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Long and short isoforms of *Neurospora* clock protein FRQ support temperature compensated circadian rhythms

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Abstract

The large (l) and small (s) isoforms of FREQUENCY (FRQ) are elements of interconnected feedback loops of the *Neurospora* circadian clock. The expression ratio of l-FRQ versus s-FRQ is regulated by thermosensitive splicing of an intron containing the initiation codon for l-FRQ. We show that this splicing is dependent on light and temperature and displays a circadian rhythm. Strains expressing only l-FRQ or s-FRQ support short and long temperature-compensated circadian rhythms, respectively. The thermosensitive expression ratio of FRQ isoforms influences period length in *wt*. Our data indicate that differential expression of FRQ isoforms is not required for temperature compensation but rather provides a means to fine-tune period length in response to ambient temperature.

Keywords

circadian clock; control of period length; *Neurospora*

1. Introduction

Most organisms display daily rhythms in biological functions. These are supported by circadian clocks that regulate expression of a large number of genes. Circadian clocks in eukaryotes are composed of interconnected positive and negative feedback loops operating on a transcriptional, translational and posttranslational level. Even under constant conditions (e.g. constant darkness), these feedback loops support a self-sustained endogenous oscillation that mimics an astronomical day. In nature, clocks are precisely synchronized to the 24 h period of the earth's rotation by external cues such as light and temperature (reviewed in: [1–5]).

The transcription factor White Collar Complex (WCC), consisting of WC-1 and WC-2 subunits [6], and FREQUENCY (FRQ) [7] are crucial elements of the circadian clock of *Neurospora* [4]. The WCC activates transcription of *frq*, which encodes the central regulator of the WCC. FRQ inhibits in a negative feedback loop the activity of the WCC by promoting its phosphorylation and in a positive loop it supports accumulation of high levels of WCC [8,9,

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10]. The conflicting functions of FRQ in the negative and positive loops are coordinated in temporal and spatial fashion [11]. In the course of a day FRQ is progressively phosphorylated at multiple sites, which triggers its degradation during the night [4,5]. Towards the end of the night negative feedback is relieved and new *frq* RNA is synthesized. As a result *frq* RNA and FRQ protein display circadian rhythms in abundance. The FRQ rhythm promotes rhythmic inactivation and reactivation of the WCC, thus driving circadian outputs such as the synthesis of carotenoids and the formation of asexual spores (conidia) [17,18].

The *frq* gene encodes a large (l) and a small (s) isoform of FRQ protein, which start at codons 1 and 100 of the *frq* ORF, respectively [12]. Expression levels of l-FRQ increase significantly with increasing temperature [13,14,15], while levels of s-FRQ do not. FRQ abundance as well as the ratio of l-FRQ vs. s-FRQ are critical for a self-sustained rhythm [12–14]. *frq* RNA is transcribed by two promoters [15]. The long 5′-UTR of *frq* RNA contains several upstream open reading frames (uORFs) [13]. The uORFs, which are in non-consensus context for translation initiation, are more efficiently translated at lower temperatures resulting in trapping of scanning ribosomes and reduction of translation initiation at the *frq* ORF [14]. Accordingly, FRQ levels decrease with decreasing temperature. In addition, the l-FRQ vs. s-FRQ ratio is regulated by temperature dependent splicing of an intron encompassing the translation initiation site of l-FRQ [15,14]. This intron is referred to as intron 2 in [15] and intron 6 (*frq-I6*) in [14] and hereafter in this report. The splice-sites of *frq-I6* deviate from consensus and the intron is spliced with higher efficiency at lower temperatures [14]. Accordingly, the fraction of s-FRQ increases with decreasing temperature. Both thermosensitive mechanisms together, trapping of scanning ribosomes and splicing of *frq-I6*, regulate abundance and ratio of l- vs. s-FRQ in a temperature-dependent fashion.

Here we have addressed functional differences of the FRQ isoforms. We show that strains expressing either l-FRQ or s-FRQ alone display fairly well temperature-compensated circadian rhythms, so that the basis of compensation in wild type does not lie in the relative levels at which the two forms are expressed. However, s-FRQ supports a longer period than l-FRQ, and in *wt* strains, the free-running period decreases slightly with increasing temperature, in part reflecting the expression ratio l-FRQ vs. s-FRQ. Our data suggest that the thermosensitive l-FRQ vs. s-FRQ ratio provides a molecular means to facilitate control of period length at different temperatures.

