Title: **A conserved chronobiological complex times *C. elegans* development**

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Abstract

The mammalian PAS-domain protein PERIOD (PER) and its *C. elegans* orthologue LIN-42 have been proposed to constitute an evolutionary link between two distinct, circadian and developmental, timing systems. However, while the function of PER in animal circadian rhythms is well understood molecularly and mechanistically, this is not true for LIN-42's function in timing rhythmic development. Here, using targeted deletions, we find that the LIN-42 PAS domains are dispensable for the protein's function in timing molts. Instead, we observe arrhythmic molts upon deletion of a distinct sequence element, conserved with PER. We show that this element, designated CK1δ-binding domain (CK1BD), mediates stable binding to KIN-20, the *C. elegans* CK1δ/ε orthologue. We demonstrate that CK1δ phosphorylates LIN-42 and define two conserved helical motifs in the CK1BD, CK1BD-A and CK1BD-B, that have distinct roles in controlling CK1δ-binding and kinase activity *in vitro*. KIN-20 and the LIN-42 CK1BD are required for proper molting timing *in vivo*, and loss of LIN-42 binding changes KIN-20 subcellular localization. The interactions mirror the central role of a stable circadian PER–CK1 complex in setting a robust ~24-hour period. Hence, our results establish LIN-42/PER – KIN-20/CK1δ/ε as a functionally conserved signaling module of two distinct chronobiological systems.

Introduction

Chronobiology is the study of biological rhythms. Circadian rhythms which enable organisms to anticipate daily cycles of light, temperature, and other environmental variables are one of the best studied examples. [1] PERIOD (PER) proteins are central to this timekeeping mechanism. Their cellular abundance, stability, and nuclear localization changes rhythmically over a ~24-hour cycle. In mammals, the paralogs PERIOD1 and PERIOD2 (PER1 and PER2) associate with Casein kinase 1 δ/ε (CK1δ/ε) as well as the CRYPTOCHROME proteins, CRY1 and CRY2, to form nuclear transcriptional complexes which repress the transcriptional activators circadian locomotor output cycles protein kaput (CLOCK) and brain and muscle ARNT-like 1 (BMAL1)(Fig. S1A).[2–5] Since the targets of the CLOCK-BMAL1 heterodimer include PER and CRY themselves, PER and CRY thus eventually repress their own production, permitting subsequent re-expression of CLOCK-BMAL1 to start a new cycle.[2,6]

To maintain the 24-hour period of circadian rhythms, a delay between the activation and the repression of CLOCK-BMAL1 transcription is required. Central to this delay is the post-translational modification of PER by one of the two closely related CK1δ and CK1ε kinases. For convenience and because of the redundancy in their function, we will henceforth refer to the two isoforms generically as CK1. Anchored through a CK1-Binding Domain (CK1BD)[7], CK1 is associated with PER for its entire existence in the cell, even translocating from the cytoplasm to the nucleus together (Fig. S1A)[4,8], allowing PER to deliver CK1 to other targets at clock-controlled promoters.[8,9] CK1-mediated phosphorylation of a PER degron licenses PER ubiquitylation and subsequent degradation, thereby limiting PER abundance and its period of activity (Fig. S1B).[10–12] In addition to the degron, CK1 phosphorylates multiple serines in another PER sequence element known as FASP ("Familial Advanced Sleep Phase")(Fig. S1B).[13,14] Phosphorylation of the FASP site turns PER into an inhibitor of CK1 kinase activity.[15,16] In humans, a single residue mutation in this region that blocks all FASP phosphorylation results in a short circadian period that manifests as the eponymous Familial Advanced Sleep Phase, where affected individuals wake up very early in the morning.[17,18]

In contrast to circadian timing, the chronobiology of development is mechanistically less well understood. In *C. elegans*, a single PER orthologue, LIN-42, appears to diverge substantially in structure and function from the mammalian and fly PER proteins. At 598 amino acids for its longest isoform, LIN-42 is substantially shorter than mouse PER2 at 1225 amino acids.[19] LIN-42 lacks a CRY-binding domain, consistent with a lack of a CRY orthologue in *C. elegans*, and in its tandem PER-ARNT-SIM (PAS) domains, only PAS-B is well conserved, adopts a canonical fold, and mediates dimerization in a mode identical to mammalian PER.[20,21] Finally, two stretches of sequence, previously termed SYQ and LT according to their first amino acids[22], bear sequence homology to the two PER CK1BD subdomains, CK1BD-A and CK1BD-B, but their function has remained unexplored. *lin-42* further differs from canonical PERs in its expression dynamics and functions: rather than exhibiting a ~24-hour, temperature-invariant period, *lin-42* expression cycles exhibit a temperature-dependent length which can be as short as ~7 hours at 25˚C.[19,23] Indeed,

94 *lin-42* was identified as a developmental gene whose mutation causes heterochronic
95 phenotypes, i.e., defects in temporal cell fate specification where cells adopt adult cell
96 fates precociously, in larvae.[24,25] Hence, it was proposed that *lin-42/PER* constitute an
97 evolutionary link between two distinct, circadian and developmental, timing systems.[19]
98
99 The extent of functional similarity between these timing systems generally, and LIN-42
100 and PER function specifically, has remained uncertain, despite the realization that
101 additional orthologues of mammalian clock genes exist and cause heterochronic
102 phenotypes when mutated.[19,21,23,26–28] Conceptually, whereas mutations of circadian
103 clock genes such as *PER* change the tempo and/or robustness of circadian rhythms,
104 heterochronic mutations are defined by their ability to alter the sequence of
105 developmental events, such that certain events are skipped.[29,30] Accordingly, and despite
106 its rhythmic expression, the heterochronic function of LIN-42 does not appear to involve
107 recurring activity but rather the stage-specific repression of the *let-7* miRNA prior to the
108 third larval stage.[31–33]
109
110 LIN-42 is also required for the rhythmic occurrence of molts. Wild-type animals molt, i.e.,
111 regenerate a new collagenous apical extracellular matrix (cuticle), at the end of each of
112 the four larval stages.[34] Under constant environmental conditions, individually grown
113 animals enter and exit molts with great temporal uniformity, revealing robust temporal
114 control. *lin-42(ok2385)* mutant animals were shown to cause a slow-down of development
115 as well as an arrhythmic molting phenotype where this uniformity is lost such that
116 individual animals molt at different times.[35,36] It is unknown how LIN-42 contributes to
117 rhythmic molting mechanistically. In addition to *CRY*, the *C. elegans* genome lacks
118 obvious orthologues of *BMAL1* and *CLOCK*, arguing for a mechanism that differs from
119 that of the circadian clock at least in the identity of several core components.[27] In a yeast
120 two-hybrid assay, LIN-42 was shown to be capable of binding to numerous transcription
121 factors.[37] Although the functional relevance of such binding for molting has remained
122 unexplored, LIN-42 binding to the REV-ERB orthologue NHR-85 appears important for
123 robust periodic transcription of the heterochronic *lin-4* miRNA. Finally, loss of KIN-20, the
124 *C. elegans* orthologue of CK1δ/ε, replicates some heterochronic phenotypes of *lin-42*

125 mutation and slows development[26], but its heterochronic functions were argued not to
126 involve LIN-42[38], and it is unknown whether KIN-20 is required for rhythmic molting.
127
128 Here, we set out to further characterize the molecular and developmental functions of
129 LIN-42. Using targeted mutations, we find that its PAS domains are largely dispensable
130 both for heterochronic pathway activity and rhythmic molting. By contrast, the SYQ/LT
131 regions are specifically required for rhythmic molting. We demonstrate that these domains
132 function as a CK1BD, mediating stable KIN-20/CK1 binding to LIN-42 *in vivo* and *in vitro*.
133 CK1 phosphorylates LIN-42 *in vitro* and this activity is distinctly modulated through the
134 two CK1BD subdomains: CK1BD-B is particularly important for stable CK1 binding,
135 whereas the CK1BD-A element inhibits CK1 enzymatic activity. Moreover, loss of LIN-42
136 binding impairs nuclear localization of KIN-20. Our results identify LIN-42/PER–KIN-
137 20/CK1 as a conserved chronobiological complex utilized by two distinct biological
138 oscillators. Although CK1 activity in the circadian clock has previously been viewed mostly
139 through the lens of its effects on PER, our findings align well with the growing notion that
140 PER-mediated regulation of CK1 may be an additional important mechanism to support
141 robust circadian rhythms.
142
143 **Results**
144 **The LIN-42 CK1BD mediates a subset of developmental functions of LIN-42**
145 Previous analysis of the two partial deletion alleles of *lin-42*, *n1089* and *ok2385* (Fig. 1A),
146 have suggested the possibility that different developmental functions could be genetically
147 separable. Although both deletions caused precocious heterochronic phenotypes, only
148 *ok2385* showed evidence of slow and arrhythmic molting.[22,35,39] To confirm these findings,
149 we quantified molt, intermolt, and larval stage duration for mutant and wild-type animals
150 at high temporal resolution, using a luciferase assay that monitors entry and exit from the
151 lethargus state associated with molting on animals grown in isolation.[36,40] Indeed, we
152 found that *lin-42(ok2385)* mutant larvae developed more slowly than wild-type animals
153 and became increasingly arrhythmic (Fig. 1B, S2A-C). Moreover, and as observed
154 previously[36], most mutant animals underwent only three molts (33/39 animals) within the
155 duration of this assay, suggesting either a precocious exit from the molting cycle, or a

very delayed or abnormal fourth molt (Fig. 1B, S2D). By contrast, essentially all *lin-42(n1089)* animals completed a normal number of molts (42/42 animals) and maintained robust synchrony, although they developed significantly slower than wild-type animals (Fig. 1B). At the same time, both alleles caused robust heterochronic phenotypes, illustrated by precocious formation of alae (Fig. 1C) in L3 to early L4 larvae. Alae are a cuticular structure normally secreted by the terminally differentiated epidermal seam cells at the L4-adult transition.

