Cellular calcium levels influenced by NCA-2 impact circadian period determination in *Neurospora*

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Running title: *Neurospora* clock and calcium-regulated phosphorylation

Abstract

Intracellular calcium signaling has been implicated in control of a variety of circadian processes in animals and plants but its role in microbial clocks has remained largely cryptic. To examine the role of intracellular Ca^{2+} in the *Neurospora* clock we screened knockouts of calcium transporter genes and identified a gene encoding a calcium exporter, *nca-2*, uniquely as having significant period effects. Loss of NCA-2 results in an increase in cytosolic calcium level, and this leads to hyper-phosphorylation of core clock components, FRQ and WC-1, and a short period as measured by both the core oscillator and overt clock. Genetic analyses showed that mutations in certain *frq* phospho-sites, and in *Ca*²⁺-calmodulin-dependent kinase (*camk-2*), are epistatic to *nca-2* in controlling the pace of the oscillator. These data are consistent with a model in which elevated intracellular Ca^{+2} leads to increased activity of CAMK-2 leading to enhanced FRQ phosphorylation, accelerated closure of the circadian feedback loop, and a shortened circadian period length. At a mechanistic level some CAMKs undergo more auto-phosphorylations in $\Delta nca-2$, consistent with high calcium in the $\Delta nca-2$ mutant influencing the enzymatic activity of CAMKs. NCA-2 interacts with multiple proteins including CSP-6, a protein known to be required for circadian output. Most importantly, expression of *nca-2* is circadian clock-controlled at both the transcriptional and translational levels, and this in combination

29 with the period effects seen in strains lacking NCA-2, firmly places calcium signaling within the larger
30 circadian system where it acts as both an input to and output from the core clock.

31 **Importance**

32 Circadian rhythms are based on cell-autonomous, auto-regulatory, feedback loops formed by
33 interlocked positive and negative arms, and they regulate myriad molecular and cellular processes in
34 most eukaryotes including fungi. Intracellular calcium signaling is also a process that impacts a broad
35 range of biological events in most eukaryotes. Clues have suggested that calcium signaling can
36 influence circadian oscillators through multiple pathways; however, mechanistic details have been
37 lacking in microorganisms. Building on prior work describing calcium transporters in the fungus
38 *Neurospora*, one such transporter, NCA-2, was identified as a regulator of circadian period length.
39 Increased intracellular calcium levels caused by loss of NCA-2 results in over-activation of calcium-
40 responsive protein kinases, in turn leading to a shortened circadian period length. Importantly,
41 expression of NCA-2 is itself controlled by the molecular clock. In this way calcium signaling can be
42 seen as providing both input to and output from the circadian system.

43 **Key words:** *nca-2*, calcium, FRQ phosphorylation, Ca²⁺-CaM-dependent kinases (CAMKs)

44 **Introduction**

45 In most eukaryotes and certain prokaryotes, circadian clocks link environmental cues such as
46 temperature and light to metabolism to regulate various physiological and molecular events ranging
47 from virulence and immunity to cell cycle control (1–3). In fungi and mammals, the core circadian
48 machinery is built based on a transcriptional-translational feedback mechanism in which the positive
49 arm drives transcription of components comprising the negative arm which, in turn, feeds back to
50 repress the positive arm, terminating its own expression. *Neurospora crassa* has been widely used as
51 a model eukaryote for circadian studies for decades. In *Neurospora*, the White Collar Complex
52 (WCC), formed from WC-1 and WC-2, serves as the positive arm transcriptional activator for the core
53 clock gene *frequency* (*frq*) by binding to one of two DNA elements, the *Clock box* (*C box*) in the dark
54 or the *Proximal Light-Response Element* (*PLRE*) in the light (4–6). FRQ, the gene product of *frq*,
55 interacts with FRH (FRQ-interacting RNA Helicase) (7, 8) and CK1 (casein kinase 1) (9) to form the
56 FFC complex, the negative arm that represses WCC activity by promoting its phosphorylation at a
57 group of residues (10).

58 Protein phosphorylation has been shown to control protein functions via protein-protein/DNA
59 associations, protein stability and activity, and subcellular localization, all of which have been proven

60 or suggested to regulate functions of circadian components (11–14). In *Neurospora*, FRQ is intricately
61 regulated by over 100 time-specific phosphorylation events (9, 15); multiple kinases, such as CKI,
62 CKII, PKA, and CAMK-1, and phosphatases, like PP2A, have been reported to directly or indirectly
63 control FRQ phosphorylation status (16–18). Extensive phosphorylation has also been observed on
64 WCC in light and dark conditions (10, 16, 19, 20). Recently, over 90 phosphoresidues have been
65 mapped on WC-1 and WC-2, governing their circadian repression and controlling circadian output,
66 and a small subset of these has been shown to be essential for the feedback loop closure (10).