2. Materials and methods

2.1 Strains, growth conditions and racetube assays

s-frq, *l-frq* and wt^{cont}., a *Δfrq* strain transformed with a *wt frq* gene, were constructed as described previously [14]. The plasmid used for mutagenesis was pBM60. It contains the *frq* locus and a portion of the *his-3* gene, which complements histidine deficiency of the background strain by homologuous recombination. $frq\text{-}ATG1^{mut}$ was created by site directed mutagenesis, changing the l-FRQ translation initiation site from AAC ATG GCG to AAT ATT GCG. In *frq-I6-DM* the splice donor was changed from C-GTGAGT to C-ACTAGT. The *frq* ORFs and part of the UTRs were sequenced prior to transformation. Construction of *hvc-16* was described previously [15]. Media for liquid cultures and racetubes were prepared as described [14]. Racetube evaluation was performed using the Chrono II software (Roenneberg, LMU Munich).

2.2 RT-PCR

Quantitative real-time RT-PCR was performed as described previously [14] using the same primers and fluorescent probes.

2.3 Western blot analysis

For all experiments shown 200 μg whole cell protein extract was treated with 100 units calf intestinal phosphatase (NEB) for 1 h at 37°C. Western blots were decorated with affinity purified antibodies against the C-terminus of FRQ.

3. Results

The ratio of l-FRQ to s-FRQ increases with increasing temperature due to thermosensitive splicing of *frq* RNA at intron 6 (*frq-I6*) [15,14]. To examine whether splicing of *frq-I6* is affected by the circadian clock, RNA samples obtained from light grown tissues and from circadian time courses at 22°C, 25°C and 28°C were analyzed by quantitative RT-PCR (Fig. 1A, B). At each temperature the fraction of spliced *frq-I6* oscillated in circadian fashion (Fig. 1B). The mean fractions of spliced *frq-I6* were about 11%, 9% and 6,4% at 22, 25 and 28°C, respectively while the amplitude of the oscillation remains relatively constant. The highest fraction of spliced *frq-I6* was present in the late subjective afternoon, i.e. approximately 4 h after levels of *frq* RNA have reached their circadian maximum. Thus, the fraction of spliced *frq-I6* is high when *frq* RNA levels are declining. The data indicate that the mean splicing efficiency of *frq-I6* is set by temperature, while splice isoforms accumulate in a clock dependent fashion. Interestingly, at lower temperatures even the maximal fraction of spliced *frq-I6* was lower in DD than in LL (Fig. 1A and B), suggesting that the abundance of spliced *frq-I6* RNA is light-dependent (see below).

We asked whether the two FRQ isoforms differ in function. As reported previously [14], mutation of the splice sites of *frq-I6* towards consensus ($I6^{opt}$) and towards non-splice sites ($I6^{mut}$) results in expression of s-FRQ and l-FRQ, respectively (Fig. 2A). To functionally characterize the FRQ isoforms, conidiation rhythms were analyzed under free-running conditions on race tubes at different temperatures. A comprehensive assessment revealed that rhythms in *l-frq* and *s-frq* strains are temperature-compensated with slight tendencies of *s-frq* and *l-frq* strains towards over- and undercompensation, respectively (Fig. 2B, C, Table 1). Furthermore, both strains entrain to 12 h light- 12 h dark cycles (not shown). These data indicate that thermosensitive splicing of *I6* is not required for temperature compensation of the clock per se. However, the period of *s-frq* was at each temperature 1 to 2 h longer than the period of *l-frq*. Due to the large number of conidial bands evaluated (>500) the differences in period length of *l-frq* and *s-frq* at 25°C and 28°C were highly significant with p values $< 4 \times 10^{-10}$ in the ANOVA test (Table 1B). The period lengths of *wt*-strains (*wt* and $wt^{cont.}$) were between those of *s-frq* and *l-frq* (Fig. 2B, C, Table 1A). The period decreased slightly with increasing temperature, correlating with the temperature dependent change of the expression ratio of s- vs. l-FRQ.

In summary, the period of strains expressing a single FRQ isoform is temperature compensated, indicating that expression of both FRQ isoforms is not required for temperature compensation per se. Rather, the temperature dependent expression ratio of l-FRQ vs. s-FRQ appears to modulate the period in *wt* strains in a systematic fashion and may thus serve to fine-tune the circadian oscillation in response to different ambient temperatures.