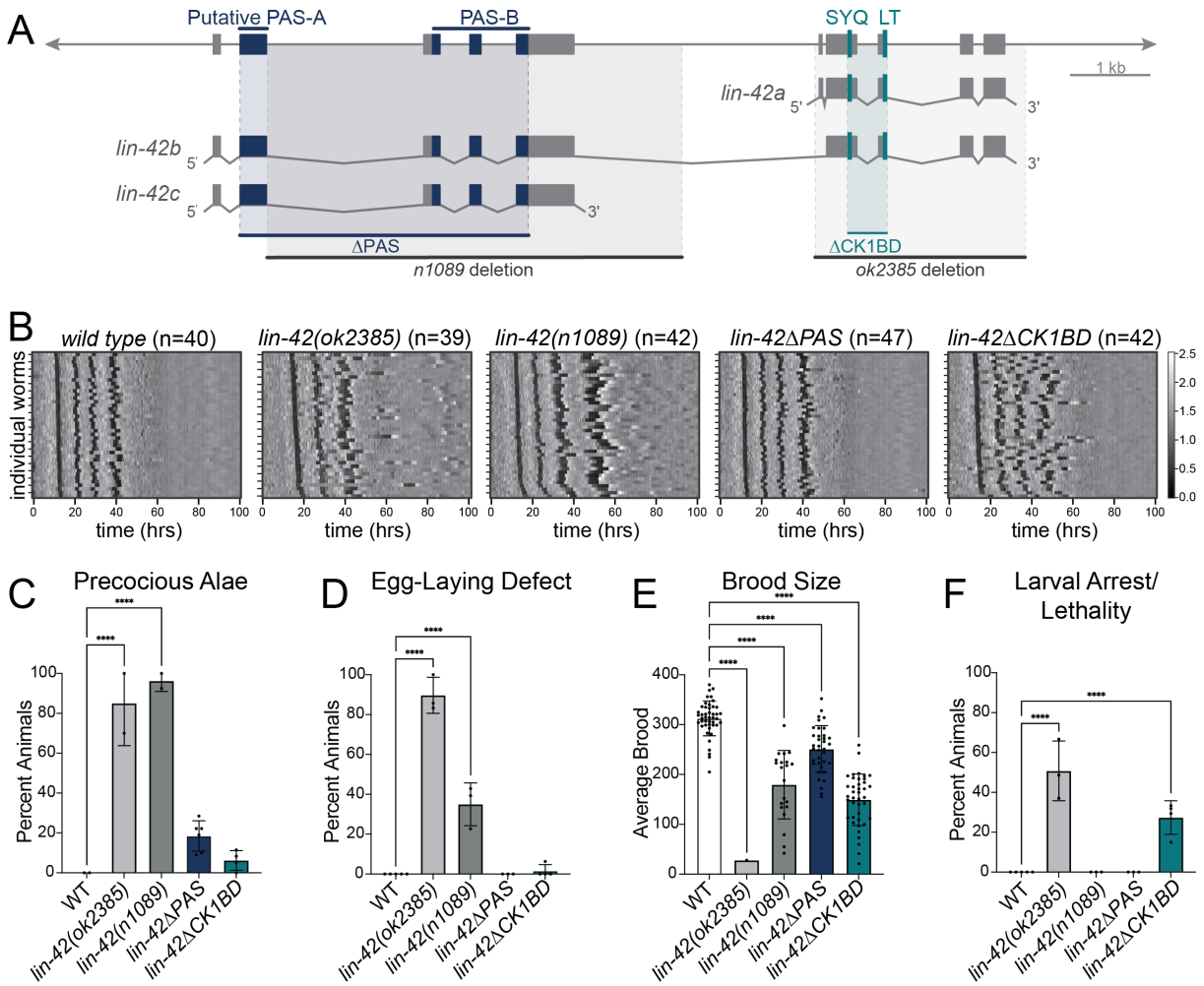

Figure 1. *lin-42ΔPAS* mutations cause mild heterochronic phenotypes while *lin-42ΔCK1BD* mutations cause asynchronous molting (A) Schematic depiction of the *lin-42* genomic locus. The gene structure of the *lin-42 a, b*, and *c* isoforms are shown. The location of sequences encoding putative PAS-A, PAS-B, SYQ, and LT sequence motifs are indicated. Published *lin-42* deletion alleles *n1089* and *ok2385* are indicated in dark and light grey, respectively, newly generated targeted Δ*PAS* and Δ*CK1BD* deletions in navy and teal, respectively. **(B)** Heatmaps showing trend-corrected luminescence traces from the indicated genotype. Each horizontal line represents one animal. Traces are sorted by entry into the first molt. Darker color indicates low luminescence signal and corresponds to the molts. **(C)** Bar plot quantifying the percentage of animals of the indicated genotype with precocious, complete, or partial alae at the L3-L4 molt. **(D)** Bar plot quantifying the percentage of animals of the indicated genotype that exhibited egg-laying defects as determined by the presence of hatched larvae in the animal. **(E)** Bar plot depicting the average number of live progeny from hermaphrodites of the indicated genotype. **(F)** Bar plot quantifying the percentage of animals of the indicated genotype that arrested or died as larvae. (C-F) Statistical significance was determined using an ordinary one-way ANOVA. $p<0.05$ was considered statistically significant. **** indicates < 0.0001. Error bars in C-F represent standard deviation.

163

164 The complex nature of the alleles, each with at least one breakpoint in a noncoding region,
165 precludes a straightforward attribution of the phenotypes to specific features of the LIN-
166 42 protein. Therefore, we decided to generate precise deletions of the two conserved
167 regions, PAS and SYQ/LT (Fig. 1A). Henceforth, and based on the functional
168 characterization which we describe below, we will refer to SYQ/LT as CK1BD for CK1-
169 Binding Domain. To our surprise, *lin-42(wrd67[ΔPAS])* mutant animals exhibited
170 essentially wild-type molting patterns in the luciferase assay, resembling neither *lin-*
171 *42(n1089)* nor *lin-42(ok2385)* mutant animals. By contrast, *lin-42(wrd63[ΔCK1BD])*
172 mutant animals exhibited increasing loss of molting synchrony over time (Fig. 1B).
173 However, unlike *lin-42(ok2385)*, *lin-42(ΔCK1BD)* mutant animals executed four
174 detectable molts during the assay (Fig. 1B, S2D).

175

176 To get a better understanding of the developmental relevance of each domain, we
177 examined additional phenotypes (Fig. 1D-F; Table S1). We found that all four alleles
178 affected brood sizes, albeit to varying degrees, in the order
179 *ok2385>>ΔCK1BD≈n1089>ΔPAS* of decreasing severity (Fig. 1E, Table S1). Only
180 *ok2385* and *n1089* caused highly penetrant egg-laying (Egl) and precocious alae
181 defects[22,35,39], whereas ΔPAS and ΔCK1BD mutants had completely wild-type egg laying
182 and only modest precocious alae (Fig. 1C and D, Table S1). Finally, both *ok2385* and, to
183 a lesser extent, ΔCK1BD caused larval arrest and lethality, a phenotype interestingly not
184 observed in the luciferase assay, suggesting an environmental dependency (Fig. 1F,
185 Table S1). Taken together, these results indicate surprisingly mild phenotypes upon loss
186 of the LIN-42 PAS region and highlight an important function of the CK1BD for rhythmic
187 molting.

188

189 **LIN-42 and KIN-20 interact *in vivo***

190 Since the genetic results suggested separable functions of LIN-42, we hypothesized that
191 these could depend on specific interaction partners. To identify interaction partners we
192 performed anti-FLAG immunoprecipitations on endogenously tagged *lin-*
193 *42(xe321[3xFLAG::lin-42b+c])* mixed-stage animal lysates followed by mass-

194 spectrometry. As a control for non-specific binding, we also performed an anti-FLAG
195 immunoprecipitation in an unrelated strain (*sart-3::GFP::3xFLAG*).[41] We observed a
196 single highly enriched interaction partner for LIN-42: KIN-20 – the orthologue of
197 mammalian CK1δ/ε (Fig. 2A).

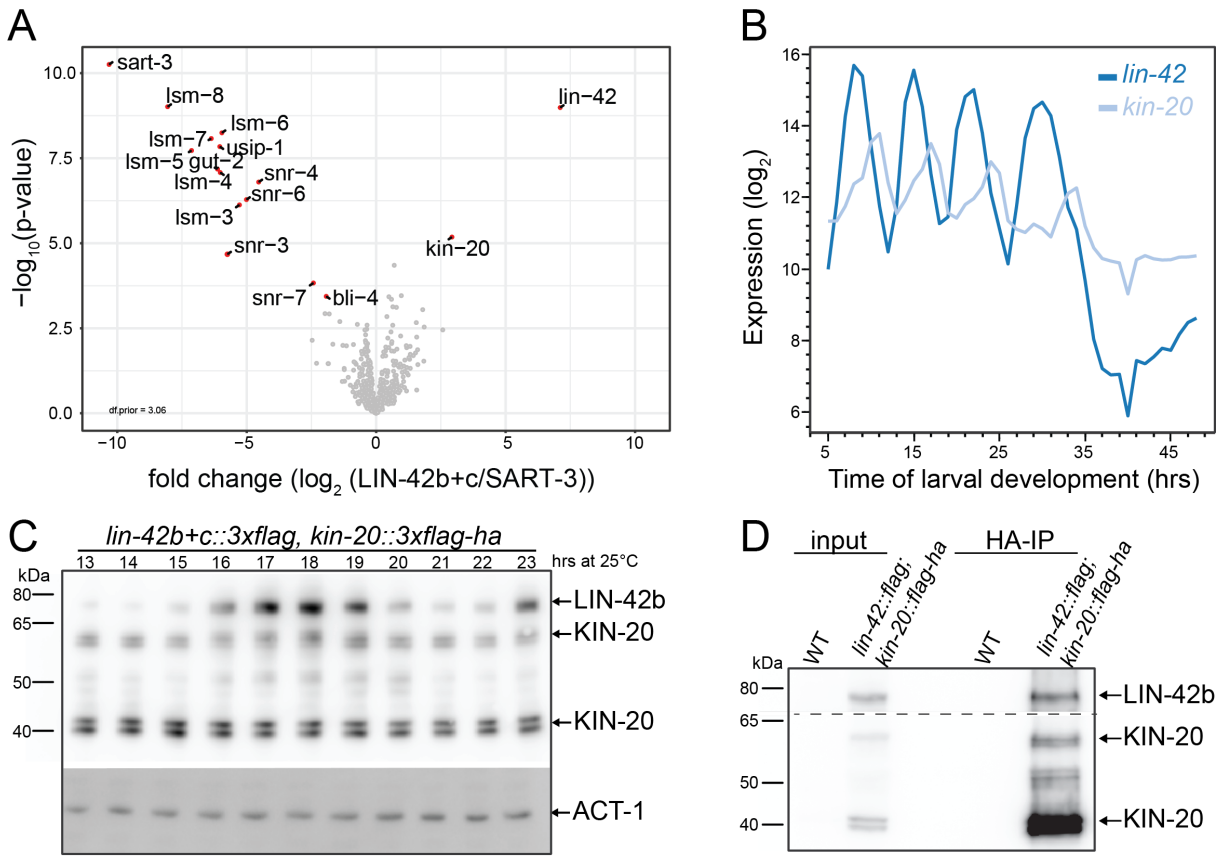

Figure 2 LIN-42 and KIN-20 interact *in vivo* (A) Volcano blot comparing protein enrichments in 3xFLAG::LIN-42b+c and 3xFLAG::SART-3 (control) immunoprecipitations, determined by mass spectrometry. **(B)** mRNA expression profile for *lin-42* (dark blue) and *kin-20* (light blue) mRNAs throughout larval development. Data Source: Meeuse et al., 2020. **(C)** Western blot with extracts from *3xflag::lin-42b+c; 3xflag-ha::kin-20* (HW3479) larvae collected hourly for 11 hours, starting at 13 h after plating synchronized L1 stage animals on food at 25°C. Top part probed with anti-FLAG-HRP (1:1,000), lower part with anti-actin-1 (1:7,500). Arrows indicate bands for LIN-42b, KIN-20 and ACT-1 (see Fig. S5A). **(D)** Anti-HA pulldown from *3xflag::lin-42b+c; 3xflag-ha::kin-20* animals. Blot probed with anti-FLAG (1:1,000).