67 Calcium as a second messenger regulates a wide variety of cellular pathways: for example, elevated
68 Ca^{2+} in the cytosol and mitochondria of neurons is required to synchronize neuronal electrical activity
69 (e.g., reviewed in (21)); all muscle fibers use Ca^{2+} as their main regulatory and signaling molecule
70 (e.g., reviewed in (22)); during mammalian fertilization, Ca^{2+} influx induces oocyte development in
71 many species (23). At the molecular level, enzymes and other proteins can be regulated by calcium
72 via allosteric regulatory effects (24). Indeed, diverse evidence also connects calcium signaling with
73 circadian regulation. In *Arabidopsis thaliana*, the concentration of cytosolic Ca^{2+} oscillates over time
74 (25, 26), which regulates circadian period length through action of a CALMODULIN-LIKE protein on
75 the core circadian oscillator (27). Circadian oscillation of Ca^{2+} has been observed in hypothalamic
76 suprachiasmatic nucleus (SCN) neurons, driving daily physiological events (28). In addition a small
77 body of literature has described effects of calcium ionophores and calmodulin antagonists on the
78 *Neurospora* clock (29–33). Although this research was published before there was sufficient
79 understanding of basic cellular physiology to fully interpret the work, it provides a rich context for
80 studies on the role of calcium signaling in the *Neurospora* clock.

81 Despite the paucity of recent data on circadian effects of calcium in fungi, the cellular physiology of
82 calcium metabolism in fungi including *Neurospora* is well understood (34–40) and is consistent with
83 general knowledge of animal cells. The resting concentration of Ca^{2+} in the cytoplasm of fungal and
84 mammalian cells is normally maintained at 50-200 nM (41–45), which is 20,000- to 100,000-fold lower
85 than that in a typical extracellular environment (46). To be maintained at this low level in the cell, Ca^{2+}
86 is actively pumped out from the cytosol to the extracellular space, reticulum, vacuole, and/or
87 mitochondria (34, 35, 47–51); bearing binding affinity to Ca^{2+} , certain proteins in the cell can also
88 contribute to lowering the level of free cytosolic Ca^{2+} (52).

89 To elicit signaling events, the cell releases Ca^{2+} from organelles or Ca^{2+} enters the cell from
90 extracellular environments. When stimulated by certain signals, cytoplasmic Ca^{2+} can be suddenly
91 increased to reach ~500-1,000 nM through activation of certain ion channels in the ER and plasma

membrane or indirect signal transduction pathways, such as G protein-coupled receptors (e.g., reviewed in (53, 54)). Cytosolic calcium bursts lead to activation of Ca^{2+} -calmodulin-dependent protein kinases (CAMKs) (55–59). In mammals, the CAMK cascade includes three kinases: CaM-kinase kinase (CaMKK), CaMKI, and CaMKIV; CaMKI and CaMKIV are phosphorylated and activated by CaMKK (55, 60–65). CaMKK and CaMKIV reside in the nucleus and cytoplasm while CaMKI is only located in cytosol; nuclear CaMKIV promotes phosphorylation of several transcription factors, such as CREB and CBP, to regulate gene expression (60, 66, 67). The *Neurospora* genome encodes four CAMK genes that are subject to diverse regulation, although little is known about their intracellular localization (18, 37).

By impacting a wide range of cellular processes, circadian clocks and calcium signaling are two classic regulatory mechanisms evolved to coordinate environmental factors, cellular responses, and metabolism. In this study, a screen of calcium regulators identified *nca-2*, a calcium pump gene, as a regulator of circadian period length in *Neurospora*. In $\Delta nca-2$ strains, FRQ and WC-1 become hyperphosphorylated; deletion of *camk-2* individually blocks the period shortening effect and FRQ hyperphosphorylations in $\Delta nca-2$. NCA-2 interacts with multiple proteins, which suggests that it might function in other cellular processes in addition to the circadian clock.

Results

Identification of *nca-2* as a regulator of the *Neurospora* circadian clock

Calcium signaling impacts circadian processes (e.g., 18, 30, 31) and directly controls a wide range of cellular and physiological events, but the means through which it impacts the circadian system is not fully described. *Neurospora* encodes several calcium transporter genes including *nca-1* (a SERCA-type ATPase), two closely related genes, *nca-2* and *nca-3* (PMCA-type ATPases), *pmr-1* (a SPCA-type Ca^{2+} -ATPase), and *cax* (a vacuolar $\text{Ca}^{2+}/\text{H}^{+}$ exchanger (35)). To facilitate monitoring circadian phenotypes, individual knockouts of these calcium pump genes were backcrossed to *ras-1^{bd}* and *frq C box*-driven *luciferase* and analyzed by race tube and luciferase assays. Of these deletion mutants tested, $\Delta pmr-1$ shows an extremely slow growth rate on race tubes (Figure 1A) but is nicely rhythmic with a slightly shorter period in the luciferase assay (Figure 1B); disruption of *nca-2*, a plasma membrane-located calcium pump, leads to a ~2-hr shorter period than that in wild type (WT) by race tube (Figure 1A) and luciferase (Figure 1B) analyses. (Of note, although on any given day the period estimates of strains bearing mutated calcium pumps showed normal precision, period length assays done on different days were more variable than is typical. For this reason, comparisons within figures always reflect assays of different strains done on the same day with the same medium.) Appearing at