In order to generate the *s-frq* and *l-frq* strains, splice-donor, -branch (Lariat) and -acceptor sites of *frq-I6* had been mutated [14]. The Lariat sequence and acceptor site of *frq-I6* are located in the coding sequence of l-FRQ (Fig. 3A). Although the amino acid sequence of FRQ was not altered by the mutations, the RNA sequence contained 10 exchanges in the ORF (Fig. 3A). To address whether the period length was indeed affected by the FRQ isoforms rather than by the sequence alterations per se that were introduced to create the mutant *frq* alleles, we generated and analyzed additional strains that expressed only a single FRQ isoform due to different means. An additional s-FRQ expressing strain was constructed by mutating the translation

initiation site of l-FRQ (Fig. 3A). Splice sites of *frq-I6* were not affected in the mutant *frq-ATG1^{mut}* gene. The corresponding *frq-ATG1^{mut}* strain expressed predominantly s-FRQ at the three temperatures investigated (Fig. 3B). At 35°C low levels of a longer FRQ isoform, l*-FRQ, were detected, which could correspond to an inefficient translation initiation at AUG2 of the *frq* ORF (Fig. 3A). Since only a very minor fraction of *frq-I6* is spliced above 30°C [14], the high levels of s-FRQ indicate that ribosomes were efficiently scanning through the non-consensus translation initiation site at AUG2 (Fig. 3A). *frq-ATG1^{mut}* displayed a long period rhythm on racetubes, very similar to that of the *s-frq* strain generated by optimization of the *I6*-splice sites (Fig. 3C; Table 1A). In contrast to the slightly over-compensated period of *s-frq*, the period length of *frq-ATG1^{mut}* decreased slightly with rising temperature. This effect might be due to the low levels of the l*-FRQ isoform (initiating at AUG2), which may, similar to the l-FRQ isoform, support a shorter rhythm. Thus, the data confirm that s-FRQ supports a longer free-running rhythm.

To show that the short period of the *l-frq* strain is due to l-FRQ rather than to the sequence alterations in the transcript we analyzed two additional strains where only the splice donor of *frq-I6* was mutated. These strains did not carry sequence alterations in the ORF. In the first strain, *hvc16* [15], the splice donor had been mutated from C-GTGAGT to C-ATGACT and in the second strain, *frq-I6-DM*, the donor was changed to C-ACTAGT. Both strains expressed predominantly l-FRQ but low levels of s-FRQ were still detectable, especially if examined in the light (Fig. 4A and data not shown). The free running period of *hvc16* was very similar to that of *wt* (Fig. 4B, [15]) but was different from the period of the s-FRQ expressing strains, *s-frq* and *frq-ATG1^{mut}* at 25°C and 28°C (data not shown). The free-running period of *frq-I6-DM* was at all temperatures similar to the *l-frq* period (Table 1A). One interpretation is that the donor-only mutations allow expression of low levels of s-FRQ that can affect period length. The differences between *hvc16* and *frq-I6-DM* may be due to the low but different levels of s-FRQ that are still expressed in these strains.

4. Discussion

FRQ is a central component of positive and negative feedback loops of the circadian clock of *Neurospora*. Expression of FRQ is regulated in a considerably complex fashion [16]. *frq* is rhythmically transcribed by two promoters, P_{prox} and P_{dist} that are both controlled by WCC [15]. Intron 6 of *frq* RNA (*frq-I6*) is spliced in a thermosensitive manner leading to RNA species that encode l-FRQ and s-FRQ, respectively [15,14]. We show here that the fraction of spliced *frq-I6* is dependent on light, temperature, and on the circadian time. These observations could be accounted for if P_{prox} transcripts were spliced with higher efficiency than P_{dist} transcripts. P_{prox} transcripts are low in DD but efficiently induced by light [15]. Accordingly, overall splicing of *frq-I6* would be more efficient in LL. P_{dist} transcripts display a high amplitude rhythm in darkness. When P_{dist} transcripts decrease in a circadian manner the fraction of P_{prox} transcripts, and thus overall splicing of *frq-I6*, would increase.