198

199 The mRNA levels of both *lin-42* and *kin-20* oscillate.[40,42] Further analysis of the published
200 sequencing data revealed that the peak phases of the two proteins differ, but that there
201 are periods where both transcripts accumulate (Fig. 2B). This is also true for the proteins:
202 we observed by Western blotting that the levels of endogenously tagged KIN-20 protein
203 were largely invariant over time, whereas those of endogenously tagged LIN-42b protein

204 levels changed rhythmically (Fig. 2C). Finally, we confirmed a physical interaction with a
205 reciprocal anti-HA pulldown from *lin-42(xe321[3xFLAG::lin-42b+c]); kin-*
206 *20(xe328[3xFLAG-HA::kin-20])* animal lysates, again using endogenously tagged
207 proteins (Fig. 2D). Taken together, we conclude that LIN-42 and KIN-20 form a complex
208 *in vivo*.
209
210 **The LIN-42 SYQ-LT regions form a functional CK1BD capable of binding**
211 **mammalian CK1**
212 Mammalian CK1 binds PER through its kinase domain. This region is highly conserved,
213 with CK1δ and KIN-20 exhibiting 79% and 100% sequence identity within the kinase
214 domain and active sites, respectively (Fig. S3).[43,44] By contrast, the CK1BD is less well
215 conserved in LIN-42 with only ~30% sequence identity and a different spacing of the
216 relative LIN-42 CK1BD-A and -B sites (Fig. 3A). Hence, it was unclear whether this region
217 could function as a CK1BD in LIN-42.
218
219 We produced recombinant proteins to test this possibility. We were unable to express
220 soluble KIN-20 from a bacterial or insect system, so we used recombinant human CK1δ
221 kinase domain (hereafter referred to as CK1), similar to previous studies of KIN-20.[45] *In*
222 *vitro* pulldown assays with biotinylated LIN-42 protein containing the SYQ/CK1BD-A and
223 LT/CK1BD-B domains as well as C-terminal tail (residues 402-589) revealed that CK1
224 bound to wild-type LIN-42 protein. Deletion of either the SYQ/CK1BD-A or LT/CK1BD-B
225 motifs reduced this interaction (Fig. 3B).
226 To test whether the CK1BD is sufficient to bind CK1, we purified a truncated fragment of
227 LIN-42 lacking the C-terminal tail (CK1BD) and performed bio-layer interferometry (BLI)
228 assays with biotinylated CK1 and titrating LIN-42 fragments. This optical technique
229 measures macromolecular interactions in real-time, providing k_{on} and k_{off} rates from which
230 affinity constants (K_D) can be calculated. The smaller size of the CK1BD compared to the
231 CK1BD+Tail (residues 402-589; same construct used in Fig. 3B) resulted in a lower
232 response signal, however, both proteins bound to biotinylated CK1 with comparable,
233 nanomolar affinities (348 ± 123 nM for CK1BD and 209 ± 78 nM for CK1BD + Tail; Fig.

234 3C-E). These results demonstrate that the SYQ and LT motifs encompass a minimal
235 CK1BD.

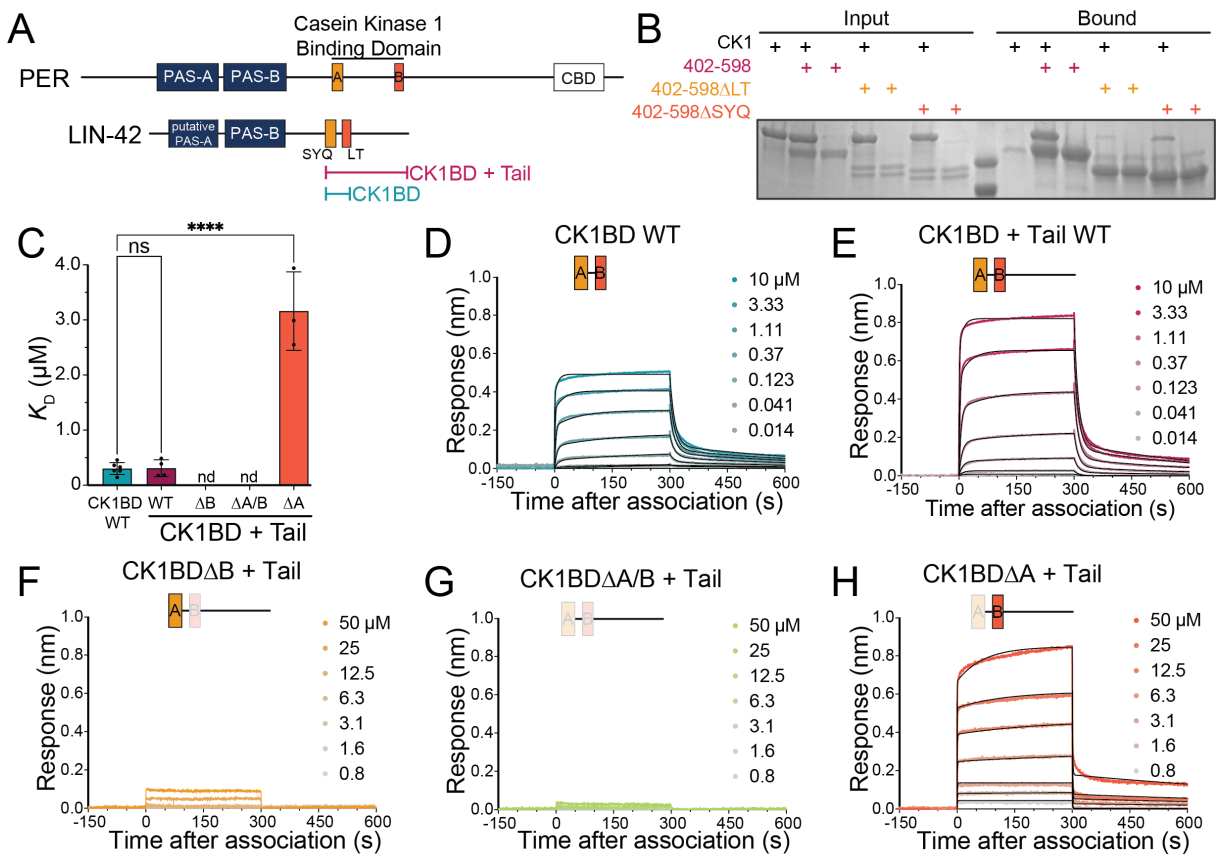

Figure 3 The LIN-42 SYQ and LT domains constitute a functional CK1-binding domain (A) Schematic representing PER2 and LIN-42 protein domains. Protein constructs used in this study, CK1BD + Tail and CK1BD, are indicated. CK1BD, Casein Kinase 1-Binding Domain; CBD, CRY-binding domain. **(B)** Representative pulldown assay of human CK1 and biotinylated LIN-42 CK1BD + Tail proteins using the indicated protein variants. **(C)** Values for K_D from kinetic analysis of BLI data (D-H) based on a 2:1 heterogeneous ligand binding model and global analysis (Octet). Mean ± SEM, ordinary one-way ANOVA. $p<0.05$ was considered statistically significant. **** indicates $p< 0.0001$. **(D-H)** Bio-layer interferometry (BLI) data for indicated LIN-42 protein binding to immobilized, biotinylated CK1. Inset values represent the concentrations of LIN-42 for individual binding reactions. Model fit to association and dissociation over time is represented by black lines. Data shown from one representative experiment of n ≥ 3 assays.

236

237 We next performed BLI experiments to quantitatively explore the significance of the
238 CK1BD-A and -B motifs in binding to CK1. LIN-42-binding to CK1 was not detectable
239 when CK1BD-B was deleted, either singly (*CK1BD-ΔB*) or in combination with CK1BD-A
240 (*CK1BD-ΔA/B*) even at higher concentrations (Fig. 3C,F,G). In contrast, deletion of the
241 CK1BD-A domain reduced the affinity of LIN-42 for CK1 approximately 10-fold (K_D = ~3
242 μM)(Fig. 3C,H), necessitating higher concentrations of the CK1BD-ΔA +Tail protein to
243 determine an accurate K_D (Fig. 3H). These data indicate that CK1BD-A and B both

contribute to high-affinity binding of CK1 *in vitro*, while revealing a more important contribution of the CK1BD-B motif, consistent with recent work that demonstrated an essential role of the human PER2 CK1BD-B motif for stable association with CK1 in a mouse model and mammalian cells.[46] Collectively, our data establish that the LIN-42 CK1BD is functionally conserved and mediates stable binding to the CK1 kinase domain.

Phosphorylation of the LIN-42 C-terminus by CK1 exhibits a conserved mode of feedback inhibition Mammalian CK1 regulates PER abundance by controlling its degradation post-translationally. Mutations on CK1 or its PER phosphorylation sites can induce changes in circadian period as great as ~4-hours.[16,18,47,48] To test whether LIN-42 is a CK1 substrate, we performed *in vitro* ^{32}P-ATP kinase assays using the CK1BD alone or the CK1BD+Tail as substrates. CK1BD protein contains 4 serine and 6 threonine residues while the CK1BD + Tail construct contains an additional 22 serine and 15 threonine residues. We observed phosphorylation for both constructs, yet more robustly on LIN-42 CK1BD+Tail than on CK1BD alone (Fig. 4A-C). Hence, CK1 appears capable of phosphorylating both CK1BD and the extended C-terminal tail. Deletion of CK1BD-B reduced phosphorylation relative to the wild-type protein, whereas phosphorylation of the CK1BD-ΔA + Tail mutant protein remained largely unaltered (Fig. 4A-C). Thus, in the two assays, loss of CK1BD-B impacts both binding and phosphorylation by CK1 more strongly than loss of CK1BD-A (Fig. 3C, 4A-C).