DD12, newly synthesized FRQ in $\Delta nca-2$ is slightly more abundant than that in WT (Figure 1C, left) and *frq* mRNA levels in $\Delta nca-2$ are substantially higher in the subjective circadian night phase (DD4, 8, 24, 28) as compared to WT (Figure 1C, right), consistent with a faster running circadian clock in $\Delta nca-2$ (Figure 1A and 1B). The cytosolic calcium level in $\Delta nca-2$ is increased about 9.3 fold as compared to WT (36) suggesting a basis for this period change. To verify that the period shortening in $\Delta nca-2$ was due to this increased intracellular Ca^{2+} , the $\Delta nca-2$ strain was examined on race tubes prepared without calcium in the medium. Interestingly, $\Delta nca-2$ displays a WT period on race tubes using Ca^{2+} -free medium while its clock becomes ~4-hr shorter than WT with normal levels of calcium in the medium (Figure 1D), confirming that the role of *nca-2* in regulating the pace of the circadian oscillator is through controlling the cytosolic calcium level. These data indicate that *nca-2* is required for keeping calcium in cytosol at reduced levels to maintain a normal circadian period.

WC-1 and FRQ are hyper-phosphorylated in $\Delta nca-2$

WC-1 and FRQ are essential components in the positive and negative arms respectively of the *Neurospora* feedback loop and their phosphorylation has been proven to play an essential role in the determining their circadian functions (9, 10, 15, 16, 19). In addition to serving as the main transcription factor driving expression of *frq*, WC-1 is also the principal blue light photoreceptor for the organism, forming a homodimer (4) and getting hyper-phosphorylated (20) upon light exposure. To probe WC-1 and FRQ in $\Delta nca-2$, amounts and phosphorylation profiles of WC-1 and FRQ were analyzed by Western blotting using specific antibodies. The stability of FRQ in $\Delta nca-2$ is very similar to that in WT (Figure S1), and although WC-1 appeared slightly less stable, the cellular levels of WC-1 were even above those of WT, altogether suggesting that the stability of the core clock components does not determine the shortened period in $\Delta nca-2$ and the WC-1 level and stability are not consistent with the period length shortening in $\Delta nca-2$. Following a light-pulse, WC-1 is more abundant and hyperphosphorylated in $\Delta nca-2$ as compared to WT (Figure 2A) whereas, surprisingly, expression of *wc-1* is significantly lower than that in WT (Figure 2B); consistent with the data from the light-pulse experiment, in the dark, $\Delta nca-2$ contains a higher level of WC-1 with more phosphorylations (Figure 2C) despite a low mRNA level (~20-50% as in WT) (Figure 2D). These data suggest that *nca-2* regulates *wc-1* expression at both transcriptional and post-transcriptional levels independent of light and dark conditions. The hyper-phosphorylation of WC-1 in $\Delta nca-2$ was confirmed by a more sensitive assay (Figure 2E) using Phos-tag gels (68) such as have been applied to resolve single phosphoresidues on WC-1 and WC-2 (10). Similar to WC-1, FRQ in $\Delta nca-2$ is also more heavily phosphorylated than that in WT at DD14, 16, and 18 (Figure 2F) when newly synthesized FRQ is the

dominant form in the cell, and at DD24 (Figure 2G) when all FRQ becomes extensively phosphorylated prior to its turnover (see also Figure 1A). Altogether, these data demonstrate that WC-1 and FRQ become hyper-phosphorylated in $\Delta nca-2$, suggesting that the elevated calcium in $\Delta nca-2$ might lead to over-activation of kinase(s) or repression of phosphatase(s) targeting FRQ and WC-1, thereby altering their activities in the clock.

Epistasis analysis is consistent with an effect of $\Delta nca-2$ on FRQ but not on WCC

FRQ is phosphorylated in a time-specific manner at over 100 sites, and elimination of certain phospho-sites in different domains can cause opposite phenotypes on period lengths (9, 15). Because loss of *nca-2* elicits FRQ hyperphosphorylation at almost all time points examined (Figure 2F and 2G), we reasoned that this enhanced FRQ phosphorylation in $\Delta nca-2$ might contribute to the short period length in this strain. If this is so, then circadian period lengths in *frq* mutants encoding proteins that cannot be phosphorylated at key residues should not display the period shortening. To this end, several *frq* phospho-mutants displaying long circadian periods from (9) were individually backcrossed to $\Delta nca-2$ and *frq-luc*, and assayed by tracking bioluminescent signals in real-time in darkness. While circadian periods of *frq*^{S541A, S545A} and *frq*^{S548A}, and *frq*⁷ responded to loss of *nca-2* as did WT (Figure 3 and Figure S2A), absence of *nca-2* does not significantly influence the period length of *frq*^{S72A, S73A, S76A}, *frq*^{S538A, S540A}, or *frq*^{S632A, S634A} (Figure 3). These are all residues whose inability to be phosphorylated results in period lengthening (9), so the epistasis of these *frq* alleles is consistent with NCA-2 influencing FRQ phosphorylation at these sites.

To examine the effect of $\Delta nca-2$ on WCC phosphorylation and period length in the same manner, $\Delta nca-2$ was backcrossed to several *wcc* mutants eliminating key phosphoresidues that have been identified and shown to determine the circadian feedback loop closure (10), and monitored by the luciferase assay. The absence of *nca-2* further shortens the periods of *wc-1*^{S971A, S988A, S990A, S992A, S994A, S995A} and *wc-2*^{S433A} respectively (Figure S2A), suggesting that *nca-2* regulates the core oscillator independent of WCC phosphorylation at the sites essential for its repression. Consistent with this, in $\Delta nca-2$ the phosphorylation levels of WC-1 S971 and S990, two key sites required for FFC-mediated WCC repression, are similar to that in WT (Figure S2B), further suggesting that altered phosphorylation of the positive arm in the oscillator is not the cause of the short period of $\Delta nca-2$.