In addition to the regulation of the l-FRQ vs. s-FRQ ratio by splicing of *frq-I6*, the abundance of both isoforms is controlled in a thermosensitive fashion at the level of translation [14]. These elaborate mechanisms obviously control the expression of l-FRQ and s-FRQ and the question arises why this might be so important. Overall levels of FRQ have been shown to be crucial for the amplitude of circadian oscillations [7,9,13,14] but what might be the function of the isoforms? The initiation codon of s-FRQ is conserved in *frq* genes of other filamentous fungi (Fig. 4C) along with the pattern of splicing [15], suggesting that l- and s-FRQ isoforms fulfill non-redundant functions. We show here that the free-running period of *wt* strains, though fairly well temperature compensated, decreases slightly with increasing temperature. *l-frq* supports a shorter and *s-frq* a longer rhythm than *wt*. However, the period length is slightly undercompensated in *l-frq* and slightly overcompensated in *s-frq*, suggesting the same

molecular mechanisms for temperature compensation (e.g. protein phosphorylation and/or turnover) affect the two FRQ isoforms in different manner. This antidromic behavior might become more pronounced when the mechanism for temperature compensation is challenged by unfavorable environmental conditions. In a *wt* strain, expressing both isoforms, the temperature-dependent change in period length reflects in part the thermosensitive expression ratio of the FRQ isoforms. Accordingly, the period in *wt* would be less affected by a partial loss of the temperature compensating mechanism than it would in strains expressing only one FRQ isoform. Thus, the ratio of l-FRQ and s-FRQ isoforms is not required for temperature compensation per se but rather provides a means to control and fine-tune period length in response to ambient temperature.

Acknowledgments

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References

1. Liu Y. Molecular mechanisms of entrainment in the Neurospora circadian clock. J Biol Rhythms 2003;18(3):195–205. [PubMed: 12828277]
2. Roenneberg T, Merrow M. The network of time: understanding the molecular circadian system. Curr Biol 2003;13:R198–207. [PubMed: 12620213]
3. Gachon F, Nagoshi E, Brown SA, Ripperger J, Schibler U. The mammalian circadian timing system: from gene expression to physiology. Chromosoma 2004;113:103–112. [PubMed: 15338234]
4. Dunlap JC, Loros JJ. The *Neurospora* circadian system. J Biol Rhythms 2004;19:414–424. [PubMed: 15534321]
5. Brunner M, Schafmeier T. Transcriptional and post-transcriptional regulation of the circadian clock of cyanobacteria and Neurospora. Genes Dev 2006;20:1061–1074. [PubMed: 16651653]
6. Talora C, Franchi L, Linden H, Ballario P, Macino G. Role of a white collar-1-white collar-2 complex in blue-light signal transduction. EMBO J 1999;18:4961–4968. [PubMed: 10487748]
7. Aronson BD, Johnson KA, Loros JJ, Dunlap JC. Negative feedback defining a circadian clock: autoregulation of the clock gene *frequency*. Science 1994;263:1578–1584. [PubMed: 8128244]
8. Lee K, Loros JJ, Dunlap JC. Interconnected feedback loops in the *Neurospora* circadian system. Science 2000;289:107–110. [PubMed: 10884222]
9. Cheng P, Yang Y, Liu Y. Interlocked feedback loops contribute to the robustness of the *Neurospora* circadian clock. Proc Natl Acad Sci USA 2001;98:7408–7413. [PubMed: 11416214]
10. Schafmeier T, Haase A, Káldi K, Scholz J, Fuchs M, Brunner M. Transcriptional feedback of *Neurospora* circadian clock gene by phosphorylation-dependent inactivation of its transcription factor. Cell 2005;122:235–246. [PubMed: 16051148]
11. Schafmeier T, Káldi K, Diernfellner A, Mohr C, Brunner M. Phosphorylation dependent maturation of *Neurospora* circadian clock protein from a nuclear repressor towards a cytoplasmic activator. Genes Dev 2006;20:297–306. [PubMed: 16421276]
12. Garceau NY, Liu Y, Loros JJ, Dunlap J. Alternative initiation of translation and time-specific phosphorylation yield multiple forms of the essential clock protein FREQUENCY. Cell 1997;89:469–476. [PubMed: 9150146]
13. Liu Y, Garceau N, Loros JJ, Dunlap JC. Thermally regulated translational control of FRQ mediates aspects of temperature responses in the *Neurospora* circadian clock. Cell 1997;89:477–486. [PubMed: 9150147]
14. Diernfellner ACR, Schafmeier T, Merrow MW, Brunner M. Molecular mechanism of temperature-sensing by the circadian clock of *Neurospora crassa*. Genes Dev 2005;19:1968–1973. [PubMed: 16107616]
15. Colot HV, Loros JJ, Dunlap J. Temperature-modulated alternative splicing and promoter use in the circadian clock gene *frequency*. Mol Biol Cell 2005;16:5563–5571. [PubMed: 16195340]

16. Brunner M, Diernfellner A. How temperature affects the circadian clock of *Neurospora crassa*. Chronobiol Int 2006;23(1–2):81–90. [PubMed: 16687282]