To explore the kinetics of CK1-mediated phosphorylation of the LIN-42 extended C-terminus, we performed substrate titration experiments using an ADP-Glo enzymatic assay. Here, we observed a decrease in kinase activity with high levels of wild-type LIN-42 CK1BD+Tail (Fig. 4D). This result mirrors the ability of phosphorylated PER as well as the apoptosis substrate p63, to inhibit the activity of CK1 (DBT in Drosophila) in both mammalian and *Drosophila* systems through product inhibition.[14]

Closer examination revealed that deletion of the LIN-42 CK1BD-A motif relieved feedback inhibition (Fig. 4D), explaining why the ^{32}P-ATP data had shown little decrease in the overall phosphorylation level (Fig. 4A-C) despite having reduced affinity for the kinase

275 (Fig. 3C). Conversely, deletion of the B motif either in isolation or with the A motif, caused
276 a significant reduction in overall phosphorylation of CK1BD+Tail. No serine and only 4
277 threonine residues are removed in this deletion, so this effect was likely not due to loss
278 of phosphosites. The catalytic efficiency (k_{cat}/K_m) of CK1 for each LIN-42 substrate
279 decreased significantly with deletion of the CK1BD motifs relative to wild-type, with activity
280 most severely impacted upon loss of the CK1BD-B motif (Fig. 4D,E). Taken together,
281 these results suggest that while both motifs are involved in binding to CK1, deletion of the
282 CK1BD-A region predominantly relieves feedback inhibition without fully disrupting the
283 anchoring interaction seen with the deletion of the CK1BD-B domain. Moreover, and
284 similar to the mammalian PER–CK1 complex[49], loss of the anchoring interaction
285 compromises, but does not fully abrogate phosphorylation.
286
287 **Identification of potential LIN-42 phosphosites**
288 To determine potential CK1-dependent LIN-42 phosphorylation sites, we performed *in*
289 *vitro* kinase reactions followed by phosphoenrichment and mass spectrometry to identify
290 phosphopeptides.There are a total of 26 serine and 21 threonine residues in the CK1BD
291 + Tail construct. Of these, 12 serine and 3 threonine sites in the C-terminus were
292 phosphorylated upon incubation of LIN-42 CK1BD + Tail with CK1 at least once from
293 three replicates *in vitro* (Fig. 4F). Although we detected low levels of phosphorylation on
294 LIN-42 constructs lacking the tail in the ^{32}P-ATP assay (Fig. 4A), no serine and only 1
295 threonine phosphorylation within the CK1BD was detected *in vitro,* reflecting possible
296 limitations in the assays.
297
298 To test whether LIN-42 was phosphorylated *in vivo*, we immunoprecipitated
299 endogenously tagged 3xFLAG::LIN-42b+c at various time points from a population of
300 synchronized L2 stage larvae and subjected precipitates to mass spectrometry. We
301 identified 15 serine residues, 1 threonine residue, and 1 tyrosine residue that were
302 phosphorylated on LIN-42. All but four of these phosphosites (all serine residues) are
303 located on the CK1BD + Tail (Fig. 4F). Among these, 6 serine residues overlap with the
304 13 phosphoserines identified after the *in vitro* reaction while no threonine or tyrosine
305 residues were phosphorylated in both the *in vitro* and *in vivo* datasets (Fig. 4F). The

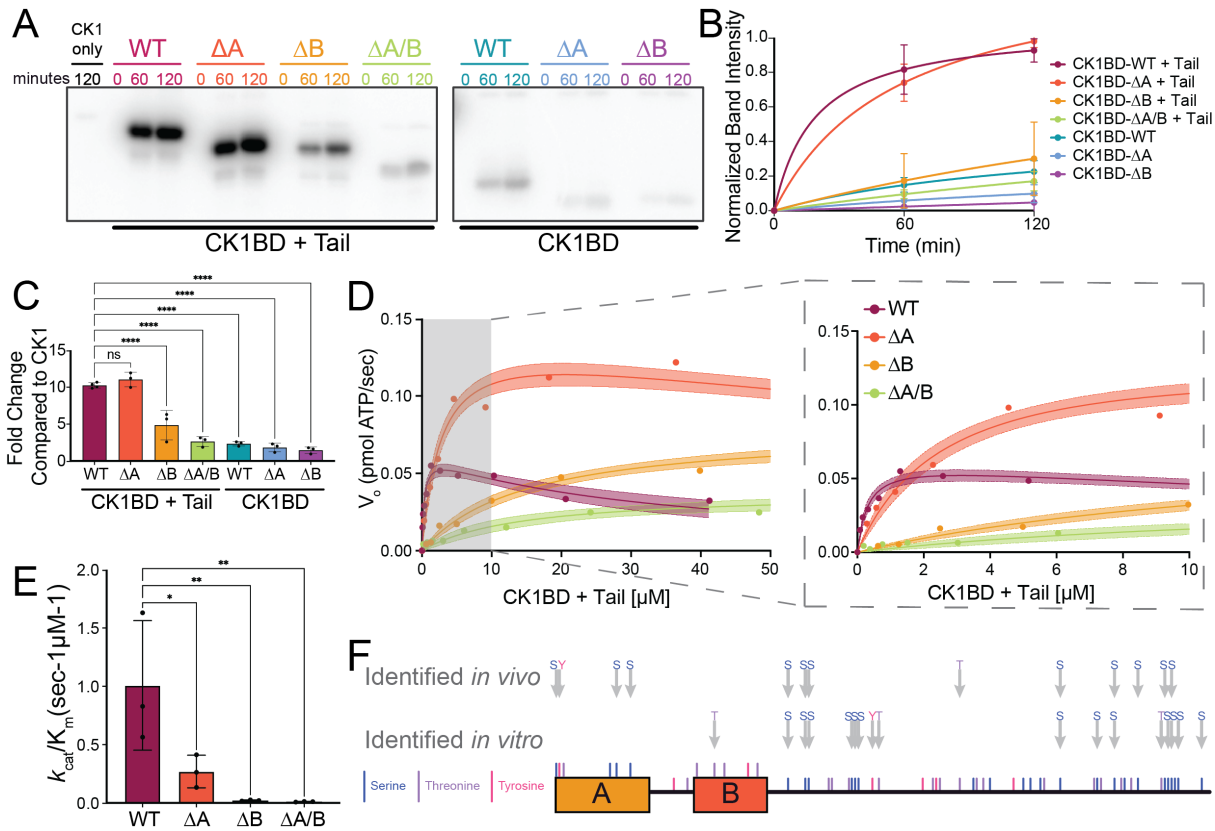

Figure 4 The extended C-terminus of LIN-42 is phosphorylated by CK1 (A) Representative ^{32}P-radiolabeled ATP enzymatic assay of CK1 activity on LIN-42 CK1BD + Tail and CK1BD wild-type and indicated domain deletion mutants. **(B)** Quantification of band intensity depicted in (A) and replicates. Model fit to Michaelis- Menten, n = 3. **(C)** Fold-change quantification of band intensity from panels A & B compared to CK1 autophosphorylation at 120 minutes. Ordinary one-way ANOVA. p<0.05 was considered statistically significant. ****p < 0.0001, n ≥ 3, mean ± SEM. (D) Representative ADP-Glo enzymatic assay comparing activity of CK1 on LIN-42 CK1BD + Tail wild-type (maroon), CK1BD-ΔA (orange), CK1BD-ΔB (yellow), and CK1BD-ΔA/B (green). **(E)** Values of k_{cat}/K_m calculated from (D). n=3, mean ± SEM, Ordinary one-way ANOVA. p<0.05 was considered statistically significant. * indicates p<0.03 and ** indicates p<0.002. **(F)** LIN-42 phosphorylation sites identified via mass spectrometry from *in vivo* samples from whole worm lysate collected hourly from synchronized L1 animals from 14 hrs (early L2) to 25 hrs (late L3) after plating at 25°C (top arrows) and *in vitro* kinase reactions of CK1 activity on LIN-42 CK1BD + Tail (bottom arrows). Serine, threonine, and tyrosine residues are indicated via vertical blue, purple, and pink ticks, respectively, along the CK1BD + Tail construct schematic.

overlap of *in vitro* and *in vivo* results is consistent with the possibility that LIN-42 is a CK1 substrate *in vivo*. Whether incomplete overlap reflects differences in kinase activities in the activity of additional kinases and/or specificity factors *in vivo*, or technical differences in the experimental approaches remains to be determined.

LIN-42 C-terminal tail deletion causes a heterochronic phenotype but leaves molting timing largely unaffected

Given the extensive phosphorylation of the LIN-42 tail *in vitro* and *in vivo*, we sought to explore its functional relevance. Unexpectedly, *lin-42(ΔTail)* mutant animals did not

315 recapitulate the *lin-42(ΔCK1DB)* arrhythmic molting phenotype but instead resembled *lin-*
316 *42(n1089)* animals phenotypically (Fig. 1C-F; Fig. 5). Specifically, *lin-42(ΔTail)* mutants
317 exhibited regular timing of the first three molts, yet an extended and irregular fourth molt
318 (Fig. 5A, S4A-C, S5). The severity of this extended 4th molt was variable and could reflect
319 unaccounted environmental differences during assay runs. The mutant animals also
320 exhibited a modest reduction in brood size, egg-laying defects and precocious alae but
321 no larval arrest (Fig. 5B-E). These data indicate that the LIN-42 C-terminus is largely
322 dispensable for timing of larval molting and suggest instead that it plays a previously
323 unrecognized role in the heterochronic functions of LIN-42.
324
325 ***kin-20* null and catalytically dead mutants exhibit arrhythmic molting**
326 Deletion of a major phosphorylation target, the LIN-42 tail, did not recapitulate *lin-*
327 *42(ΔCK1BD)* mutant molting timing defects which prompted the question whether KIN-20
328 was at all required for rhythmic molting. To address this question, we monitored molt
329 timing for *kin-20(ok505)* null mutant animals (*kin-20(0))*. *kin-20* loss recapitulated both the
330 slow development and arrhythmic molt phenotypes of *lin-42(ok2835)* and *lin-42(ΔCK1DB)*
331 mutations (Fig. 1B; Fig. 5A, S4A-C). Like *lin-42(ok2835)* and *lin-42(ΔCK1DB)* mutations,
332 the *kin-20(0)* mutation also caused larval arrest when animals were grown on plate but
333 not in the liquid culture luciferase assay (Fig. 1F; Fig. 5E). Additionally, we found that *kin-*
334 *20(0)* mutant animals resembled *lin-42(ok2835)* mutants in their severe egg-laying
335 defects and brood size reduction, phenotypes which are weaker or even absent from *lin-*
336 *42(ΔCK1DB)* animals (Fig. 1D,E; Fig. 5B,C). Finally, like *lin-42(ΔCK1DB)* animals but
337 unlike *lin-42(ok2385)* mutants, *kin-20* null mutants did not develop precocious alae (Fig.
338 1C; Fig. 5D), consistent with earlier work.[26]
339
340 To assess the relevance of KIN-20's enzymatic activity for rhythmic molting, we
341 engineered a D310A point mutation into the endogenous *kin-20* locus to abrogate
342 catalytic activity. This mutation disrupts the HRD motif in the catalytic loop that
343 coordinates phosphorylated residues (Fig. S3).[50,51] The resulting *kin-20(xe355[D310A])*
344 animals recapitulated the *kin-20(0)* mutant arrhythmic molting phenotype, although KIN-
345 20 levels were not decreased (Fig. S6A-D). Indeed, we observed a trend towards