$\Delta camk-2$ does not further shorten the period of $\Delta nca-2$

Data in Figures 2 and 3 are consistent with NCA-2 acting through kinases or phosphatases on FRQ, and the elevated calcium in $\Delta nca-2$ (36) might activate Ca²⁺-responsive kinases to over-phosphorylate

FRQ (Figure 2F and 2G). CAMKs have been well documented to be activated by the elevated intracellular Ca^{2+} and calmodulin. There are four *camk* genes (*camks-1-4*) annotated in the *Neurospora* genome and their catalytic domains are conserved despite a low overall identity of amino acid sequences (37). Expression of *camk-1-4* genes moderately increases in $\Delta nca-2$ compared to those in WT across 28 hrs in the dark (Figure S3). Among the four CAMKs, CAMK-1 has been reported to directly phosphorylate FRQ at multiple sites *in vitro* although only a very subtle period defect was observed in $\Delta camk-1$ (18); however, in our hands the $\Delta camk-1$ strain showed greatly reduced growth and was arrhythmic on race tubes (Figure S4A), suggesting that prior data may have reflected a revertant strain. To further evaluate this and characterize roles for CAMKs we made all combinations of $\Delta camk$ mutants, transformed these with the *C-box luc* reporter, and assayed their clocks. We found that circadian periods in individual or combinational knockouts of *camks* are indeed quite similar to WT (Figure S4B). To test whether $\Delta nca-2$ regulates the clock through *camks-1-4*, $\Delta nca-2$ was backcrossed to $\Delta camks-1-4$ respectively and circadian periods were assayed by luciferase analyses. Interestingly, $\Delta camks-1$, -3 , and -4 each showed the characteristic period shortening when in combination with $\Delta nca-2$; however, $\Delta camk-2 \Delta nca-2$ showed the same circadian period as the $\Delta camk-2$ single mutant with no additional shortening due to $\Delta nca-2$ (Figure 4A), suggesting that *nca-2* and *camk-2* function in the same pathway to regulate the circadian period. Because in certain cases activated kinases not only phosphorylate their substrates but also actuate autophosphorylation in *cis* or in *trans*, phosphorylation on these kinases can be indicative of their activities. To test this, the phosphorylation status of CAMKs-1-4 were measured by Western blotting using the 149:1 (acrylamide/bisacrylamide) Phos-tag gel that has been used to resolve single phosphorylation events on WC-1 and WC-2 (10). CAMKs-2 and -4 display similar phospho-profiles in the presence or absence of *nca-2*, while, interestingly, CAMKs-1 or -3 in $\Delta nca-2$ undergo more phosphorylations than those in the WT background (Figure 4B), suggesting that their activities might be stimulated due to elevated calcium resulted from the absence of *nca-2*. Taken together, these data suggest that the elevated calcium concentration in $\Delta nca-2$ directly or indirectly activates CAMKs, which leads to hyper-phosphorylation of FRQ, thereby shortening the circadian period. The data further indicate that although intracellular calcium can influence periodicity through CAMKs, phosphorylation by CAMKs is not required for rhythmicity; it is modulatory.

Characterization of *nca-2*

In the *Neurospora* genome, transcription of ~40% coding genes are circadianly controlled directly or indirectly by the WCC-FFC oscillator (69, 70). We used transcriptional and translational fusions with

the *luciferase* gene to see whether *nca-2* is a *ccg*: (1) the *nca-2* promoter was fused to the *luciferase* gene and transformed to the *csr* locus for real-time analysis of *nca-2* transcription, showing that transcription driven by the *nca-2* promoter is clearly rhythmic (Figure 5A); (2) after fusing the *nca-2* coding sequence with the *luciferase* open reading frame (ORF), tracking the bioluminescent signal of NCA-2-LUC protein reveals that the NCA-2-LUC signal also oscillates in a typical circadian manner (Figure 5B). These data indicate that calcium signaling in the cell might be regulated by the circadian clock through rhythmically transcribing and translating a calcium pump gene, *nca-2*. These data place NCA-2 in the larger cellular circadian system: *nca-2* and NCA-2 expression are clock-regulated, and NCA-2 activity, or lack thereof, impacts circadian period length. To identify potential DNA elements conferring circadian transcription of *nca-2*, we searched rhythmic motifs derived from (69). These were identified as sequences that were over-represented among rhythmically expressed genes. Interestingly, the first three of the four types of motifs identified in the paper (69) are found in the *nca-2* promoter (1.7 kb upstream of “ATG”) (data not shown). However, we do not know what TFs bind to these motifs; they do not appear in available databases, including the extensive catalogue of inferred sequence preferences of DNA-binding proteins (Cis-BP) (<http://cisbp.ccbr.utoronto.ca>) (71) that covers >1000 TFs from 131 species including *Neurospora*. Although there were weak matches to the motifs, none of the matches were from *Neurospora* (data not shown).

