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 Δ*nca-2*, consistent with high calcium in the Δ*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

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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.

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, Ca^{2+}-CaM-dependent kinases (CAMKs)

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

3

92 membrane or indirect signal transduction pathways, such as G protein-coupled receptors (e.g.,

93 reviewed in (53, 54)). Cytosolic calcium bursts lead to activation of Ca^{2+}–calmodulin-dependent

94 protein kinases (CAMKs) (55–59). In mammals, the CAMK cascade includes three kinases: CaM-

95 kinase kinase (CaMKK), CaMKI, and CaMKIV; CaMKI and CaMKIV are phosphorylated and activated

96 by CaMKK (55, 60–65). CaMKK and CaMKIV reside in the nucleus and cytoplasm while CaMKI is

97 only located in cytosol; nuclear CaMKIV promotes phosphorylation of several transcription factors,

98 such as CREB and CBP, to regulate gene expression (60, 66, 67). The *Neurospora* genome encodes

99 four CAMK genes that are subject to diverse regulation, although little is known about their

100 intracellular localization (18, 37).

101 By impacting a wide range of cellular processes, circadian clocks and calcium signaling are two

102 classic regulatory mechanisms evolved to coordinate environmental factors, cellular responses, and

103 metabolism. In this study, a screen of calcium regulators identified *nca-2*, a calcium pump gene, as a

104 regulator of circadian period length in *Neurospora.* In Δ*nca-2* strains, FRQ and WC-1 become hyper-

105 phosphorylated; deletion of *camk-2* individually blocks the period shortening effect and FRQ hyper-

106 phosphorylations in Δ*nca-2*. NCA-2 interacts with multiple proteins, which suggests that it might

107 function in other cellular processes in addition to the circadian clock.

108 **Results**

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

110 Calcium signaling impacts circadian processes (e.g., 18, 30, 31) and directly controls a wide range of

111 cellular and physiological events, but the means through which it impacts the circadian system is not

112 fully described. *Neurospora* encodes several calcium transporter genes including *nca-1* (a SERCA-

113 type ATPase), two closely related genes, *nca-2* and *nca-3* (PMCA-type ATPases), *pmr-1* (a SPCA-

114 type Ca^{2+}-ATPase), and *cax* (a vacuolar Ca^{2+}/H^+ exchanger (**35**)). To facilitate monitoring circadian

115 phenotypes, individual knockouts of these calcium pump genes were backcrossed to *ras-1^{bd}* and *frq C*

116 *box*-driven *luciferase* and analyzed by race tube and luciferase assays. Of these deletion mutants

117 tested, Δ*pmr-1* shows an extremely slow growth rate on race tubes (Figure 1A) but is nicely rhythmic

118 with a slightly shorter period in the luciferase assay (Figure 1B); disruption of *nca-2*, a plasma

119 membrane-located calcium pump, leads to a ~2-hr shorter period than that in wild type (WT) by race

120 tube (Figure 1A) and luciferase (Figure 1B) analyses. (Of note, although on any given day the period

121 estimates of strains bearing mutated calcium pumps showed normal precision, period length assays

122 done on different days were more variable than is typical. For this reason, comparisons within figures

123 always reflect assays of different strains done on the same day with the same medium.) Appearing at

4

124   DD12, newly synthesized FRQ in Δnca-2 is slightly more abundant than that in WT (Figure 1C, left)

125   and frq mRNA levels in Δnca-2 are substantially higher in the subjective circadian night phase (DD4,

126   8, 24, 28) as compared to WT (Figure 1C, right), consistent with a faster running circadian clock in

127   Δnca-2 (Figure 1A and 1B). The cytosolic calcium level in Δnca-2 is increased about 9.3 fold as

128   compared to WT (36) suggesting a basis for this period change. To verify that the period shortening in

129   Δnca-2 was due to this increased intracellular Ca^{2+}, the Δnca-2 strain was examined on race tubes

130   prepared without calcium in the medium. Interestingly, Δnca-2 displays a WT period on race tubes

131   using Ca^{2+}-free medium while its clock becomes ~4-hr shorter than WT with normal levels of calcium

132   in the medium (Figure 1D), confirming that the role of nca-2 in regulating the pace of the circadian

133   oscillator is through controlling the cytosolic calcium level. These data indicate that nca-2 is required

134   for keeping calcium in cytosol at reduced levels to maintain a normal circadian period.

135   **WC-1 and FRQ are hyper-phosphorylated in Δnca-2**

136   WC-1 and FRQ are essential components in the positive and negative arms respectively of the

137   Neurospora feedback loop and their phosphorylation has been proven to play an essential role in the

138   determining their circadian functions (9, 10, 15, 16, 19). In addition to serving as the main

139   transcription factor driving expression of frq, WC-1 is also the principal blue light photoreceptor for the

140   organism, forming a homodimer (4) and getting hyper-phosphorylated (20) upon light exposure. To

141   probe WC-1 and FRQ in Δnca-2, amounts and phosphorylation profiles of WC-1 and FRQ were

142   analyzed by Western blotting using specific antibodies. The stability of FRQ in Δnca-2 is very similar

143   to that in WT (Figure S1), and although WC-1 appeared slightly less stable, the cellular levels of WC-

144   1 were even above those of WT, altogether suggesting that the stability of the core clock components

145   does not determine the shortened period in Δnca-2 and the WC-1 level and stability are not consistent

146   with the period length shortening in Δnca-2. Following a light-pulse, WC-1 is more abundant and

147   hyperphosphorylated in Δnca-2 as compared to WT (Figure 2A) whereas, surprisingly, expression of

148   wc-1 is significantly lower than that in WT (Figure 2B); consistent with the data from the light-pulse

149   experiment, in the dark, Δnca-2 contains a higher level of WC-1 with more phosphorylations (Figure