346 increased accumulation of the mutant KIN-20 protein, possibly indicating autoregulation
347 but not further pursued by us (Fig. 5A; S4A-C; S6B). We conclude that KIN-20 and
348 especially its enzymatic activity are required for rhythmic molting.
349

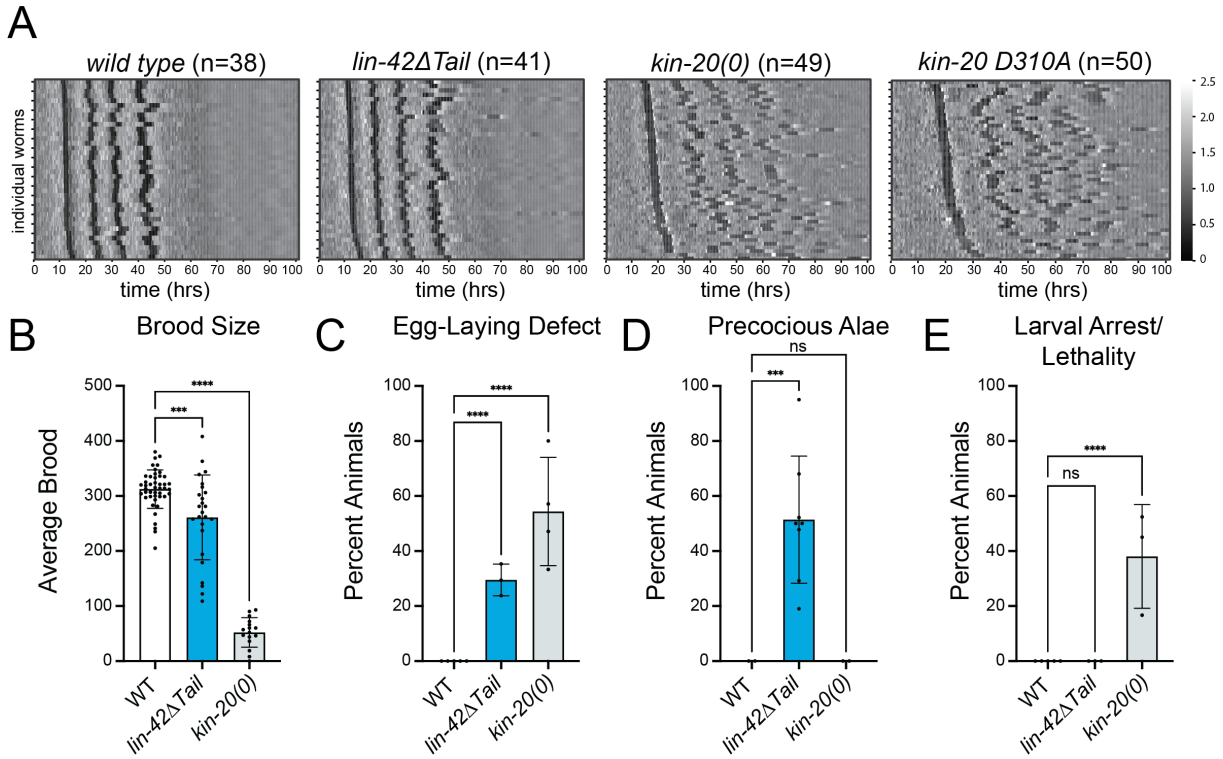

Figure 5 *kin-20* null and catalytically dead alleles exhibit larval arrest and asynchronous molting similar to *lin-42(ΔCK1BD)* mutants (A) Heatmaps showing trend-corrected luminescence traces from the indicated genotype. Each horizontal line represents one animal. Traces are sorted by entry into the first molt. Darker color indicates low luminescence signal and corresponds to the molts. **(B)** Bar plot depicting the average number of live progeny from hermaphrodites of the indicated genotype. **(C)** Bar plot quantifying the percentage of animals of the indicated genotype that exhibited egg-laying defects as determined by the presence of hatched larvae in the animal. **(D)** Bar plot quantifying the percentage of animals of the indicated genotype with precocious complete or partial alae at the L3-L4 molt. **(E)** Bar plot quantifying the percentage of animals of the indicated genotype that arrested or died as larvae. (**B-E)** Statistical significance was determined using ordinary one-way ANOVA. p<0.05 was considered statistically significant. ** indicates p<0.0001****, indicates p<0.0001. Error bars in B-E represent standard deviation.

350

351 **KIN-20 exhibits dynamic changes in subcellular localization**

352 Although KIN-20 kinase activity and its binding to the LIN-42 CK1BD are required for
353 rhythmic molting, LIN-42 tail phosphorylation appeared dispensable. Moreover, *kin-20*
354 deletion or inactivation appeared to cause more pronounced defects than deletion of the
355 LIN-42-CK1BD. Hence, we wondered whether the significance of the interaction between
356 the two proteins could lie in regulation of KIN-20 by LIN-42, rather than vice versa. We
357 therefore examined KIN-20 levels and localization. Using GFP::KIN-20 (with a split-GFP

358 system to enhance the signal; Methods), we observed both nuclear and cytoplasmic
359 signal in the epidermis. Strikingly, the relative distribution between these compartments
360 varied with developmental stage. Microfluidics-based observation of early larvae[52]
361 revealed substantial cytoplasmic GFP::KIN-20 signal during molts and at the beginning
362 of larval stages but a predominantly nuclear signal in the middle of the larval stage (Fig.
363 S7). We recapitulated these dynamics in L4 stage animals grown on plates (Fig. 6A),

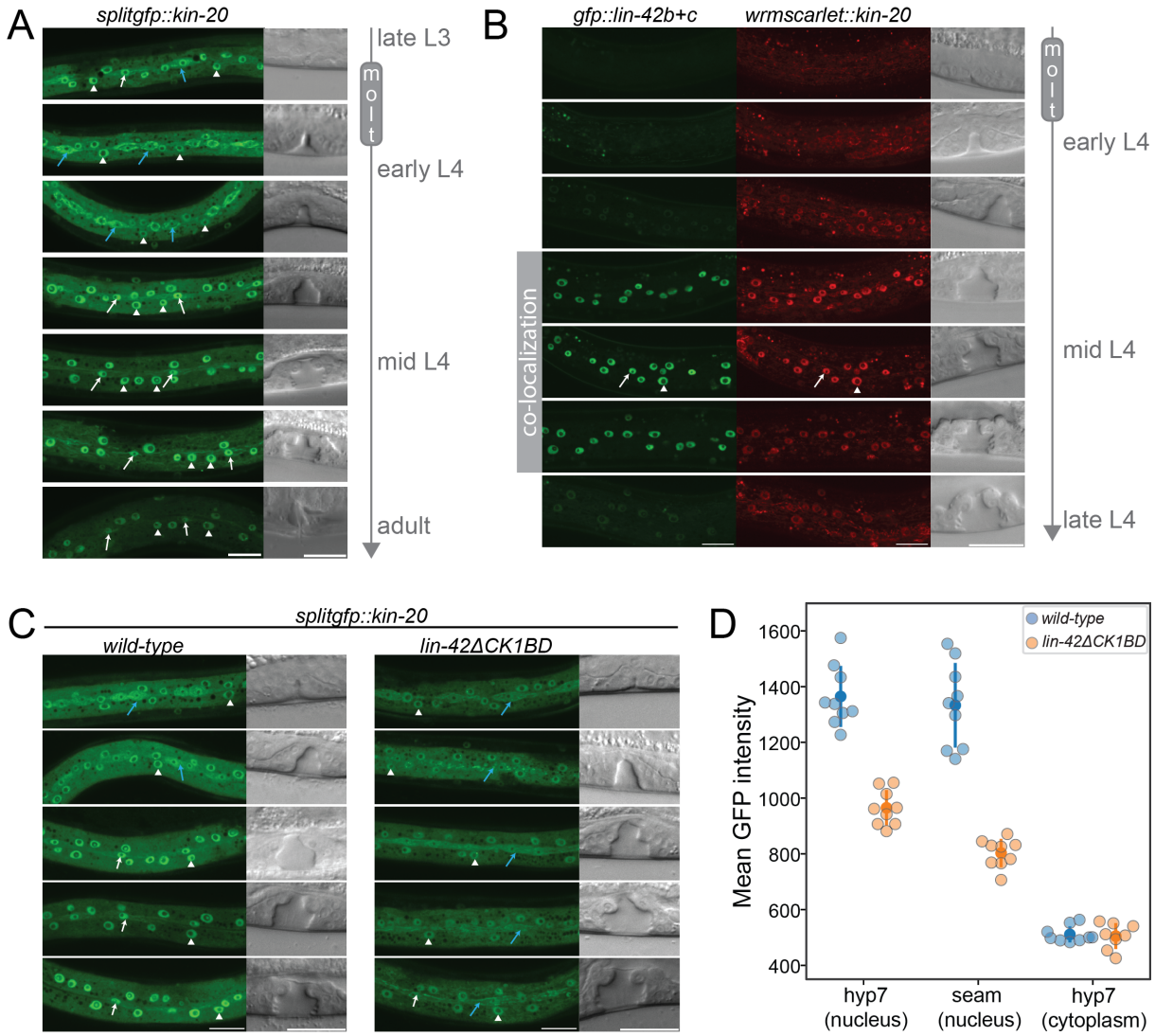

Figure 6. The LIN-42 CKBD is required for KIN-20 nuclear localization. (A) Confocal images of *splitgfp::kin-20* animals staged according to vulva morphology (Mok et al., 2015) from late L3 to adult. Arrows indicate seam cell nuclear (white) and cytoplasmic (blue) localization; white arrowheads indicate nuclear hyp7 localization. Scale bar=20 µm. **(B)** Confocal images of *gfp::lin-42b+c; wrmscarlet::kin-20* staged according to vulva morphology. An arrow indicates seam cell, an arrowhead hyp7 nuclear localization. Scale bar=20 µm. **(C)** Confocal images of *splitgfp::kin-20* in wild type and *lin-42ΔCK1BD* mutant animals during early - mid L4 stage based on the vulvae morphology. Arrows indicate seam cell nuclear (white) and cytoplasmic (blue) localization, white arrowheads indicate hyp7 nuclear localization. Scale bar=20 µm. **(D)** Quantification of GFP::KIN-20 signal in hyp7 (nucleus), seam cells (nucleus) and hyp7 (cytoplasm) of wild-type (blue) and *lin-42ΔCK1BD* (orange) animals. Each condition with n=9 mid-L4 stage worms.