Consistent with its role as a calcium exporter, NCA-2 is predicted to contain two calcium ATPase domains and a haloacid dehalogenase (HAD) domain (Figure S5A). To understand the role of NCA-2 at a mechanistic level, we mapped the NCA-2 interactome by affinity purification. C-terminal V5-10xHis-3xFLAG (VHF)-tagged NCA-2 was affinity-purified under a non-denaturing condition (Figure 5C), and its interacting proteins identified by mass spectrometry. Among NCA-2’s interactors identified (Table S1) was the phosphatase CSP-6 whose interaction with NCA-2 was confirmed by immuno-precipitation (Figure 5D). CSP-6 has been shown to control circadian output and WCC phosphorylations independent of the circadian feedback loop (72), suggesting that NCA-2 might have other roles relevant to CSP-6. Both $\Delta csp-6$ and the double mutant $\Delta csp-6; \Delta nca-2$ display an arrhythmic overt clock on race tubes (Figure S5B), indicating that $\Delta nca-2$ is unable to rescue the output defect in $\Delta csp-6$. Interestingly however, while growing more slowly than $\Delta csp-6$, the double mutant $\Delta csp-6; \Delta nca-2$ shows a period similar to $\Delta nca-2$ by the luciferase assay (Figure S5C), suggesting that *nca-2* does not act through *csp-6* in controlling the pace of the core oscillator. Altogether, these data demonstrate that *nca-2* is a *ccg* and suggest that cellular calcium signaling might be regulated by the circadian clock via rhythmic expression of *nca-2*.

Downregulation of *calcineurin* does not influence the circadian period

In a wide variety of eukaryotes, a prolonged increase in intracellular Ca^{2+} activates a calcium and calmodulin dependent serine/threonine protein phosphatase, calcineurin, which mediates dephosphorylation of transcription factors, such as NFAT to regulate gene expression (73–86). *calcineurin* (*ncu03833*) is an essential gene in *Candida albicans* and *Neurospora* (87, 88), so to determine whether *calcineurin* influences the circadian clock, we downregulated its expression by replacing its native promoter with the *qa-2* promoter, an inducible promoter activated by quinic acid (QA). In the absence of QA, WC-1 is undetectable and FRQ is barely seen in *qa-2*-driven *calcineurin* (Figure S6A), consistent with a short period/arrhythmic clock observed in the strain (Figure S6B). To better examine this, we assayed rhythmicity at extremely low levels of inducer just sufficient for rhythmicity (10^{-8} M QA) or at high levels at or above WT expression (10^{-2} M QA). We found that period length was not proportional to the level of *calcineurin* expression at levels supporting any rhythmicity, and that even at vanishingly low *calcineurin* expression the core oscillator displays a period similar to that in WT, suggesting that the level of *calcineurin* does not determine the pace of the clock. This said, the severe reduction in WC-1 levels in the *qa-2*-driven *calcineurin* strain cultured without QA would be consistent with at least an indirect role for *calcineurin* in controlling WC-1 expression.

Discussion

In this study, we have identified *nca-2* as a calcium pump involved in regulating circadian period length through CAMK-mediated FRQ phosphorylations. These data confirm that calcium signaling, a crucial regulatory pathway in mediating cellular and biochemical processes, must be well controlled for normal circadian period length determination. Most significantly, calcium signaling is now placed as an ancillary feedback loop within the larger circadian oscillatory system: The clock controls the expression of *NCA-2* and thereby intracellular calcium levels and intracellular calcium, in turn, modulates the period length of the clock. In this regard the larger *Neurospora* circadian system is regulated by calcium in a manner reminiscent of that seen in the mammalian brain (e.g. (89)). As prolonged activation of signaling pathways is wasteful and harmful to the cell, the elevated cytosolic calcium in $\Delta nca-2$ over-activates CAMKs, leading to FRQ hyperphosphorylation and thereby causing a period defect (Figure 5E). The involvement of intracellular Ca^{2+} in the circadian system is further nuanced by the finding that expression of some *camk* genes is clock-controlled (Figure S3 and (69, 70)), so both the activator and effectors of calcium-induced regulation are clock-modulated and clock-affecting. This emphasizes the pervasive nature of both circadian and calcium control of the biology of the cell (Figure 5E).

Among calcium trafficking genes, *nca-2* encodes the major Ca^{2+} exporter (34). *Neurospora* encodes three transporter *nca* genes as well as the vacuolar calcium importer gene *cax*, but interestingly, only disruption of *nca-2* leads to a significant period change (Figure 1A), suggesting that NCA-2 plays a major role in lowering cytosolic calcium. Consistent with this, the calcium level in $\Delta nca-2$ has been reported to rise ~9.3 times while it remains normal in $\Delta nca-1$ or $\Delta nca-3$ (36). It is possible that NCA-2 has higher affinity for Ca^{2+} , is more abundant on the plasma membrane, or more efficient in transporting calcium than the other two NCAs.