150   2C) despite a low mRNA level (~20-50% as in WT) (Figure 2D). These data suggest that nca-2

151   regulates wc-1 expression at both transcriptional and post-transcriptional levels independent of light

152   and dark conditions. The hyper-phosphorylation of WC-1 in Δnca-2 was confirmed by a more

153   sensitive assay (Figure 2E) using Phos-tag gels (68) such as have been applied to resolve single

154   phosphoresidues on WC-1 and WC-2 (10). Similar to WC-1, FRQ in Δnca-2 is also more heavily

155   phosphorylated than that in WT at DD14, 16, and 18 (Figure 2F) when newly synthesized FRQ is the

156   dominant form in the cell, and at DD24 (Figure 2G) when all FRQ becomes extensively

157   phosphorylated prior to its turnover (see also Figure 1A). Altogether, these data demonstrate that

158   WC-1 and FRQ become hyper-phosphorylated in Δ*nca-2*, suggesting that the elevated calcium in

159   Δ*nca-2* might lead to over-activation of kinase(s) or repression of phosphatase(s) targeting FRQ and

160   WC-1, thereby altering their activities in the clock.

161   **Epistasis analysis is consistent with an effect of Δ*nca-2* on FRQ but not on WCC**

162   FRQ is phosphorylated in a time-specific manner at over 100 sites, and elimination of certain

163   phospho-sites in different domains can cause opposite phenotypes on period lengths (9, 15).

164   Because loss of *nca-2* elicits FRQ hyperphosphorylation at almost all time points examined (Figure 2F

165   and 2G), we reasoned that this enhanced FRQ phosphorylation in Δ*nca-2* might contribute to the

166   short period length in this strain. If this is so, then circadian period lengths in *frq* mutants encoding

167   proteins that cannot be phosphorylated at key residues should not display the period shortening. To

168   this end, several *frq* phospho-mutants displaying long circadian periods from (9) were individually

169   backcrossed to Δ*nca-2* and *frq-luc*, and assayed by tracking bioluminescent signals in real-time in

170   darkness. While circadian periods of $frq^{S541A, S545A}$ and frq^{S548A} , and frq^7 responded to loss of *nca-2* as

171   did WT (Figure 3 and Figure S2A), absence of *nca-2* does not significantly influence the period length

172   of $frq^{S72A, S73A, S76A}$, $frq^{S538A, S540A}$, or $frq^{S632A, S634A}$ (Figure 3). These are all residues whose inability to

173   be phosphorylated results in period lengthening (9), so the epistasis of these *frq* alleles is consistent

174   with NCA-2 influencing FRQ phosphorylation at these sites.

175   To examine the effect of Δ*nca-2* on WCC phosphorylation and period length in the same manner,

176   Δ*nca-2* was backcrossed to several *wcc* mutants eliminating key phosphoresidues that have been

177   identified and shown to determine the circadian feedback loop closure (10), and monitored by the

178   luciferase assay. The absence of *nca-2* further shortens the periods of $wc-1^{S971A, S988A, S990A, S992A, S994A,}$

179   ^{S995A} and $wc-2^{S433A}$ respectively (Figure S2A), suggesting that *nca-2* regulates the core oscillator

180   independent of WCC phosphorylation at the sites essential for its repression. Consistent with this, in

181   Δ*nca*-2 the phosphorylation levels of WC-1 S971 and S990, two key sites required for FFC-mediated

182   WCC repression, are similar to that in WT (Figure S2B), further suggesting that altered

183   phosphorylation of the positive arm in the oscillator is not the cause of the short period of Δ*nca-2*.

184   **Δ*camk-2* does not further shorten the period of Δ*nca-2***

185   Data in Figures 2 and 3 are consistent with NCA-2 acting through kinases or phosphatases on FRQ,

186   and the elevated calcium in Δ*nca-2* (36) might activate Ca^{2+}-responsive kinases to over-phosphorylate

187   FRQ (Figure 2F and 2G). CAMKs have been well documented to be activated by the elevated

188   intracellular Ca^{2+} and calmodulin. There are four *camk* genes (*camks-1-4*) annotated in the

189   *Neurospora* genome and their catalytic domains are conserved despite a low overall identity of amino

190   acid sequences (37). Expression of *camk-1-4* genes moderately increases in Δ*nca-2* compared to

191   those in WT across 28 hrs in the dark (Figure S3). Among the four CAMKs, CAMK-1 has been

192   reported to directly phosphorylate FRQ at multiple sites *in vitro* although only a very subtle period

193   defect was observed in Δ*camk-1* (18); however, in our hands the Δ*camk-1* strain showed greatly

194   reduced growth and was arrhythmic on race tubes (Figure S4A), suggesting that prior data may have

195   reflected a revertant strain. To further evaluate this and characterize roles for CAMKs we made all

196   combinations of Δ*camk* mutants, transformed these with the *C-box luc* reporter, and assayed their

197   clocks. We found that circadian periods in individual or combinational knockouts of *camks* are indeed

198   quite similar to WT (Figure S4B). To test whether Δ*nca-2* regulates the clock through *camks-1-4*,

199   Δ*nca-2* was backcrossed to Δ*camks-1-4* respectively and circadian periods were assayed by

200   luciferase analyses. Interestingly, Δ*camks-1, -3, and -4* each showed the characteristic period

201   shortening when in combination with Δ*nca-2*; however, Δ*camk-2* Δ*nca-2* showed the same circadian

202   period as the Δ*camk-2* single mutant with no additional shortening due to Δ*nca-2* (Figure 4A),

203   suggesting that *nca-2* and *camk-2* function in the same pathway to regulate the circadian period.