364 which additionally revealed an apparent membrane-bound pool of KIN-20 in seam cells
365 (Fig. 6A).
366
367 **LIN-42 binding promotes nuclear localization of KIN-20**
368 The timing of increased nuclear KIN-20 in the middle of a larval stage coincides with peak
369 accumulation of LIN-42, especially LIN-42b (Fig. 2C). Moreover, using endogenously
370 tagged LIN-42::GFP and wrmScarlet::KIN-20, we found that the two proteins co-localized
371 in the nuclei of epidermal seam and hyp7 cells (Fig. 6B). Hence, we wondered if LIN-42
372 contributed to the change in KIN-20 localization. To test this notion, we examined how
373 lack of LIN-42 binding affected KIN-20 localization dynamics. Specifically, we visualized
374 GFP::KIN-20 in *lin-42(ΔCK1BD)* mutant animals during mid-L4 stage. We observed
375 reduced nuclear GFP signal relative to wild-type animals (Fig. 6C,D) in both hypodermal
376 and seam cell nuclei with no significant change in cytoplasmic levels. Together, these
377 data indicate that LIN-42 promotes nuclear accumulation of KIN-20.
378
379 **Discussion**
380 PER and its nematode orthologue LIN-42 control two distinct types of biological timing:
381 mammalian and fly circadian rhythms (PER) and *C. elegans* developmental progression
382 (LIN-42). The identification of additional orthologous pairs functioning in these respective
383 pathways, ROR/NHR-23, CK1/DBT/KIN-20, Timeless/TIM-1, and REV-ERB/NHR-85,
384 suggests that there is a conserved set of biological timing genes, whose function can be
385 exploited in different contexts. The wiring among these components, in the few cases
386 where this has been explored, appeared strikingly different, making it unclear why and
387 how these genes would be evolutionarily selected for distinct timing functions. Our work
388 shows the first example of a conserved module in these clocks: the LIN-42/PER – KIN-
389 20/CK1 interaction.
390
391 **The PAS domains are largely dispensable for LIN-42 developmental timing function**
392 The PAS domains are the most notable feature of LIN-42 sequence conservation and
393 were thought to be an interaction platform for a developmental stage-specific binding
394 partner.[19] Previous work characterizing the *lin-42(ok2385)* and *n1089* alleles suggested

395 that the PAS domains were necessary for the heterochronic functions of LIN-42.[22,35] Yet,
396 our precise deletion of the LIN-42 PAS domains caused only a mild precocious alae
397 phenotype with significantly less penetrance than in *lin-42(n1089)* mutants, suggesting
398 that this model is incorrect. While *lin-42(n1089)* removes the sequence of the PAS
399 domains, it also results in a truncation of *lin-42b* and removal of part of the *lin-42a*
400 promoter sequence.[22,35] The *n1089* phenotype could reflect loss of the PAS domains
401 combined with potential reduction in expression of other elements such as the C-terminal
402 tail or an NHR-85 interaction site.[37]
403
404 The weak phenotypes seen with our precise deletion are particularly striking when
405 considering that the PAS domains, and specifically the highly conserved PAS-B domain,
406 mediate LIN-42 dimerization[20] - a function that is important for PER activity in circadian
407 rhythms[53]. Although it remains to be determined whether the PAS domains are necessary
408 for LIN-42 dimerization *in vivo*, it seems possible that LIN-42's molting timing function
409 does not require dimerization.
410
411 **LIN-42 contains a CK1BD that is important for rhythmic molting**
412 Contrasting with the PAS domain, we found the LIN-42 SYQ/LT regions to be required
413 for rhythmic molting, and we could demonstrate *in vitro* that they constitute a functional
414 CK1BD that anchors KIN-20/CK1, promotes LIN-42 phosphorylation, and mediates
415 kinase inhibition. Moreover, the patterns of phosphorylation that CK1 generates on LIN-
416 42 *in vitro* overlap with those that we observed on LIN-42 *in vivo*. We note that it remains
417 to be demonstrated to which extent the *in vivo* phosphorylation events depend on KIN-
418 20. Our attempts to address this question by examining LIN-42 phosphorylation in strains
419 lacking active KIN-20 were thwarted by the slow growth, sickness, and arrhythmic molting
420 observed in these strains.
421
422 The two CK1BD subdomains have separable functions *in vitro*. The CK1BD-B plays a
423 more prominent role in kinase binding, mirroring the anchoring function of its mammalian
424 and *Drosophila* equivalents.[14,46,49] Anchoring allows CK1 to target lower affinity, non-
425 consensus motifs, which are key regulatory sites in fly/mammalian circadian

426 clocks.[14,46,49,54,55] However, without the PER CK1BD-B not all capacity for
427 phosphorylation is lost, which suggests that high-affinity binding is not essential for
428 phosphorylation but promotes it.[49] For LIN-42-ΔCK1BD-B, we also see low levels of
429 phosphorylation by CK1 *in vitro* (Fig. 4), suggesting that it may play a similar role in
430 controlling KIN-20 activity.
431
432 The LIN-42 CK1BD-A plays a less prominent role in binding but rather is involved in CK1
433 inhibition. CK1δ-dependent phosphorylation of both mammalian and *Drosophila* PER at
434 sites near the CK1BD leads to feedback inhibition of CK1 through conserved anion
435 binding sites.[14] Our *in vitro* LIN-42 phosphorylation data reveal a decrease in enzyme
436 catalytic activity as substrate concentration increases, dependent on the CK1BD-A,
437 consistent with feedback product inhibition of KIN-20 (Fig. 4). Alternatively, this result
438 could reflect substrate inhibition, where increasing amounts of LIN-42 CK1BD+Tail result
439 in a non-productive enzyme-substrate complex. Such substrate inhibition has also been
440 reported in several recent studies of CK1 showing phosphorylated substrates binding the
441 enzyme and regulating its activity.[43,56–58] Further enzymatic and structural studies will be
442 required to distinguish between these modes of regulation.
443
444 **Function of the LIN-42-CK1 interaction**
445 Many models of circadian clock function highlight phosphorylation of PER by CK1 as the
446 central consequence of their interaction, controlling PER stability and thereby setting the
447 period length. Intriguingly, although CK1 heavily phosphorylates the LIN-42 C-terminal
448 tail, including on serine and threonine residues that are conserved among *Caenorhabditis*
449 nematodes, but not on more distantly related nematodes (Fig. S8), deletion of this
450 sequence does not affect molting rhythmicity. These data suggest either phosphosite
451 redundancy, or that regulated phosphorylation of LIN-42 is not the key function of this
452 complex for controlling molting timing.
453
454 For PER–CK1, several lines of evidence show that reciprocal to CK1-mediated regulation
455 of PER, PER also regulates CK1 activity, both on itself and potentially other targets such
456 as CLOCK. Indeed, PER and CRY function as a bridging complex to provide CK1 access

457 to CLOCK[8,9], and mutation of the CK1BD causes defects in PER and CK1 nuclear
458 localization as well as CK1-mediated phosphorylation of CLOCK.[9,59] Product inhibition
459 through phosphorylated PER may thus limit phosphorylation not only of PER itself, but
460 potentially also of other substrates, including CLOCK. Strikingly, KIN-20 also exhibits
461 dynamic subcellular localization, with nuclear accumulation depending on LIN-42 binding.
462 Whether this nuclear accumulation reflects co-transport, nuclear retention, or stabilization
463 of the nuclear KIN-20 pool is not known. However, together with the fact that the LIN-42
464 tail, despite being heavily phosphorylated, is dispensable for rhythmic molting, this
465 localization data indeed supports a model where a key function of the LIN-42–KIN-20
466 complex in the context of molting could be regulation of KIN-20 rather than LIN-42. Such
467 a scenario may also explain the previous observation that forced expression of the short
468 LIN-42a isoform, largely comprising the CK1BD + Tail, caused extended molt durations[35],
469 as this might sequester KIN-20 and/or inhibit its kinase activity.
470

471 **Distinct functions of LIN-42 and KIN-20 in molting versus heterochronic timing?**

472 Our data not only support the existence of LIN-42 and KIN-20 in a stable complex that is
473 important for molting timing, but also suggest that LIN-42 and KIN-20 can function
474 independently of one another in some contexts. Neither *kin-20(lf)* nor *lin-42(ΔCK1DB)*
475 mutations cause precocious alae, a phenotype observed with several other *lin-42* mutant
476 alleles. These findings agree with previous genetic data that suggested independent
477 functions of *lin-42* and *kin-20* in regulating the expression of the heterochronic *let-7*
478 microRNA.[38] A recent study on LIN-42's function in regulating another heterochronic
479 miRNA, *lin-4*, indicated that physical interaction with the NHR-85 transcription factor may
480 support LIN-42's activity in this process, and perhaps the heterochronic pathway more
481 generally.[37] Relatively mild effects upon *nhr-85* deletion, and LIN-42's ability to interact
482 with additional transcription factors in a yeast two-hybrid assay suggest that additional
483 functionally relevant interactions partners remain to be identified. The apparent
484 differences in wiring among components of the heterochronic pathway and the molting
485 timer may reflect their different modes of timekeeping where the heterochronic pathway
486 is chiefly concerned with the order of events and the molting timer - similar to the circadian
487 clock - with the tempo and/or robustness of their execution.

488

489 Indeed, although LIN-42/PER have previously been suggested to provide an evolutionary
490 link between circadian (PER) and heterochronic (LIN-42) timing systems[19], our data
491 suggest that the link primarily derives from functional conservation between circadian and
492 molting timing. Hence, we may speculate that the heterochronic function of LIN-42, which
493 appears to involve neither rhythmic activity nor conserved interactions with orthologues
494 of other circadian clock [19,21,23,26–28] arose secondarily to, or independent from, a rhythmic
495 timing function. Finally, we note with interest that the phosphorylation of PER by CK1 is
496 an important component of temperature compensation in the circadian clock[60], which
497 allows this clock to maintain its period despite variation in temperature. By contrast, the
498 period of *C. elegans* molting and oscillatory genes expression changes with temperature.
499 Our data that the LIN-42 tail, where the bulk of CK1 mediated phosphorylation occurs, is
500 dispensable for rhythmic molting may thus reflect the lack of robust temperature
501 compensation in the *C. elegans* developmental clock.