Temporal FRQ phosphorylation, the core pacemaking mechanism in the circadian feedback loop, is mediated by multiple kinases including at least CKI, CKII, and CAMK-1 (9, 16, 18). Deletion of *camk-2* gene prevents the high intracellular Ca^{2+} from shortening the circadian period, indicating the dominant role in mediating the effect of calcium on the clock and making it a likely addition to the CAMKs active on the clock. Periods of several *frq* phosphorylation mutants, *frq*^{S72A, S73A, S76A}, *frq*^{S538A, S540A} and *frq*^{S632A, S634A} (Figure 3), were not significantly altered in the background of $\Delta nca-2$, and the domain where FRQ S72, S73, and S76 are located bears CAMK motifs (9), consistent with calcium-activated CAMK acting through these residues. Interestingly, although it is CAMK-1 that has been shown to directly phosphorylate FRQ *in vitro* (18) its loss here did not abrogate the effects of loss of NCA-2. It may be that the phosphosites targeted by different CAMKs on FRQ are distinct and have different effects on rhythmicity. Freshly germinated $\Delta camk-1$ displays a developmental defect (18, 37), whereas knockouts of the other three *camks* individually grow as robustly as WT (Figure S4A); however, the growth defect of $\Delta camk-1$ strains appears to rapidly revert back to normal after a few rounds of inoculation of $\Delta camk-1$ on new slants (18), suggesting that other CAMKs might be able to gradually compensate for the loss of *camk-1* over time.

WCC can be phosphorylated at over 90 sites and a small group of these is required for the closure of the circadian feedback loop (10). Interestingly, in $\Delta nca-2$, WC-1 is hyperphosphorylated and more abundant than in WT despite a reduced *wc-1* RNA level (Figure 1A); this finding is consistent with a “black widow” model in which site-specific phosphorylation of transcription activators makes them inactive in driving transcription but more stable (90). However, lacking key phosphoresidues determining the feedback loop closure, *wc-1* mutants, such as *wc-1*^{S971A, S988A, S990A, S992A, S994A, S995A}, *wc-2*^{S394A, S428A, S429A, S433A, S435A}, show rhythms with short circadian period lengths due to elevated activity of WCC (10), whereas $\Delta nca-2$ strains, bearing hyperphosphorylated and more stable WC-1, also display a short period (Figure 1 A and 1B; Figure 2C and 2E). One possible explanation is that the hyper-phosphorylation of WC-1 in $\Delta nca-2$ occurs at residues regulating the circadian

amplitude/output instead of those required for the feedback loop closure while the period shortening effect in $\Delta nca-2$ is caused by enhanced FRQ phosphorylation. WCC phosphoresidues can be briefly classified into two categories: the ones involved in the feedback loop closure and the other ones regulating the robustness of *frq* transcription (amplitude reflecting peak to trough in circadian cycles) (10). Key phosphomutants of *wcc* showed an additive effect with $\Delta nca-2$ on period length, suggesting that NCA-2 is not directly involved in the regulation of sites participating in the feedback loop closure but instead regulates WCC phosphoresidues relevant to the circadian amplitude.

Material and methods

Strains and culture conditions

328-4 (*ras-1^{bd} A*) was used as a wild-type strain in the race tube analyses and, 661-4a (*ras-1^{bd} A*), which bears the *frq C-box* fused to codon-optimized *luciferase* gene at the *his-3* locus, served as the wild type in luciferase assays. *Neurospora* transformation was performed as previously reported (91, 92). Medium in the race tube analyses contains 1 x Vogel's salts, 0.17% arginine, 1.5% agar, 50 ng/mL biotin, and 0.1% glucose and liquid culture medium (LCM) contains 1 x Vogel's, 0.5% arginine, 50 ng/mL biotin, and 2% glucose. Unless otherwise specified, race tubes were cultured in constant light for 16–24 hrs at 25 °C to synchronize strains and then transferred to the dark at 25 °C. The Vogeloid (10X) used to make the Ca^{2+} -free medium in Figure 1D contains 100 mM NH_4Cl , 20 mM $MgCl_2 \cdot 6H_2O$, 100 mM KCl, 20 mM methionine, 50 ng/mL biotin, and 0.1% glucose (36).

Bioluminescence assays

Luciferase assays were conducted as previously described (10). Briefly, strains with the *frq C-box luciferase* transcriptional reporter at the *his-3* locus were grown in 96 well plates bearing 0.1% glucose race tube medium having luciferin in constant light overnight (16-24 hrs) at 25 °C and then transferred to the dark at 25 °C to start circadian cycles. Bioluminescent signals were tracked by a CCD-camera every hour for 5 or more days. Luciferase data were extracted using the NIH ImageJ software with a custom macro, and circadian period lengths were manually determined.

Protein lysate and Western blot (WB)

For WB, 15 mgs of whole-cell protein lysate were loaded per lane on a 3-8% tris-acetate or 6.5% tris-glycine (bearing Phos-tag) SDS gel (92). Custom-raised antibodies against WC-1, WC-2, FRQ, and FRH have been described previously (93–95). V5 antibody (Thermo Pierce) and FLAG antibody (M2, Sigma-Aldrich) were diluted 1:5000 for use as the primary antibody. To analyze the phosphorylation

345 profiles of CAMKs, 20 μ M Phos-tag chemical (ApexBio) was added to the homemade 6.5% Tris-
 346 glycine SDS-PAGE gel bearing a ratio of 149:1 acrylamide/bisacrylamide (10).
 347 Immunoprecipitation (IP)
 348 IP was performed as previously described (91, 92). Briefly, 2 mgs of total protein were incubated with
 349 20 μ L of V5 agarose (Sigma-Aldrich) rotating at 4 °C for 2 hr. The agarose beads were washed with 1
 350 mL of protein extraction buffer (50 mM HEPES [pH 7.4], 137 mM NaCl, 10% glycerol, 0.4% NP-40)
 351 twice and eluted with 50 μ L of 5 x SDS sample buffer at 99 °C for 5 min.