17. Correa A, Lewis ZA, Greene AV, March IJ, Gomer RH, Bell-Pedersen D. Multiple oscillators regulate circadian gene expression in *Neurospora*. Proc Natl Acad Sci U S A 2003;100(23):13597–602. [PubMed: 14597725]

18. Dong W, Tang X, Yu Y, Griffith J, Nilsen R, Choi D, Baldwin J, Hilton L, Kelps K, Mcguire J, Morgan R, Smith M, Case M, Arnold J, Schuttler HB, Wang Q, Liu J, Reeves J, Logan D. Systems biology of the neurospora biological clock. IET Syst Biol 2007;1(5):257–65. [PubMed: 17907673]

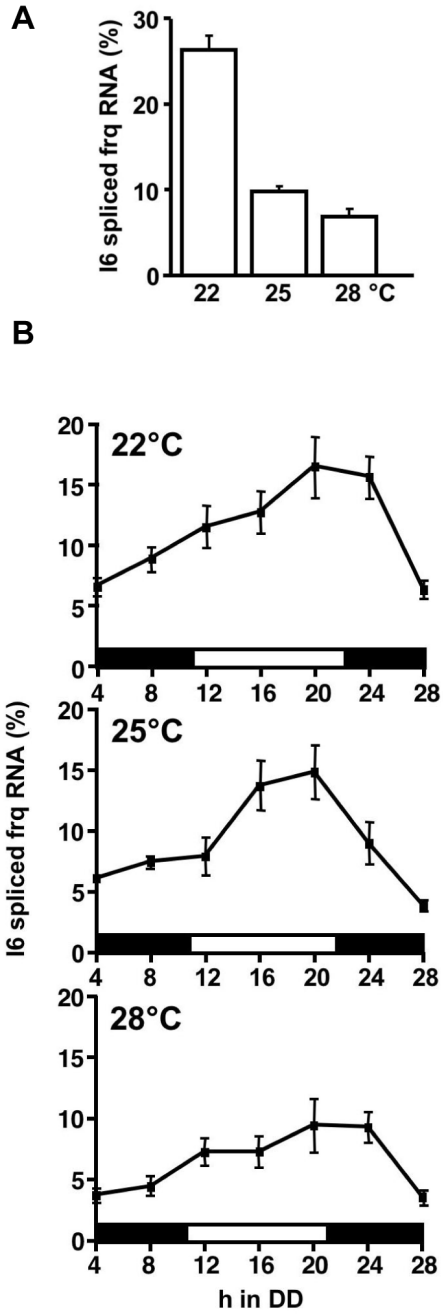


Fig. 1.

Splicing efficiency of *frq-I6* is set by temperature and modulated in a circadian and temperature dependent fashion. *Neurospora* cultures were grown at the indicated temperatures and harvested (**A**) in constant light (LL) and (**B**) after the indicated time periods in darkness (DD). RNA was prepared and quantified by RT-PCR using fluorescent probes specific to spliced *frqI6* and to total frq RNA [14]. The fraction (in %) of *frq* RNA spliced at I6 (spliced/total *frq* RNA) was plotted versus the time in DD. Black and white bars indicate subjective night and day, respectively.