204   Because in certain cases activated kinases not only phosphorylate their substrates but also actuate

205   autophosphorylation in *cis* or in *trans*, phosphorylation on these kinases can be indicative of their

206   activities. To test this, the phosphorylation status of CAMKs-1-4 were measured by Western blotting

207   using the 149:1 (acrylamide/bisacrylamide) Phos-tag gel that has been used to resolve single

208   phosphorylation events on WC-1 and WC-2 (10). CAMKs-2 and -4 display similar phospho-profiles in

209   the presence or absence of *nca-2*, while, interestingly, CAMKs-1 or -3 in Δ*nca-2* undergo more

210   phosphorylations than those in the WT background (Figure 4B), suggesting that their activities might

211   be stimulated due to elevated calcium resulted from the absence of *nca-2*. Taken together, these data

212   suggest that the elevated calcium concentration in Δ*nca-2* directly or indirectly activates CAMKs,

213   which leads to hyper-phosphorylation of FRQ, thereby shortening the circadian period. The data

214   further indicate that although intracellular calcium can influence periodicity through CAMKs,

215   phosphorylation by CAMKs is not required for rhythmicity; it is modulatory.

216   **Characterization of *nca-2***

217   In the *Neurospora* genome, transcription of ~40% coding genes are circadianly controlled directly or

218   indirectly by the WCC-FFC oscillator (69, 70). We used transcriptional and translational fusions with

7

219 the *luciferase* gene to see whether *nca-2* is a *ccg*: (1) the *nca-2* promoter was fused to the *luciferase*

220 gene and transformed to the *csr* locus for real-time analysis of *nca-2* transcription, showing that

221 transcription driven by the *nca-2* promoter is clearly rhythmic (Figure 5A); (2) after fusing the *nca-2*

222 coding sequence with the *luciferase* open reading frame (ORF), tracking the bioluminescent signal of

223 NCA-2-LUC protein reveals that the NCA-2-LUC signal also oscillates in a typical circadian manner

224 (Figure 5B). These data indicate that calcium signaling in the cell might be regulated by the circadian

225 clock through rhythmically transcribing and translating a calcium pump gene, *nca-2*. These data place

226 NCA-2 in the larger cellular circadian system: *nca-2* and NCA-2 expression are clock-regulated, and

227 NCA-2 activity, or lack thereof, impacts circadian period length. To identify potential DNA elements

228 conferring circadian transcription of *nca-2*, we searched rhythmic motifs derived from (69). These

229 were identified as sequences that were over-represented among rhythmically expressed genes.

230 Interestingly, the first three of the four types of motifs identified in the paper (69) are found in the *nca-*

231 *2* promoter (1.7 kb upstream of "ATG") (data not shown). However, we do not know what TFs bind to

232 these motifs; they do not appear in available databases, including the extensive catalogue of inferred

233 sequence preferences of DNA-binding proteins (Cis-BP) (http://cisbp.ccbr.utoronto.ca) (71) that

234 covers >1000 TFs from 131 species including *Neurospora*. Although there were weak matches to the

235 motifs, none of the matches were from *Neurospora* (data not shown).

236 Consistent with its role as a calcium exporter, NCA-2 is predicted to contain two calcium ATPase

237 domains and a haloacid dehalogenase (HAD) domain (Figure S5A). To understand the role of NCA-2

238 at a mechanistic level, we mapped the NCA-2 interactome by affinity purification. C-terminal V5-

239 10xHis-3xFLAG (VHF)-tagged NCA-2 was affinity-purified under a non-denaturing condition (Figure

240 5C), and its interacting proteins identified by mass spectrometry. Among NCA-2's interactors

241 identified (Table S1) was the phosphatase CSP-6 whose interaction with NCA-2 was confirmed by

242 immuno-precipitation (Figure 5D). CSP-6 has been shown to control circadian output and WCC

243 phosphorylations independent of the circadian feedback loop (72), suggesting that NCA-2 might have

244 other roles relevant to CSP-6. Both Δ*csp-6* and the double mutant Δ*csp-6*; Δ*nca-2* display an

245 arrhythmic overt clock on race tubes (Figure S5B), indicating that Δ*nca-2* is unable to rescue the

246 output defect in Δ*csp-6*. Interestingly however, while growing more slowly than Δ*csp-6*, the double

247 mutant Δ*csp-6*; Δ*nca-2* shows a period similar to Δ*nca-2* by the luciferase assay (Figure S5C),

248 suggesting that *nca-2* does not act through *csp-6* in controlling the pace of the core oscillator.

249 Altogether, these data demonstrate that *nca-2* is a *ccg* and suggest that cellular calcium signaling

250 might be regulated by the circadian clock via rhythmic expression of *nca-2*.

8

Downregulation of *calcineurin* does not influence the circadian period

251 In a wide variety of eukaryotes, a prolonged increase in intracellular Ca^{2+} activates a calcium and

252

253 calmodulin dependent serine/threonine protein phosphatase, calcineurin, which mediates

254 dephosphorylation of transcription factors, such as NFAT to regulate gene expression (73–86).

255 *calcineurin* (*ncu03833*) is an essential gene in *Candida albicans* and *Neurospora* (87, 88), so to

256 determine whether *calcineurin* influences the circadian clock, we downregulated its expression by

257 replacing its native promoter with the *qa-2* promoter, an inducible promoter activated by quinic acid

258 (QA). In the absence of QA, WC-1 is undetectable and FRQ is barely seen in *qa-2*-driven *calcineurin*

259 (Figure S6A), consistent with a short period/arrhythmic clock observed in the strain (Figure S6B). To

260 better examine this, we assayed rhythmicity at extremely low levels of inducer just sufficient for

261 rhythmicity (10^{-8} M QA) or at high levels at or above WT expression (10^{-2} M QA). We found that period

262 length was not proportional to the level of *calcineurin* expression at levels supporting any rhythmicity,

263 and that even at vanishingly low *calcineurin* expression the core oscillator displays a period similar to

264 that in WT, suggesting that the level of *calcineurin* does not determine the pace of the clock. This

265 said, the severe reduction in WC-1 levels in the *qa-2*-driven *calcineurin* strain cultured without QA

266 would be consistent with at least an indirect role for *calcineurin* in controlling WC-1 expression.