502

503

504 **Materials and Methods**

505 ***C. elegans* culture**

506 *C. elegans* were grown and maintained at 20°C unless indicated otherwise and cultured
507 as described[61] on MYOB[62] or NGM 2% agar plates with *Escherichia coli* OP50 bacteria.
508 Full genotypes of strains used in this work and the respective growth conditions are listed
509 in Table S2. All mutant strains were backcrossed to N2 at least 2X. The Bristol N2 isolate
510 was used as the wildtype. Below, we describe how we generated novel strains for this
511 study. In Table S3, we list oligo repair templates, plasmids and genotyping primers. In
512 Table S4 we list crRNAs used.

513

514 **Genome editing**

515 Novel *lin-42* mutant alleles (*(wrd67[ΔPAS])*, (*wrd63[ΔCK1BD)* and (*wrd107[ΔTail])*) using
516 CRISPR-Cas9 were generated by injection of Cas9 or Cas12 ribonucleoprotein
517 complexes as described.[63] Specified deletions were made in the endogenous *lin-42* locus
518 in N2 animals. Repair templates were used at 100 ng/µl along with Cas9 or Cas12 at 250

519 ng/µl, crRNA oligos at 60 ng/µl, and 10 ng/µl co-injection marker (pCFJ90).[64] Strains used
520 in the luciferase assays were generated by crossing the mutant strains with a previously
521 described luciferase reporter strain (HW1993)[40] (Table S2).
522
523 Strains containing endogenously tagged *lin-42* and *kin-20* were obtained using
524 CRISPR/Cas9 as described previously.[64] In short: 5 µg of of Alt-R S.p. Cas9 Nuclease
525 V3 (IDT, Cat # 1081058), 2 µg of Alt-R® CRISPR-Cas9 tracrRNA (IDT, Cat # 1072532)
526 and 1.5 µg of crRNA were incubated at 37°C for 15 min to form the RNP complex. 500
527 ng repair template and co-injection markers pIK127 (10 ng/µl) and pRF4 (*rol-6(su1006)*)
528 (40 ng/µl) were added to a final volume of 20 µl in water. Mix was injected into N2 animals.
529 For *lin-42*, a *gfp::3xflag* (amplified from plasmid pIK384 with primers KK41/KK42) or
530 *3xflag* tag (Ultramer DNA Oligo, KK90) was inserted at the N-terminus of LIN-42b+c
531 isoforms.
532
533 *For kin-20*, a *3x::flag-ha* (Ultramer DNA oligo, KK107), *4xgfp11::linker:.flag* (amplified
534 from plasmid pIK401 with primers KK145/KK146) or *wrmScarlet* (amplified from plasmid
535 pIK385 with primers KK102/KK103) was inserted at the first common shared exon of all
536 KIN-20 isoforms with following flanking sequence: 5′ tatattcaaattttcagCGGAGATG-
537 [insert]-GAACTTCGTGTCGGCAATCGTTTCC 3′).
538
539 To reconstitute GFP with the split-GFP system, we crossed the obtained
540 *4xgfp11::linker::flag::kin-20* line into a MosSCI strain containing the *gfp1-10* fragment
541 expressed from an *eft-3* promoter. For this strain the *gfp1-10* sequence from plasmid
542 pCZGY2254[65] for *C. elegans* was codon optimized. After adding two synthetic introns the
543 codon-optimized sequence was ordered as a gBlock from IDT. The sequence was cloned
544 into a MosSCI-compatible backbone together with *eft-3*p promoter and *tbb-2* 3'UTR
545 sequences to create plasmid pIK407 by Gibson assembly. pIK407 (*eft-3p::gfp1-*
546 *10(codon-optimized)::tbb-2* 3'UTR) was inserted into the ttTi5605 site on chromosome II
547 by MosSCI.[66]
548

549 To generate a catalytic dead KIN-20, the D310A mutation was introduced by CRISPR-
550 Cas9 into N2 and *3xflag-ha::kin-20* animals, respectively. Injection was performed as
551 described previously for the *lin-42* and *kin-20* tagged strains. The ultramer DNA oligo
552 KK137 containing the D310A mutation and two silent mutations (to abrogate crRNA
553 recognition) was used as a repair template.
554
555 **Immunoprecipitation**
556 For the 3xFLAG-LIN-42 IP, mixed stage animals were obtained by plating eggs after a
557 standard hypochlorite treatment and growth at different temperatures (15°C, 20°C and
558 25°C). 24 hrs after plating, worms were collected by pooling 30,000 worms from each
559 temperature condition per strain and replicate (triplicates for each strain). For the HA-KIN-
560 20 IP, synchronized worms were collected after 32 hrs at 25°C. For the mapping of LIN-
561 42 phosphorylation sites, synchronized worms were grown at 25°C and collected hourly
562 from 14 hrs (L2 larval stage) to 25 hrs (L4 larval stage). Worm extracts followed by
563 immunoprecipitation were done as previously described.[67] In brief, extracts were made in
564 lysis buffer (50mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% TRITON X-100, 1 mM EDTA)
565 supplemented with 1 mM PMSF and 1 tablet of cOmplete Protease inhibitor (Roche, cat.
566 No. 11 873 580 001) per 50 ml using an MP Biomedical Fast-Prep-24 5G bead beater.
567 For the FLAG IP, cleared worm extract was incubated with 50 µl of 50% bead suspension
568 of anti-FLAG M2 Magnetic Beads (Sigma. Catalog Number M8823) for 2 hours at 4°C.
569 For the HA-IP worm extract was incubated with 50 µl of bead suspension of Pierce™
570 Anti-HA Magnetic Beads (Thermo Scientific, Cat. No. 88837).
571
572 **Proteolytic digest**
573 The beads were incubated for 4 hrs at RT with 800 rpm shaking in 5 µl of digestion buffer
574 (3 M guanidine hydrochloride, 20 mM EPPS (4-(2-Hydroxyethyl)-1-
575 piperazinepropanesulfonic acid) pH 8.5, 10 mM CAA (2-Chloroacetamide), 5 mM TCEP
576 (Tris(2-carboxyethyl)phosphine hydrochloride)) and 1 µl of Lys-C (0.2 µg/µl in 50 mM[68],
577 pH 8.5). 1 µl of trypsin (0.2 µg/µl) was added and incubated at 37°C overnight. After 12
578 hrs another 1 µl of trypsin was added and incubation continued for 4 more hours at 37°C
579

580 **Mass spectrometry to identify LIN-42 interacting factors**

581 The generated peptides were acidified with 0.8% TFA (final concentration) and analyzed
582 by LC–MS/MS on an EASY-nLC 1000 [Thermo Scientific] with a two-column set-up. The
583 peptides were applied onto a peptide 75 µm x 2 cm PepMap Trap trapping column
584 [Thermo Scientific] in 0.1% formic acid, 2% acetonitrile in H2O at a constant pressure of
585 800 bar. Using a flow rate of 150 nl/min, peptides were separated on a 50 µm x 15 cm
586 PepMap C18, 2 µm, 100 A [Thermo Scientific] at 45 ˚C with a linear gradient of 2%–6%
587 buffer B in buffer A in 3 min followed by a linear increase from 6 to 22% in 40 min, 22%–
588 28% in 9 min, 28%–36% in 8 min, 36%–80% in 1 min, and the column was finally washed
589 for 14 min at 80% buffer B in buffer A (buffer A: 0.1% formic acid; buffer B: 0.1% formic
590 acid in acetonitrile). The separation column was mounted on an EASY-Spray™ source
591 [Thermo Scientific] connected to an Orbitrap Fusion LUMOS [Thermo Scientific]. The data
592 were acquired using 120,000 resolution for the peptide measurements in the Orbitrap and
593 a top T (3 s) method with HCD fragmentation for each precursor and fragment
594 measurement in the ion trap according the recommendation of the manufacturer (Thermo
595 Scientific). For the analysis protein identification and relative quantification of the proteins
596 was performed with MaxQuant v.1.5.3.8 using Andromeda as search engine, and label-
597 free quantification (LFQ). The *C. elegans* subset of the UniProt v.2021_05 combined with
598 the contaminant database from MaxQuant was searched and the protein and peptide
599 FDR were set to 0.01. The LFQ intensities estimated by MaxQuant were analyzed with
600 the einprot R package (https://github.com/fmicompbio/einprot) v0.5.4. Features classified
601 by MaxQuant as potential contaminants or reverse (decoy) hits or identified only by site,
602 as well as features identified based on a single peptide or with a score below 10, were
603 filtered out. The LFQ intensities were log2 transformed and missing values were imputed
604 using the 'MinProb' method from the imputeLCMD R package v2.0 with default settings.
605 Pairwise comparisons were performed using limma v3.50.0, considering only features
606 with at least 2 non-imputed values across all the samples in the comparison. Estimated
607 log2-fold changes and P-values (moderated t-test) from limma were used to construct
608 volcano plots. Data will be available via ProteomeXchange with identifier PXD058601
609 upon publication.
610

611 **Mass spectrometry to map *in vivo* LIN-42 phosphorylation sites**

612 The peptides from each sample were labeled with TMTpro reagents and pooled. The TMT
613 labeled peptide mixture was subjected to off-line high pH fractionation on a YMC Triart
614 C18 0.5 × 250 mm column (YMC Europe GmbH) using the Agilent 1100 system (Agilent
615 Technologies). A total of 96 fractions was collected for each experiment and
616 concatenated into 48 fractions as previously described. For each LC-MS analysis, all
617 available peptides were loaded onto a PepMap Neo trap (Thermo Fisher) using the
618 Vanquish Neo UHPLC system (Thermo Fisher). On-line peptide separation was
619 performed on a 15-cm EASY-Spray™ C18 column (ES75150PN, Thermo Fisher) by
620 applying a linear gradient of increasing ACN concentration at a flowrate of 200 nL/min.
621 Orbitrap Fusion Lumos Tribrid (Thermo Fisher) mass spectrometer was operated in the
622 data-dependent mode. The ions for the survey scan were collected for a maximum of 50
623 ms to reach the standard AGC target value and the scan recorded using an Orbitrap
624 detector at a resolution of 120,000. The topmost intense precursor ions from the Orbitrap
625 survey scan recorded every 3 sec were selected for stepped higher-energy C-trap
626 dissociation (HCD) at 29%, 32% and 35% normalized collision energy scan. To reach an
627 AGC value of 100,000 ions, the maximum ion accumulation time for the MS2 scan was
628 set to 500 ms. The TMT reporter ions were quantified using an MS2 scan recorded using
629 the Orbitrap analyzer at a resolution of 50,000. Thermo RAW files were processed using
630 Proteome Discoverer 2.4 software (Thermo Fisher) as described in the manufacturer's
631 instructions. Briefly, the Sequest search engine was used to search the MS2 spectra
632 against the *C. elegans* UniProt database (downloaded on 05/2023) supplemented with
633 common contaminating proteins. For peptide identification, cysteine
634 carbamidomethylation and TMTpro tags on lysine and peptide N-termini were set as static
635 modifications, whereas oxidation of methionine residues and acetylation protein N-termini
636 were set as variable modifications. The assignments of the MS2 scans were filtered to
637 allow 1% FDR. For reporter quantification, the S/N values were corrected for isotopic
638 impurities of the TMTpro reagent using the values provided by the manufacturer. The
639 sums across all TMTpro reporter channels were normalized assuming equal total protein
640 content in each sample. Data will be available via ProteomeXchange with identifier
641 PXD058598 upon publication.