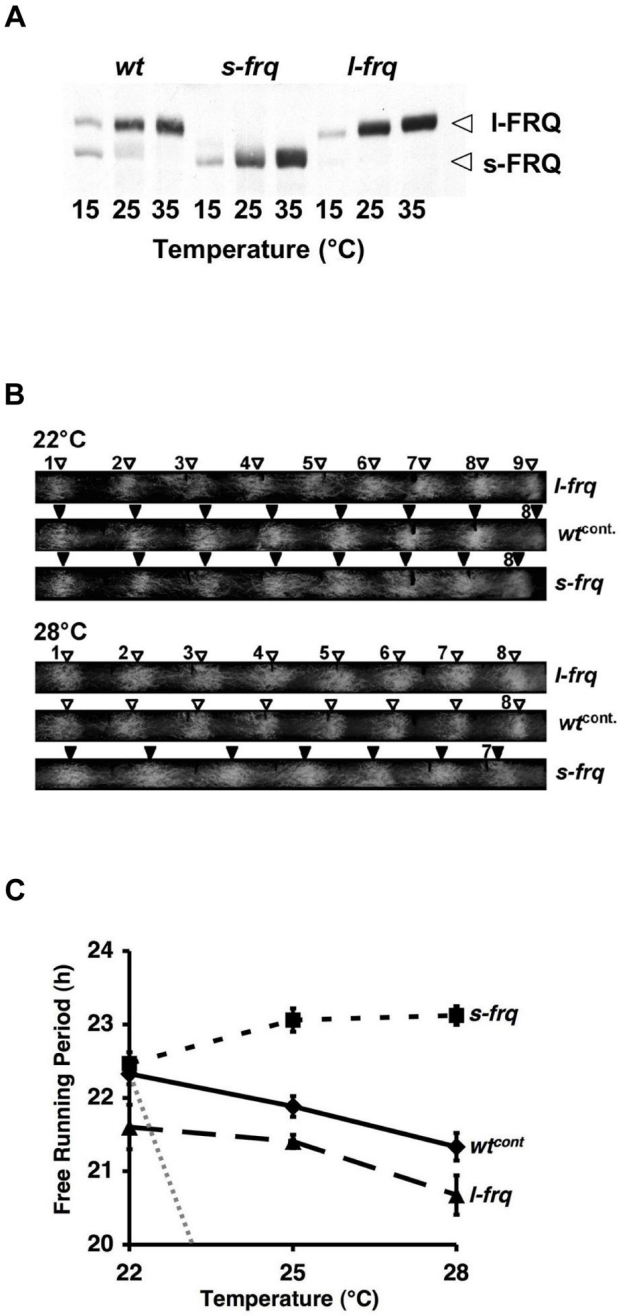


Fig. 2.

l-FRQ to s-FRQ determines the length of free running period. (**A**) Western blot analysis of protein extracts from cultures grown in LL at the indicated temperatures. Protein extracts were treated with phosphatase prior to SDS PAGE. Optimizing the splice sites of intron 6 (*s-frq*) results in the expression of s-FRQ while mutation of the splice sequences (*l-frq*) results in expression of l-FRQ. The temperature dependence of expression levels of FRQ is not affected by mutation of *frq-I6*. (**B**) Typical racetubes of *s-frq*, *l-frq* and the corresponding *wt* ($wt^{cont.}$) at 22°C and at 28°C. Conidial bands are indicated by arrow heads and numbered. The growth rate of the strains was identical. (**C**) *l-frq* and *s-frq* support considerably well temperature compensated short and long period rhythms, respectively. The period lengt of *wt* is between

that *l-frq* and *s-frq*. Data represent assessment of the temperature dependence of the free-running period of *l-frq*, *s-frq*, and $wt^{cont.}$ from 183 racetubes, each with an average of 5 conidial bands (see also Table 1A). The dotted line represents a hypothetical uncompensated reaction with a Q_{10} of 2.