Discussion

268 In this study, we have identified *nca-2* **as** a calcium pump involved in regulating circadian period

269 length through CAMK-mediated FRQ phosphorylations. These data confirm that calcium signaling, a

270 crucial regulatory pathway in mediating cellular and biochemical processes, must be well controlled

271 for normal circadian period length determination. Most significantly, calcium signaling is now placed

272 as an ancillary feedback loop within the larger circadian oscillatory system: The clock controls the

273 expression of NCA-2 and thereby intracellular calcium levels and intracellular calcium, in turn,

274 modulates the period length of the clock. In this regard the larger *Neurospora* circadian system is

275 regulated by calcium in a manner reminiscent of that seen in the mammalian brain (e.g. (89)). As

276 prolonged activation of signaling pathways is wasteful and harmful to the cell, the elevated cytosolic

277 calcium in Δ*nca-2* over-activates CAMKs, leading to FRQ hyperphosphorylation and thereby causing

278 a period defect (Figure 5E). The involvement of intracellular Ca^{2+} in the circadian system is further

279 nuanced by the finding that expression of some *camk* genes is clock-controlled (Figure S3 and (69,

280 70)), so both the activator and effectors of calcium-induced regulation are clock-modulated and clock-

281 affecting. This emphasizes the pervasive nature of both circadian and calcium control of the biology of

282 the cell (Figure 5E).

283 Among calcium trafficking genes, *nca-2* encodes the major Ca^{2+} exporter (34). *Neurospora* encodes

284 three transporter *nca* genes as well as the vacuolar calcium importer gene *cax*, but interestingly, only

285 disruption of *nca-2* leads to a significant period change (Figure 1A), suggesting that NCA-2 plays a

286 major role in lowering cytosolic calcium. Consistent with this, the calcium level in Δ*nca-2* has been

287 reported to rise ~9.3 times while it remains normal in Δ*nca-1* or Δ*nca-3* (36). It is possible that NCA-2

288 has higher affinity for Ca^{2+}, is more abundant on the plasma membrane, or more efficient in

289 transporting calcium than the other two NCAs.

290 Temporal FRQ phosphorylation, the core pacemaking mechanism in the circadian feedback loop, is

291 mediated by multiple kinases including at least CKI, CKII, and CAMK-1 (9, 16, 18). Deletion of *camk-2*

292 gene prevents the high intracellular Ca^{+2} from shortening the circadian period, indicating the dominant

293 role in mediating the effect of calcium on the clock and making it a likely addition to the CAMKs active

294 on the clock. Periods of several *frq* phosphorylation mutants, *frq*$^{S72A, S73A, S76A}$, *frq*$^{S538A, S540A}$ and

295 *frq*$^{S632A, S634A}$ (Figure 3), were not significantly altered in the background of Δ*nca-2*, and the domain

296 where FRQ S72, S73, and S76 are located bears CAMK motifs (9), consistent with calcium-activated

297 CAMK acting through these residues. Interestingly, although it is CAMK-1 that has been shown to

298 directly phosphorylate FRQ *in vitro* (18) its loss here did not abrogate the effects of loss of NCA-2. It

299 may be that the phosphosites targeted by different CAMKs on FRQ are distinct and have different

300 effects on rhythmicity. Freshly germinated Δ*camk-1* displays a developmental defect (18, 37),

301 whereas knockouts of the other three *camks* individually grow as robustly as WT (Figure S4A);

302 however, the growth defect of Δ*camk-1* strains appears to rapidly revert back to normal after a few

303 rounds of inoculation of Δ*camk-1* on new slants (18), suggesting that other CAMKs might be able to

304 gradually compensate for the loss of *camk-1* over time.

305 WCC can be phosphorylated at over 90 sites and a small group of these is required for the closure of

306 the circadian feedback loop (10). Interestingly, in Δ*nca-2*, WC-1 is hyperphosphorylated and more

307 abundant than in WT despite a reduced *wc-1* RNA level (Figure 1A); this finding is consistent with a

308 "black widow" model in which site-specific phosphorylation of transcription activators makes them

309 inactive in driving transcription but more stable (90). However, lacking key phosphoresidues

310 determining the feedback loop closure, *wc-1* mutants, such as *wc-1*$^{S971A, S988A, S990A, S992A, S994A, S995A}$;

311 *wc-2*$^{S394A, S428A, S429A, S433A, S435A}$, show rhythms with short circadian period lengths due to elevated

312 activity of WCC (10), whereas Δ*nca-2* strains, bearing hyperphosphorylated and more stable WC-1,

313 also display a short period (Figure 1 A and 1B; Figure 2C and 2E). One possible explanation is that

314 the hyper-phosphorylation of WC-1 in Δ*nca-2* occurs at residues regulating the circadian

10

315 amplitude/output instead of those required for the feedback loop closure while the period shortening

316 effect in Δ*nca-2* is caused by enhanced FRQ phosphorylation. WCC phosphoresidues can be briefly

317 classified into two categories: the ones involved in the feedback loop closure and the other ones

318 regulating the robustness of *frq* transcription (amplitude reflecting peak to trough in circadian cycles)

319 (10). Key phosphomutants of *wcc* showed an additive effect with Δ*nca-2* on period length, suggesting

320 that NCA-2 is not directly involved in the regulation of sites participating in the feedback loop closure

321 but instead regulates WCC phosphoresidues relevant to the circadian amplitude.