352 Other techniques

353 RNA extraction, reverse transcription (RT), and quantitative PCR were conducted as previous reports
 354 (72, 91). VHF-tagged NCA-2 was purified with the same method applied for isolation of C-terminal
 355 VHF-tagged WC-1 and mass spectrometry analyses were performed as previously described (72,
 356 91). Data acquisition and analysis of luciferase runs were carried out as previously described (10).

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Legends

Figure 1 Gene deletions of calcium pumps were tested for circadian phenotypes by race tube (A) and luciferase (B) analyses. Strains were cultured on 0.1% glucose race tube medium in a 96 well plate and synchronized by growth in constant light overnight (16-24 hrs) followed by transfer to darkness. Bioluminescent signals were monitored by a CCD-camera every hour, bioluminescence data were acquired using ImageJ with a custom macro, and period lengths were manually calculated. Raw bioluminescent data from three replicates were plotted with the X-axis and Y-axis representing time (hrs) and arbitrary units respectively. (C) Left, Western blot showing the expression level of FRQ in WT and $\Delta nca-2$ over 28 hr detected with FRQ-specific antibody. DD, hours after the light to dark transfer; right, RT-qPCR showing relative levels of *frq* mRNA expressed in WT and $\Delta nca-2$; *rac-1* was used as an internal control, to which *frq* expression is normalized (n = 3, mean values $\pm$ standard error of the mean). Asterisks indicate statistical significance when compared with WT as determined by a two-tailed student's *t* test. *****P<0.00001; ****P=0.00006; ***P=0.001337; **P<0.01; *P=0.010131. NS, difference is not significant. (D) Race tube assays of WT and the $\Delta nca-2$ strain using race tube media in the presence or absence of 2 mM calcium chloride. Growth fronts of the strains were marked by vertical blacklines every 24 hr. *nca-3* (NCU05154): calcium P-type ATPase; *nca-1* (NCU03305): calcium-transporting ATPase sarcoplasmic/endoplasmic reticulum type; *cax* (NCU07075): calcium/proton exchanger; *pmr-1* (NCU03292): calcium-transporting ATPase type 2C member 1; *nca-2* (NCU04736): plasma membrane calcium-transporting ATPase 3; gene names, NCU numbers, and descriptions were obtained from the FungiDB website (<https://fungidb.org/fungidb/app>). Period was determined as described in Material and Methods and is reported $\pm$ SD (n=3).

Figure 2 Circadian components WC-1 and FRQ are hyper-phosphorylated in $\Delta nca-2$. (A) Total WC-1 was followed by Western blotting. Samples were cultured in the constant dark prior to a 15, 30, 60, and 120 min light exposure. Non-specific bands in the same blot were shown for equal loading. Decreased electrophoretic mobility is indicative of phosphorylation status (7). (B) mRNAs extracted from samples cultured in the dark for 24 hrs or following a 15, 30, or 120 min light exposure as indicated were reverse-transcribed to cDNA followed by quantitative PCR with a primer set specific to *wc-1*. (C) Western blotting of WC-1 in a 24-hr time course with a 4-hr interval. (D) Similar to (C), RT-

qPCR was performed with samples harvested at the circadian conditions indicated. Phosphorylation profiles of WC-1 (**E**) and FRQ (**F**, **G**) in WT and $\Delta nca-2$ were analyzed by Western blotting using SDS-PAGE gels bearing (G) 20 μ M Phos-tag chemicals and a ratio of 149:1 acrylamide to bis-acrylamide. (**F**) Western blotting of FRQ in WT and $\Delta nca-2$ from DD14 to DD24 with a 2-hr resolution. * in **E** denotes the mobility of unphosphorylated WC-1, and the bracket the region corresponding to hyperphosphorylated WC-1. Arrows indicate hyper-phosphorylated FRQs observed in $\Delta nca-2$.

Figure 3 Some *frq* alleles are epistatic to $\Delta nca-2$. The *frq* C box promoter activity was measured using C box-luciferase at the *his-3* locus in indicated *frq* phospho-mutants in the presence or absence of *nca-2*. Strains were grown on 0.1% glucose race tube medium in constant light overnight (16-24 hrs) prior to transfer to darkness. *frq*^{S72A, S73A, S76A}, *frq*^{S541A, S545A}, *frq*^{S538A, S540A}, *frq*^{S548A}, and *frq*^{S632A, S634A} were derived from (9). Period was determined as described in Material and Methods and is reported $\pm$ SD (n=3).

