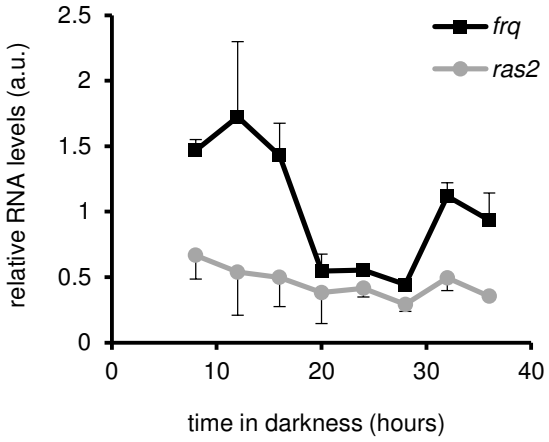

2.5
2
1.5
1
0.5
0
relative RNA levels (a.u.)
0
10
20
30
40
time in darkness (hours)
frq
ras2

Fig 4

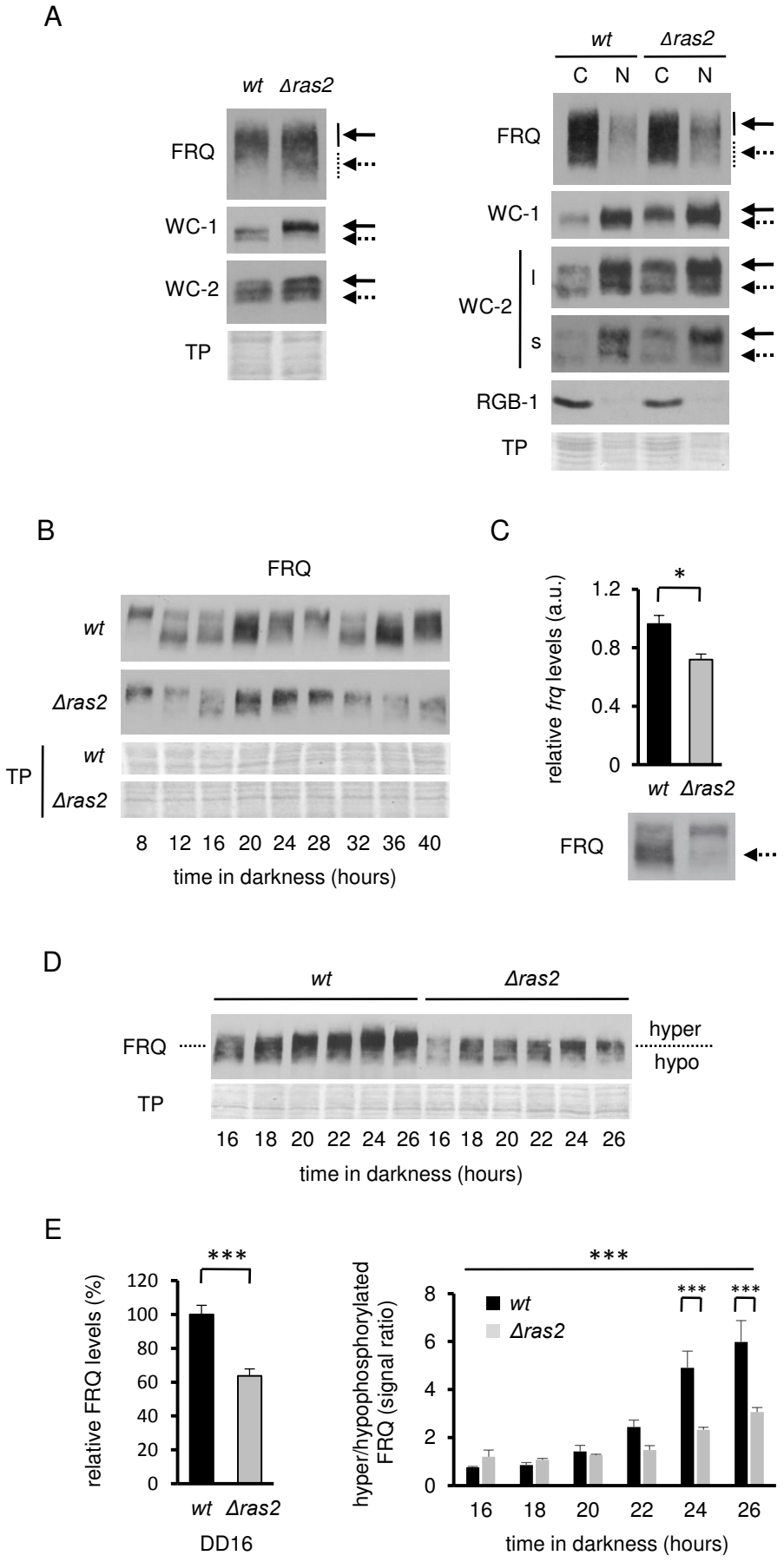
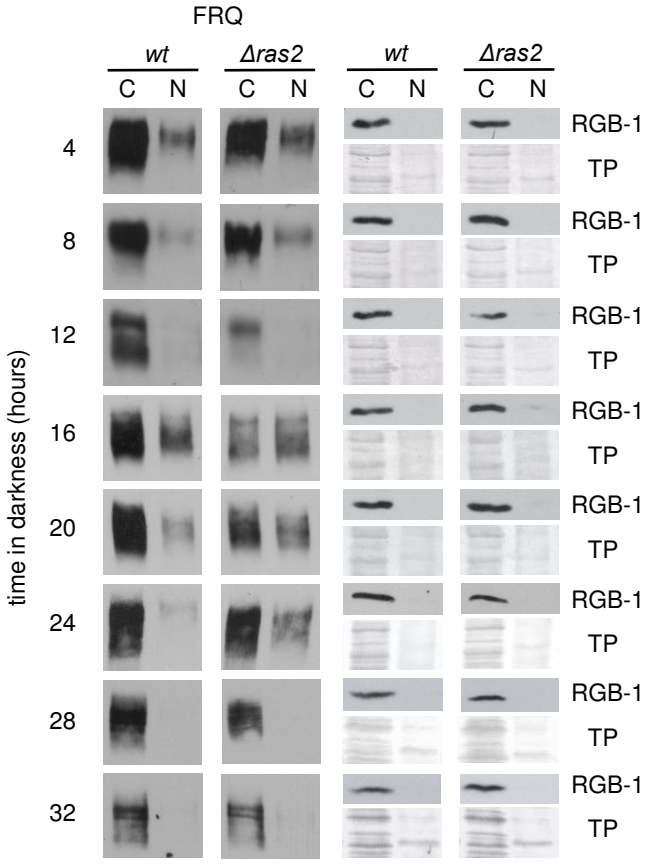
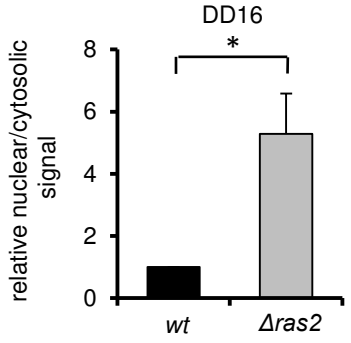