Material and methods

322

323 Strains and culture conditions

324 328-4 (*ras-1^{bd}* A) was used as a wild-type strain in the race tube analyses and, 661-4a (*ras-1^{bd}* A),

325 which bears the *frq C-box* fused to codon-optimized *luciferase* gene at the *his-3* locus, served as the

326 wild type in luciferase assays. *Neurospora* transformation was performed as previously reported (91,

327 92). Medium in the race tube analyses contains 1 x Vogel's salts, 0.17% arginine, 1.5% agar, 50

328 ng/mL biotin, and 0.1% glucose and liquid culture medium (LCM) contains 1 x Vogel's, 0.5% arginine,

329 50 ng/mL biotin, and 2% glucose. Unless otherwise specified, race tubes were cultured in constant

330 light for 16–24 hrs at 25 °C to synchronize strains and then transferred to the dark at 25 °C. The

331 Vogeloid (10X) used to make the Ca^{2+}-free medium in Figure 1D contains 100 mM NH_4Cl, 20 mM

332 $MgCl_2.6H_2O$, 100 mM KCl, 20 mM methionine, 50 ng/mL biotin, and 0.1% glucose (36).

333 Bioluminescence assays

334 Luciferase assays were conducted as previously described (10). Briefly, strains with the *frq C-box*

335 *luciferase* transcriptional reporter at the *his-3* locus were grown in 96 well plates bearing 0.1%

336 glucose race tube medium having luciferin in constant light overnight (16-24 hrs) at 25 °C and then

337 transferred to the dark at 25 °C to start circadian cycles. Bioluminescent signals were tracked by a

338 CCD-camera every hour for 5 or more days. Luciferase data were extracted using the NIH ImageJ

339 software with a custom macro, and circadian period lengths were manually determined.

340 Protein lysate and Western blot (WB)

341 For WB, 15 mgs of whole-cell protein lysate were loaded per lane on a 3-8% tris-acetate or 6.5% tris-

342 glycine (bearing Phos-tag) SDS gel (92). Custom-raised antibodies against WC-1, WC-2, FRQ, and

343 FRH have been described previously (93–95). V5 antibody (Thermo Pierce) and FLAG antibody (M2,

344 Sigma-Aldrich) were diluted 1:5000 for use as the primary antibody. To analyze the phosphorylation

11

345 profiles of CAMKs, 20 μM 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 μL 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 μL 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).

357 **Acknowledgements**

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604

605   **Legends**

606   **Figure 1** Gene deletions of calcium pumps were tested for circadian phenotypes by race tube (**A**) and

607   luciferase (**B**) analyses. Strains were cultured on 0.1% glucose race tube medium in a 96 well plate

608   and synchronized by growth in constant light overnight (16-24 hrs) followed by transfer to darkness.

609   Bioluminescent signals were monitored by a CCD-camera every hour, bioluminescence data were

610   acquired using ImageJ with a custom macro, and period lengths were manually calculated. Raw

611   bioluminescent data from three replicates were plotted with the X-axis and Y-axis representing time

612   (hrs) and arbitrary units respectively. (**C**) Left, Western blot showing the expression level of FRQ in

613   WT and Δ*nca-2* over 28 hr detected with FRQ-specific antibody. DD, hours after the light to dark

614   transfer; right, RT-qPCR showing relative levels of *frq* mRNA expressed in WT and Δ*nca-2*; *rac-1* was

615   used as an internal control, to which *frq* expression is normalized (n = 3, mean values ± standard

616   error of the mean). Asterisks indicate statistical significance when compared with WT as determined

617   by a two-tailed student's *t* test. *****P<0.00001; ****P=0.00006; ***P=0.001337; **P<0.01;

618   *P=0.010131. NS, difference is not significant. (**D**) Race tube assays of WT and the Δ*nca-2* strain

619   using race tube media in the presence or absence of 2 mM calcium chloride. Growth fronts of the

620   strains were marked by vertical blacklines every 24 hr. *nca-3* (NCU05154): calcium P-type ATPase;

621   *nca-1* (NCU03305): calcium-transporting ATPase sarcoplasmic/endoplasmic reticulum type; *cax*

622   (NCU07075): calcium/proton exchanger; *pmr-1* (NCU03292): calcium-transporting ATPase type 2C

623   member 1; *nca-2* (NCU04736): plasma membrane calcium-transporting ATPase 3; gene names, NCU

624   numbers, and descriptions were obtained from the FungiDB website (https://fungidb.org/fungidb/app).

625   Period was determined as described in Material and Methods and is reported ± SD (n=3).

626   **Figure 2** Circadian components WC-1 and FRQ are hyper-phosphorylated in Δ*nca-2.* (**A**) Total WC-1

627   was followed by Western blotting. Samples were cultured in the constant dark prior to a 15, 30, 60,

628   and 120 min light exposure. Non-specific bands in the same blot were shown for equal loading.

629   Decreased electrophoretic mobility is indicative of phosphorylation status (7). (**B**) mRNAs extracted

630   from samples cultured in the dark for 24 hrs or following a 15, 30, or 120 min light exposure as

631   indicated were reverse-transcribed to cDNA followed by quantitative PCR with a primer set specific to

632   *wc-1*. (**C**) Western blotting of WC-1 in a 24-hr time course with a 4-hr interval. (**D**) Similar to (**C**), RT-

24

633 qPCR was performed with samples harvested at the circadian conditions indicated. Phosphorylation

634 profiles of WC-1 (**E**) and FRQ (**F**, **G**) in WT and Δ*nca-2* were analyzed by Western blotting using

635 SDS-PAGE gels bearing (G) 20 μM Phos-tag chemicals and a ratio of 149:1 acrylamide to bis-

636 acrylamide. (**F**) Western blotting of FRQ in WT and Δ*nca-2* from DD14 to DD24 with a 2-hr resolution.