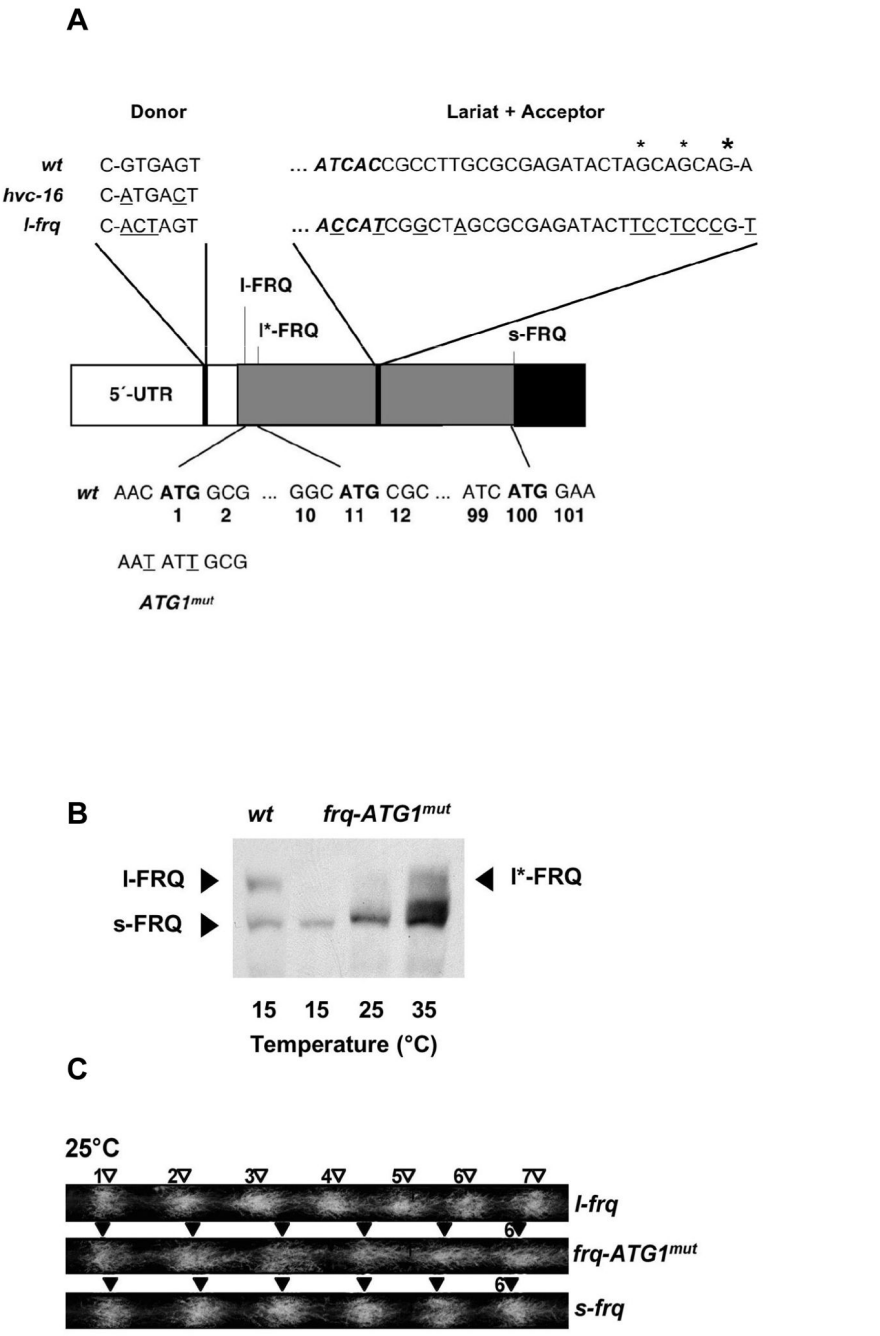
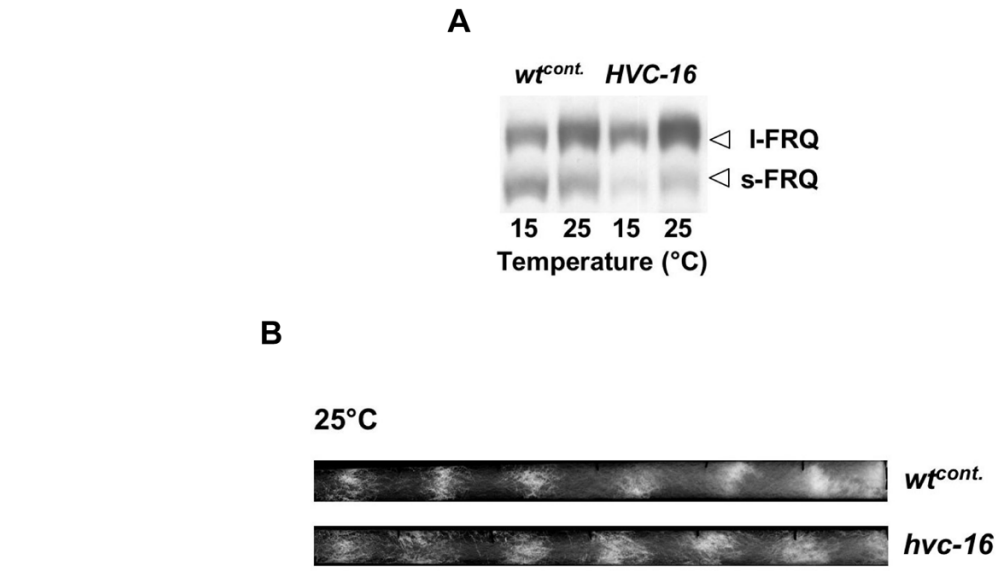


Fig. 3.

frq-ATG1^{mut} expresses predominantly s-FRQ and supports a long period circadian rhythm. (**A**) Schematic outline of the translation initiation sites of FRQ isoforms and intron 6. Top: *wt* donor, lariat (bold italic) and acceptor sites are shown above the outline. The large asterisk indicates the sequenced acceptor site, the small asterisks indicate putative cryptic acceptors. Corresponding sequences mutated in *hvc-16* and *l-frq* are also shown, sequence alterations are underlined. Bottom: Sequence context around the initiation sites of l-FRQ, l*-FRQ and s-FRQ. Numbers below the sequence refer to codons of l-FRQ. The initiation ATGs of l*-FRQ and s-FRQ correspond to codons 11 and 100 of l-FRQ, respectively. The sequence alterations introduced in the *frq-ATG1*^{mut} allele are shown.