A
wt Δras2
FRQ
WC-1
WC-2
TP
wt Δras2
C N C N
FRQ
WC-1
WC-2
l
s
RGB-1
TP
B
FRQ
wt
Δras2
TP
wt
Δras2
8 12 16 20 24 28 32 36 40
time in darkness (hours)
C
relative frq levels (a.u.)
1.2
0.8
0.4
0
*
wt Δras2
FRQ
D
wt
Δras2
FRQ
hyper
hypo
TP
16 18 20 22 24 26 16 18 20 22 24 26
time in darkness (hours)
E
relative FRQ levels (%)
120
100
80
60
40
20
0
***
wt Δras2
DD16
hyper/hypophosphorylated FRQ (signal ratio)
8
6
4
2
0
***
wt
Δras2
***
***
16 18 20 22 24 26
time in darkness (hours)

Fig 5


FRQ
wt
Δras2
wt
Δras2
C N C N C N C N
time in darkness (hours)
4
8
12
16
20
24
28
32
RGB-1
TP


DD16
*
relative nuclear/cytosolic signal
0
2
4
6
8
wt
Δras2

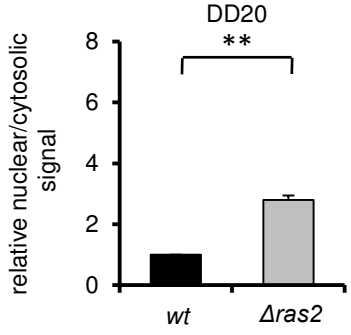
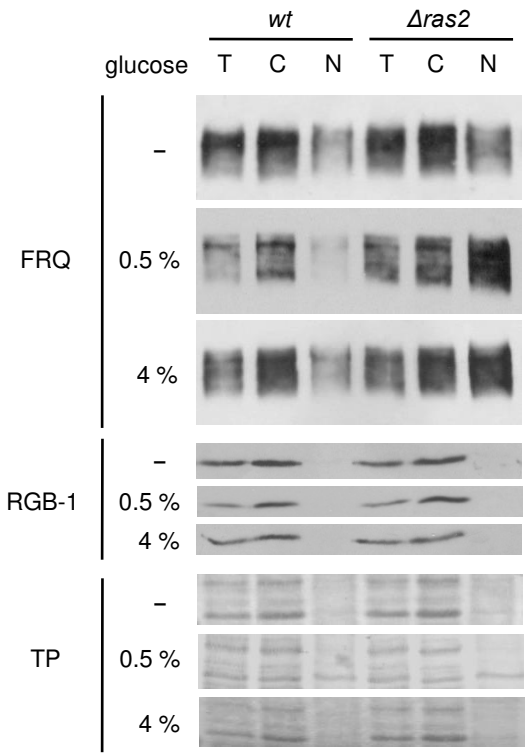
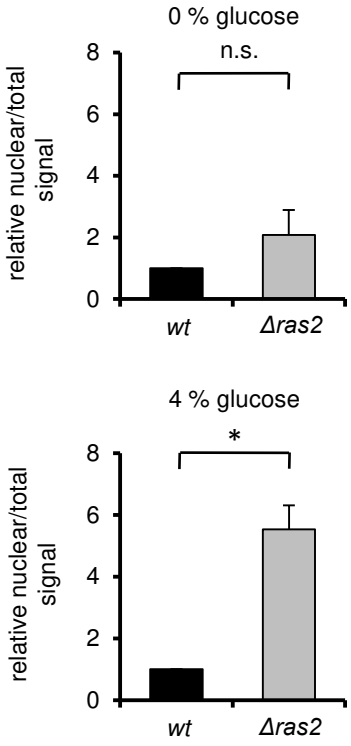
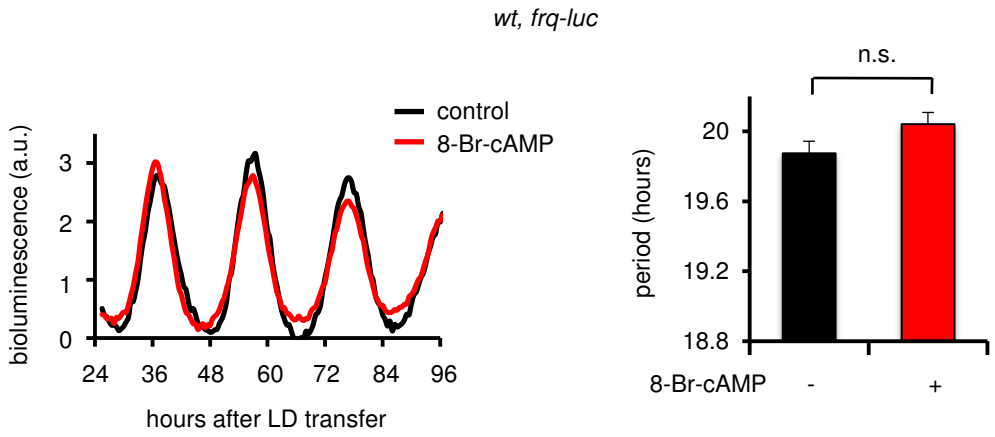

DD20
**
relative nuclear/cytosolic signal