637 * in **E** denotes the mobility of unphosphorylated WC-1, and the bracket the region corresponding to

638 hyperphosphorylated WC-1. Arrows indicate hyper-phosphorylated FRQs observed in Δ*nca-2*.

639 **Figure 3** Some *frq* alleles are epistatic to Δ*nca-2*. The *frq C box* promoter activity was measured

640 using *C box-luciferase* at the *his-3* locus in indicated *frq* phospho-mutants in the presence or absence

641 of *nca-2*. Strains were grown on 0.1% glucose race tube medium in constant light overnight (16-24

642 hrs) prior to transfer to darkness. $frq^{S72A,\ S73A,\ S76A}$, $frq^{S541A,\ S545A}$, $frq^{S538A,\ S540A}$, frq^{S548A}, and $frq^{S632A,}$

643 ^{S634A} were derived from (9). Period was determined as described in Material and Methods and is

644 reported ± SD (n=3).

645 **Figure 4** Period shortening of Δ*nca-2* is rescued by deletion of *camk-2*. (**A**) Luciferase assays were

646 performed with a *frq C box* promoter-driven *luciferase* gene at the *his-3* in individual knockouts of

647 *camks-1-4* in the presence or absence of Δ*nca-2* as indicated. Periods are reported as described in

648 Material and Methods and is reported ± SD (n=3). (**B**) Upper panel, total levels of CAMKs-1-4 that

649 have a 3xFLAG tag respectively at their C-termini in WT or the Δ*nca-2* background were assayed by

650 Western blotting with FLAG antibody; lower panel, phosphorylation profiles of CAMKs-1-4 were

651 analyzed for the same sample set with 149:1 acrylamide to bis-acrylamide SDS-PAGE gels

652 containing Phos-tag. Asterisks indicate non-specific bands. For CAMKs-1, -2, and -4, total lysates

653 were applied while CAMK-3 was first pulled down by FLAG antibody-conjugated resins and

654 subsequently assayed in WB due to an overlap between CAMK-3 phospho-isoforms and non-specific

655 bands in the Phos-tag gel.

656 **Figure 5** *nca-2* is a *ccg* and modulates both input to and output from the core clock. (**A**) The *nca-2*

657 promoter fused to the *luciferase* gene was transformed to the *csr* locus and luciferase signals were

658 followed at 25 °C in the dark. Period was determined as described in Material and Methods and is

659 reported ± SD (n=3). (**B**) The *nca-2* open reading frame was fused to the 5' end of the firefly *luciferase*

660 gene and the same assay as in (**A**) was performed to trace the luciferase signal. (**C**) A representative

661 silver-stained gel showing NCA-2^{VHF} and its interactome purified from a culture grown in the light.

662 NCA-2^{VHF} and interactors were affinity-purified, trichloroacetic acid (TCA)-precipitated, and analyzed

663 by mass spectrometry. (**D**) NCA-2 is tagged with a V5 tag and one of its interactors, CSP-6, was

664 tagged with a 3xFLAG tag; Co-immunoprecipitation was performed using V5 resin and Western

25

665   blotting was done with V5 and FLAG antibodies respectively. (**E**) Working model for the roles of

666   intracellular calcium and of *nca-2* in the circadian system, In Δ*nca-2,* increased calcium over-activates

667   CAMKs, which induces FRQ over-phosphorylations and thereby causes a faster running clock; the

668   circadian clock regulates expression of *nca-2* and *camk*s.

669   **Figure S1** Stability of WC-1 and FRQ in WT and Δ*nca-2.* (**A**) Left, cycloheximide (CHX) was added to

670   the *Neurospora* culture growing in the light at a final concentration of 40 µg/mL and tissues were

671   harvested at indicated time points and assayed by Western blotting with WC-1- and FRQ-specific

672   antibodies respectively; right, densitometric analyses of the blots on the left showing WC-1 and FRQ

673   levels in WT and Δ*nca-2* treated with 40 µg/mL CHX for 0, 4, 8, or 12 hrs as indicated. (**B**) Left, FRQ

674   stability in WT and Δ*nca-2* is measured using CHX-treated cultures sampled every 2 hrs over 14 hrs;

675   right, densitometric analysis of FRQ on the left panel.

676   **Figure S2** Δ*nca-2* shortens period length in a long period *frq* allele and in *wcc* phospho-mutants (**A**)

677   The activity of the *frq* promoter is measured by the *C-box-luc* bioluminescence in the background of

678   *frq*[7], *wc-1*[S971A, S988A, S990A ,S992A, S994A, S995A], or *wc-2*[S433A] in the presence or absence of *nca-2* as

679   indicated in the figure. (**B**) Phosphorylations of WC-1 S971 and S990 in the presence or absence of

680   *nca-2* were assayed using a 6.5% SDS-PAGE gel bearing Phos-tag (for details, see Materials and

681   Methods). Phosphorylation of WC-1 residues S971, S988, S990, S992, S994, and S995 is promoted

682   by FRQ and essential for the closure of the circadian feedback loop (10).

683   **Figure S3** Expression of *camks* in WT and Δ*nca-2.* mRNA levels of *camks-1-4* in WT and Δ*nca-2*

684   were assayed by RT-qPCR with samples grown under light or dark conditions as indicated.

685   Expression of *camks* was normalized to that of *rac-1.*

686   **Figure S4** Individual or combinational deletion of *camk* genes does not dramatically impact the

687   circadian period by the luciferase assay.(**A**) Gene knockouts of individual *camks-1-4* were assayed for

688   circadian phenotypes by race tube. Δ*camk-1* cultures in the top and middle tubes are true knockouts

689   while the one at the bottom is a typical revertant as reported by (18) (**B**) Combinations of Δ*camk*

690   mutants were tested by the luciferase assay.