(**B**) Western blot analysis of FRQ levels and isoforms expressed in *frq-ATG1*^{mut} at the indicated temperatures in constant light. (**C**) Racetube assays revealing that the free-running periods of *frq-ATG1*^{mut} and *s-frq* are similar (see also Table 1A).



C

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N.c.  MADSGDKSQGMRPPPFDSRGHPLPRRASPDKSITLENHRLARDTSSRVTSSSALGVTESQPQLKSSPTRRNSSGES------EPTNWFNQSNRNPAAAFHDESHIMEVDP
S.f.  MADSGDKSQGMRPPPFDSRGHPLPRRASPDKSITLHNHRLARDASSRVISSSALGVTGPQPQPTSSPTRRDSSGESNDTGQSDPKSWFDQSNRNPVAAFNDESNIMEVDP
L.a.    MSDLKSKRRNAP-SLAQHTYPRRVSPENSVTLRHHRLARDAS---LQAN-----SSRSQAATSSPRRNSSDESHDTGQSDANKWFDQSNLNPTANY--DSNAMDVDP
H.s.    MQPTEDLPRGTPLSSTGHPLPRRTSPEQSVTLRHHQLARDAS---LKASLGSAPPNDSNENSSSPRRASSGESHETGQSDAKKWFNQSNQNPTATF--DSNAMDVDP
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Fig. 4.

(**A**) l- and s-FRQ expressed in *hvc-16* in constant light. Western blot analysis of protein extracts from cultures grown in LL at the indicated temperatures. Protein extracts were treated with phosphatase prior to SDS PAGE. At 15 and 25°C low levels of s-FRQ can still be detected in the donor mutant *hvc-16*. (**B**) Racetube assay revealing that the free-running periods of *hvc16* (21.7) and wt^{cont} (21.5) are similar at 25°C (n=6). (**C**) Evolutionary conservation of s-FRQ in filamentous fungi. Alignment of the N-terminal part of the amino acid sequence of FRQ proteins of *N.c., Neurospora crassa; S.f., Sordaria fimicola; L.a., Leptosphaeria australiensis; H.s., Hypocrea spinulosa*. Methionine residues (M) are printed in bold.

Table 1

Table 1A Free-running periods of *wt* and mutant strains at the indicated temperatures. For *s-frq*, *l-frq* and wt^{cont} the results from ≥3 independent racetube experiments are summarized. n = number of racetubes evaluated per data point. In average 5 conidial bands were evaluated per racetube.

	22°C		25°C		28°C		
	τ (hr)	SEM	τ (hr)	SEM	τ (hr)	SEM	Q_{10}
$wt^{cont.}$	22.32	±0.14 (n=22)	21.88	±0.14 (n=26)	21.33	±0.19 (n=17)	1.08
wt	22.44	±0.08 (n=4)	21.88	±0.07 (n=4)	21.64	±0.01 (n=4)	1.06
l-frq	21.60	±0.30 (n=13)	21.41	±0.09 (n=31)	20.68	±0.27 (n=15)	1.07
s-frq	22.47	±0.15 (n=20)	23.06	±0.16 (n=20)	23.12	±0.13 (n=19)	0.95
frq-I6-DM	21.36	±0.21 (n=14)	21.66	±0.09 (n=21)	20.40	±0.23 (n=10)	1.08
frq-$ATG1^{mut}$	23.54	±0.07 (n=10)	23.33	±0.27 (n=5)	22.81	±0.30 (n=8)	1.05

Table 1B Significance of differences in period length between the indicated strains at different temperatures (see Fig. 2C and Table 1A). p-values of ANOVA test shown.

	22°C	25°C	28°C
$wt^{cont.}$*: s-frq*	0.5099	1.5939E-06	2.2801E-09
$wt^{cont.}$*: l-frq*	3.2171E-03	4.8717E-03	0.0474
s-frq: l-frq	8.5717E-03	5.1327E-13	4.0358E-10
$wt^{cont.}$*: frq-*$ATG1^{mut}$	4.8702E-06	2.2182E-04	2.4636E-04
$wt^{cont.}$*: wt*	0.7408	0.9933	0.4420
l-frq: frq-I6-DM	0.5068	0.0649	0.4536