0
2
4
6
8
wt
Δras2

Fig 6

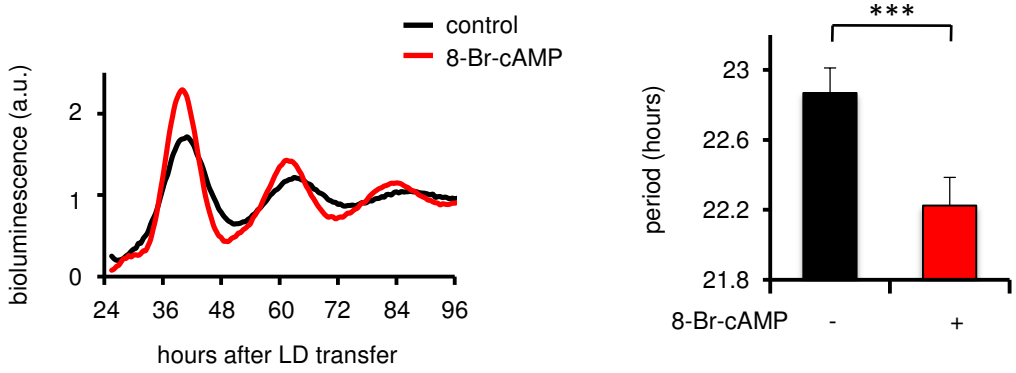

wt
Δras2
glucose
T C N T C N
FRQ
–
0.5 %
4 %
RGB-1
–
0.5 %
4 %
TP
–
0.5 %
4 %


0 % glucose
n.s.
relative nuclear/total signal
8
6
4
2
0
wt
Δras2
4 % glucose
*
relative nuclear/total signal
8
6
4
2
0
wt
Δras2

A


wt, frq-luc
control
8-Br-cAMP
bioluminescence (a.u.)
0
1
2
3
24
36
48
60
72
84
96
hours after LD transfer
n.s.
period (hours)
18.8
19.2
19.6
20
8-Br-cAMP
-
+

B


Δras2, frq-luc
control
8-Br-cAMP
bioluminescence (a.u.)
0
1
2
24
36
48
60
72
84
96
hours after LD transfer
***
period (hours)
21.8
22.2
22.6
23
8-Br-cAMP
-
+

The small G protein RAS2 is involved in the metabolic compensation of the circadian clock in the circadian model Neurospora crassa

Norbert Gyöngyösi, Anita Szöke, Krisztina Ella and Krisztina Káldi

J. Biol. Chem. published online July 20, 2017