691   **Figure S5** NCA-2 domain analyses and its relationship with *csp-6* on the clock. (**A**) Predicted

692   domains in NCA-2 analyzed by the on-line tool SMART (http://smart.embl-

693   heidelberg.de/smart/set_mode.cgi?NORMAL=1) Race tube (**B**) and luciferase (**C**) analyses of Δ*nca-2*,

694   Δ*csp-6*, and Δ*csp-6*; Δ*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.
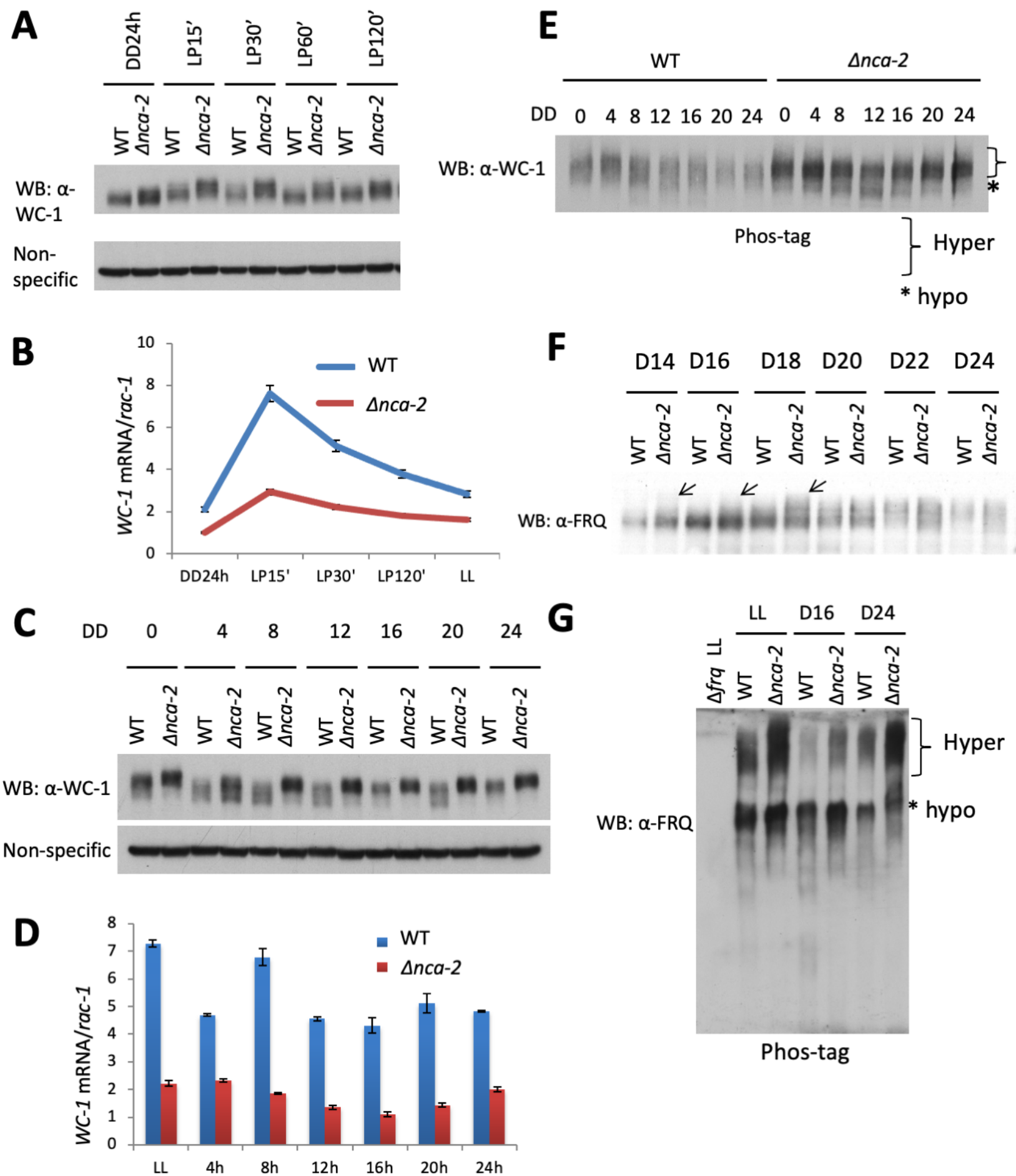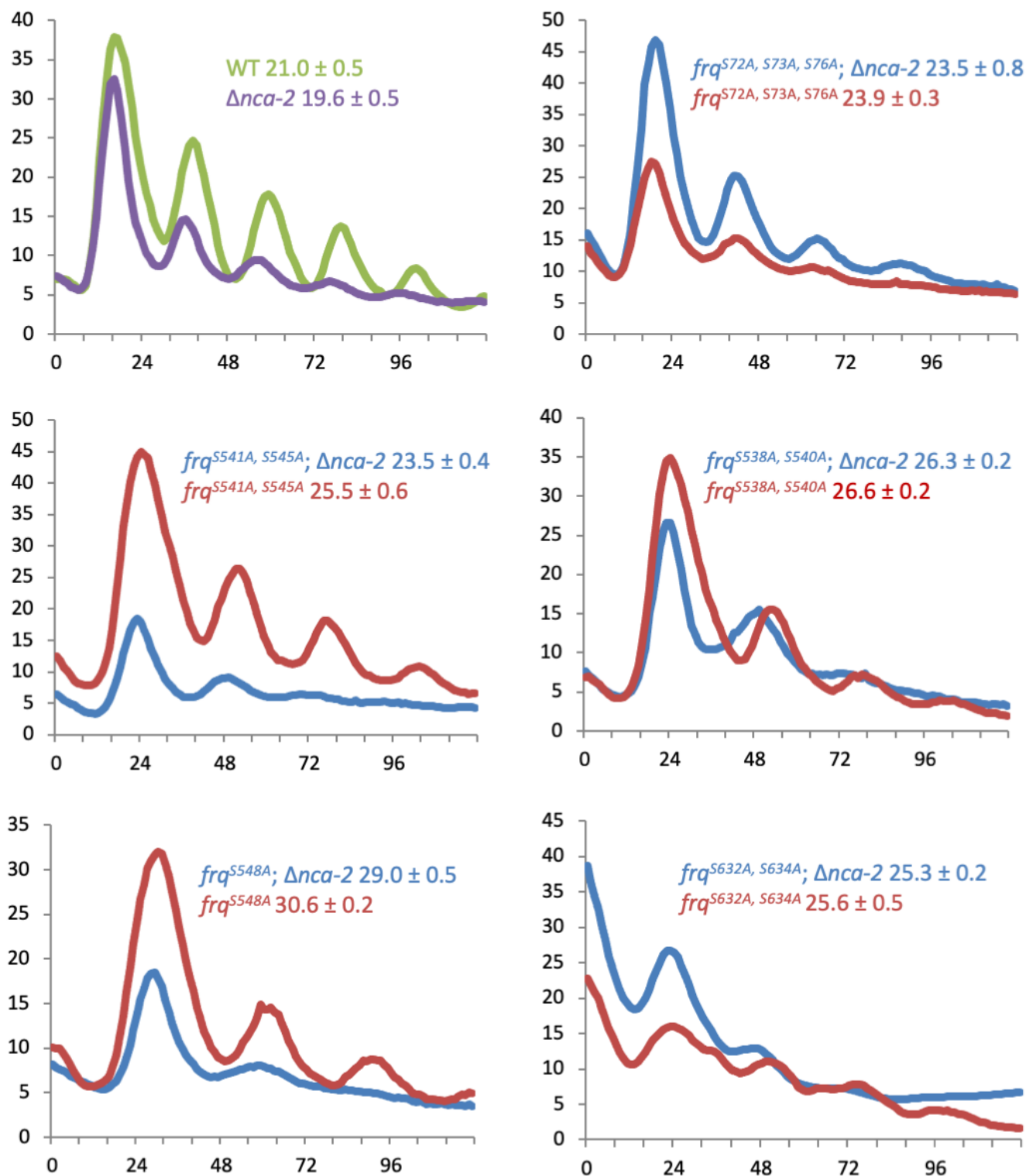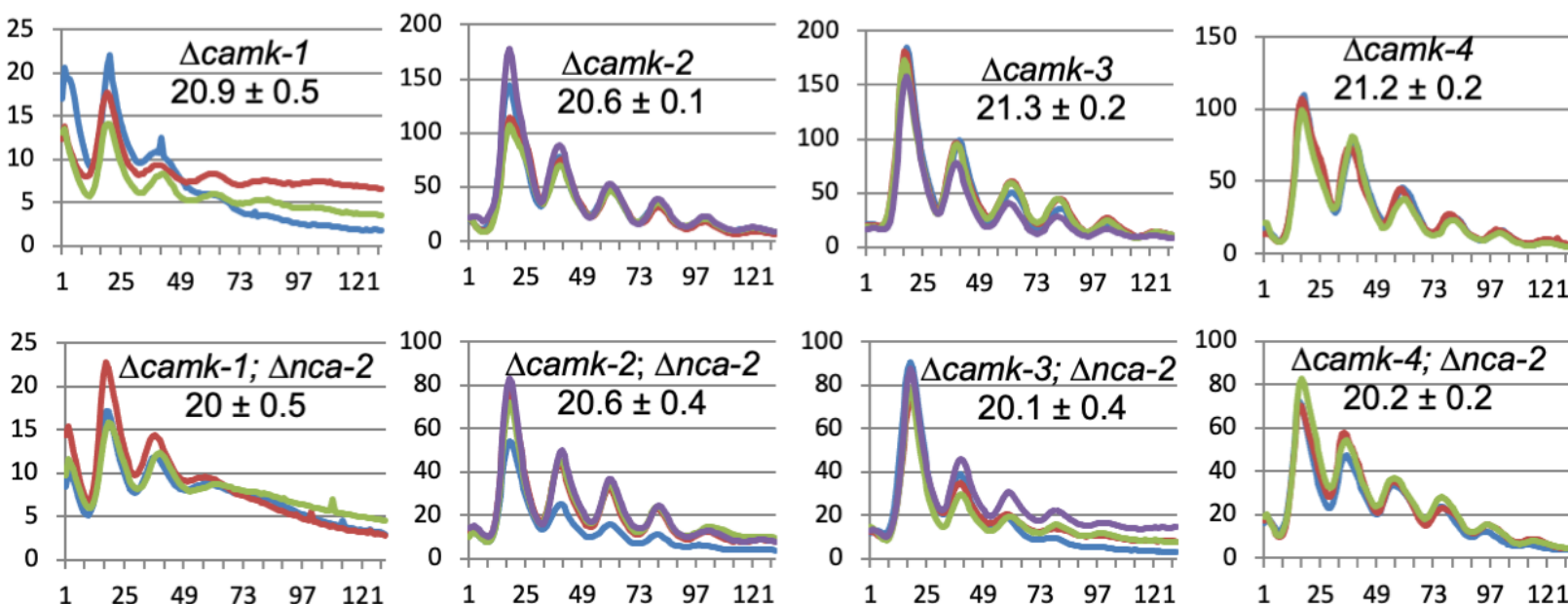
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Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

A

Luciferase gene driven by the *nca-2* promoter
20 ± 0.0

B

nca-2 fused with the *luciferase* gene under the *nca-2* promoter
20.2 ± 0.2

C

D

E