Figure 4 Period shortening of $\Delta nca-2$ is rescued by deletion of *camk-2*. (**A**) Luciferase assays were performed with a *frq* C box promoter-driven luciferase gene at the *his-3* in individual knockouts of *camks-1-4* in the presence or absence of $\Delta nca-2$ as indicated. Periods are reported as described in Material and Methods and is reported $\pm$ SD (n=3). (**B**) Upper panel, total levels of CAMKs-1-4 that have a 3xFLAG tag respectively at their C-termini in WT or the $\Delta nca-2$ background were assayed by Western blotting with FLAG antibody; lower panel, phosphorylation profiles of CAMKs-1-4 were analyzed for the same sample set with 149:1 acrylamide to bis-acrylamide SDS-PAGE gels containing Phos-tag. Asterisks indicate non-specific bands. For CAMKs-1, -2, and -4, total lysates were applied while CAMK-3 was first pulled down by FLAG antibody-conjugated resins and subsequently assayed in WB due to an overlap between CAMK-3 phospho-isoforms and non-specific bands in the Phos-tag gel.

Figure 5 *nca-2* is a *ccg* and modulates both input to and output from the core clock. (**A**) The *nca-2* promoter fused to the luciferase gene was transformed to the *csr* locus and luciferase signals were followed at 25 °C in the dark. Period was determined as described in Material and Methods and is reported $\pm$ SD (n=3). (**B**) The *nca-2* open reading frame was fused to the 5' end of the firefly luciferase gene and the same assay as in (**A**) was performed to trace the luciferase signal. (**C**) A representative silver-stained gel showing NCA-2^{VHF} and its interactome purified from a culture grown in the light. NCA-2^{VHF} and interactors were affinity-purified, trichloroacetic acid (TCA)-precipitated, and analyzed by mass spectrometry. (**D**) NCA-2 is tagged with a V5 tag and one of its interactors, CSP-6, was tagged with a 3xFLAG tag; Co-immunoprecipitation was performed using V5 resin and Western

blotting was done with V5 and FLAG antibodies respectively. (E) Working model for the roles of intracellular calcium and of *nca-2* in the circadian system, In $\Delta nca-2$, increased calcium over-activates CAMKs, which induces FRQ over-phosphorylations and thereby causes a faster running clock; the circadian clock regulates expression of *nca-2* and *camks*.

Figure S1 Stability of WC-1 and FRQ in WT and $\Delta nca-2$. (A) Left, cycloheximide (CHX) was added to the *Neurospora* culture growing in the light at a final concentration of 40 μ g/mL and tissues were harvested at indicated time points and assayed by Western blotting with WC-1- and FRQ-specific antibodies respectively; right, densitometric analyses of the blots on the left showing WC-1 and FRQ levels in WT and $\Delta nca-2$ treated with 40 μ g/mL CHX for 0, 4, 8, or 12 hrs as indicated. (B) Left, FRQ stability in WT and $\Delta nca-2$ is measured using CHX-treated cultures sampled every 2 hrs over 14 hrs; right, densitometric analysis of FRQ on the left panel.

Figure S2 $\Delta nca-2$ shortens period length in a long period *frq* allele and in *wcc* phospho-mutants (A) The activity of the *frq* promoter is measured by the *C-box-luc* bioluminescence in the background of *frq⁷*, *wc-1^{S971A, S988A, S990A, S992A, S994A, S995A}*, or *wc-2^{S433A}* in the presence or absence of *nca-2* as indicated in the figure. (B) Phosphorylations of WC-1 S971 and S990 in the presence or absence of *nca-2* were assayed using a 6.5% SDS-PAGE gel bearing Phos-tag (for details, see Materials and Methods). Phosphorylation of WC-1 residues S971, S988, S990, S992, S994, and S995 is promoted by FRQ and essential for the closure of the circadian feedback loop (10).

Figure S3 Expression of *camks* in WT and $\Delta nca-2$. mRNA levels of *camks-1-4* in WT and $\Delta nca-2$ were assayed by RT-qPCR with samples grown under light or dark conditions as indicated. Expression of *camks* was normalized to that of *rac-1*.

Figure S4 Individual or combinational deletion of *camk* genes does not dramatically impact the circadian period by the luciferase assay. (A) Gene knockouts of individual *camks-1-4* were assayed for circadian phenotypes by race tube. $\Delta camk-1$ cultures in the top and middle tubes are true knockouts while the one at the bottom is a typical revertant as reported by (18) (B) Combinations of $\Delta camk$ mutants were tested by the luciferase assay.

Figure S5 NCA-2 domain analyses and its relationship with *csp-6* on the clock. (A) Predicted domains in NCA-2 analyzed by the on-line tool SMART (http://smart.embl-heidelberg.de/smart/set_mode.cgi?NORMAL=1) Race tube (B) and luciferase (C) analyses of $\Delta nca-2$, $\Delta csp-6$, and $\Delta csp-6; \Delta nca-2$.

695 **Figure S6** Down-regulation of *calcineurin subunit B* has little influence on circadian period length
696 across expression levels compatible with rhythmicity. The endogenous promoter of *calcineurin*
697 *subunit B* was replaced by the *qa-2* promoter. **(A)** FRQ, WC-1, and WC-2 proteins were determined
698 by Western blotting with specific antibodies and **(B)** *frq* transcription was tracked by *frq C box-luc* at
699 the *his-3* locus in the absence or presence of quinic acid (QA) at the concentration indicated.

Figure 2

Figure 3

Figure 4

A

B

Figure 5