642

643 **Western Blot**

644 Synchronized animals were collected hourly from 13 hrs - 23 hrs after plating at 25°C.
645 Extracts were made by boiling at 95°C for 5 minutes in lysis buffer (63 mM Tris-HCl (pH
646 6.8), 5 mM DTT, 2% SDS, 5% sucrose) followed by sonication with a BioRupter Plus
647 (Diagnode) with the following settings: 13 cycles, 30 s on/off at 4°C. Samples were
648 cleared by centrifugation, before separating proteins by SDS-PAGE (loading: 50 µg
649 protein extract per well) and transferring them to PVDF membranes by semi-dry blotting.
650 The following antibodies were used: Monoclonal mouse anti-FLAG M2-Peroxidase
651 (HRP)(Sigma-Aldrich; A8592, dilution: 1:1000), monoclonal mouse anti-Actin clone C4
652 (Millipore; MAB1501, dilution 1:7500) and anti-mouse HRP conjugated antibody (GE
653 Healthcare #NXA931, dilution 1:2000). The membrane was incubated with ECL™ Prime
654 Western-Blot-Reagent (Cytiva, #RPN2236) and bands detected with an Amersham
655 Imager 680 (GE Healthcare).

656

657 **Phenotypic analysis**

658 For phenotypic analyses, gravid adults were bleached, and embryos were hatched at low
659 density onto MYOB plates seeded with OP50, or gravid adults were picked onto seeded
660 plates and removed after 1-2 hours. Animals were maintained at 20°C and observed daily
661 or twice daily. Bag-of-worms phenotype was determined when live progeny were
662 observed inside adult animals. For brood counts, animals were individually transferred to
663 fresh wells daily for five days after L4 and live progeny were counted and averaged.

664

665 To score premature alae, synchronized animals were collected from MYOB plates by
666 washing off plates. 1000 µl of M9 + 2% gelatin was added to the plate or well, agitated to
667 suspend animals in M9+gelatin, and then transferred to a 1.5 ml tube. Animals were spun
668 at 700xg for 1 min. The media was then aspirated off and animals were resuspended in
669 500µl M9 + 2% gelatin with 5 mM levamisole. 12 µl of animals in M9 +gel with levamisole
670 solution were placed on slides with a 2% agarose pad and secured with a coverslip.
671 Images were acquired using a Plan-Apochromat 40x/1.3 Oil DIC lens or a Plan-
672 Apochromat 63x/1.4 Oil DIC lens on an AxioImager M2 microscope (Carl Zeiss

673 Microscopy, LLC) equipped with a Colibri 7 LED light source and an Axiocam 506 mono
674 camera. Acquired images were processed through Affinity photo software (version:
675 1.9.2.1035). For the confocal microscopy, L3/L4 stage-animals were picked from OP50
676 plates grown at 25°C. Worms were mounted on a glass slide with a 2 % agarose patch
677 immobilized with 5 µl of 10 mM levamisole (Fluca Analytical, #31742). Images were
678 acquired in channels for red (561 nm laser), green (488 nm laser) and DIC with a 40x/1.3
679 immersion objective on a Zeiss LSM700 confocal microscope. Acquired images were
680 processed with FiJi.[68]
681
682 **Quantification of GFP levels**
683 For quantification, mid-L4 stage worms (defined by vulval morphology) were selected.
684 Images acquired in the GFP fluorescence channel were imported into Napari for
685 analysis.[69] The following regions of interest were manually annotated for quantification:
686 Nuclei of hyp7 and seam cells. For the cytoplasm of hyp7, the layer with the maximum
687 intensity of nuclear GFP signal in hyp7 was selected and a region between the annotated
688 hyp7 nuclei was chosen. To define the background, a region in the gonad was used
689 (where no GFP signal was detected). For the quantification, average GFP levels were
690 computed per region and worm. Background levels were subtracted to the GFP levels of
691 all other regions.
692
693 **Microfluidics**
694 Worms were prepared and loaded into the device as described in Berger et al. (2021).
695 Briefly, gravid adult hermaphrodites were harvested and treated with bleach. The
696 resulting embryos were collected by centrifugation at 1,600 g for 1 minute and washed
697 three times with S-Basal buffer. For synchronization, embryos were incubated overnight
698 in S-Basal, allowing larvae to arrest. Arrested larvae were then passed through a 10 µm
699 filter (pluriStrainer Mini 10 µm, PluriSelect), and 6,000 worms were transferred to 3 NGM
700 plates, where they were incubated for 12 hours at 25°C. Once the worms reached the
701 late L1 stage, they were harvested using M9 buffer, washed twice to remove debris, and
702 loaded into the experimental device. During the experiment, bacterial food was supplied
703 at a constant rate of 1 µl/h, with periodic increases to 100 µl/h for 5 seconds every 30

704 minutes to clear debris. Images were acquired every 10 minutes over 24 hours using a
705 spinning disk confocal scanning microscope (Yokogawa CSU W1 with Dual T2).
706 Brightfield and fluorescent signals (488 nm laser) were recorded simultaneously using
707 two sCMOS Photometrics Prime 95B cameras with a 40 x oil immersion lens (NA = 1.3).
708 Imaging was conducted with a 25 ms exposure time and a motorized z-drive, acquiring
709 z-stacks with a 1 µm step size and 20 images per stack. Analysis was performed using
710 Fiji/ImageJ software.
711
712 **Luciferase assays**
713 Assays were performed and analyzed as described.[40] Briefly, gravid adults were
714 bleached, and eggs were immediately singled into wells containing 90 µl OP50/S-
715 Basal/D-Luciferin solution per well and left in the luminometer machine and measured
716 every 10 minutes for 0.5 seconds. The assays lasted 96 hrs or 130 hrs. For statistical
717 analysis, we performed a Wilcoxon-Mann-Whitney test (implemented in the python
718 package SciPy version 1.4.1 as the function Mann-Whitney-U).
719
720 **Expression and purification of recombinant proteins**
721 All proteins were expressed from a pET22-based vector in *Escherichia coli* Rosetta (DE3)
722 cells based on the Parallel vector series.[70] All LIN-42 constructs (CK1BD + Tail, residues
723 402-598; CK1BD, residues 402-475; CK1BD-ΔA, -ΔB, and ΔAB mutants) were expressed
724 downstream of an N-terminal TEV-cleavable His-NusA tag. Human CK1δ catalytic
725 domains (CK1δ ΔC, residues 1–317) were all expressed with a TEV-cleavable His-GST
726 tag. All proteins expressed from Parallel vectors have an additional N-terminal vector
727 artifact of 'GAMDPEF' remaining after TEV cleavage. Cells were grown in LB media at
728 37°C until the O.D.600 reached ~0.8; expression was induced with 0.5 mM IPTG, and
729 cultures were grown for approximately 16–20 hr more at 18°C. Cells were centrifuged at
730 3,200 x g, resuspended in 50 mM Tris, pH 7.5, 300 mM NaCl, 20 mM imidazole, 5%
731 (vol/vol) glycerol, 1 mM tris(2-carboxyethyl)phosphine (TCEP), and 0.05% Tween-20. For
732 purification of recombinant protein, cells were lysed with a microfluidizer followed by
733 sonication and then the lysate was clarified via centrifugation at 140,500 x g for 1 hour at
734 4ºC. Ni-NTA affinity chromatography was used to extract his-tagged proteins from the

735 lysate and then the affinity/solubility tags were cleaved using His6-TEV (GST-TEV for
736 CK1δ ΔC) protease overnight at 4ºC. The cleaved protein was then separated from
737 solubility tag and TEV by a second Ni-NTA (GST for CK1δ ΔC) affinity column and further
738 purified using size exclusion chromatography (SEC) in 50 mM Tris, pH 7.5, 200 mM NaCl,
739 1 mM EDTA, 5% (vol/vol) glycerol, 1 mM TCEP, and 0.05% Tween-20. Small aliquots of
740 protein were frozen in liquid nitrogen and stored at -70ºC for long-term storage.
741
742 ***In vitro* biotinylation, pull-down assays, and bio-layer interferometry**
743 LIN-42 CK1BD + Tail constructs and CK1δ ΔC were biotinylated via Sortase A-mediated
744 reactions between a Sortase A recognition motif peptide (biotin-LPETGG) and our LIN-
745 42 CK1BD + Tail/CK1δ ΔC (N-terminal G from 'GAMDPEF' artifact). Reactions were
746 carried out in 50 mM Tris pH 7.5 and 150 mM NaCl using 5 μM His6-Sortase A, 300 μM
747 biotin-LPETGG, and 50 μM protein. Ni-NTA affinity chromatography followed by SEC was
748 used to purify labeled protein from His6-Sortase A and excess biotin, respectively. Pull
749 down assays were performed using magnetic streptavidin beads to bind biotinylated LIN-
750 42 CK1BD + Tail WT and mutants (final concentration 5 μM) in the presence and absence
751 of CK1δ ΔC (final concentration 5 μM). All BLI assays were performed in SEC buffer
752 supplemented with 7.5mM BSA as previously described[71,72] using an eight-channel Octet-
753 RED96e.
754
755 **ADP-Glo kinase assays**
756 Substrate titration kinase reactions were performed on the indicated recombinant LIN-42
757 proteins (CK1BD + Tail WT or mutants) using the ADP-Glo kinase assay kit (Promega)
758 according to the manufacturer's instructions. All reactions were performed in 30 μL
759 volumes in duplicate (n=3 independent experiments) using 1x kinase buffer (25mM Tris
760 pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, and 2 mM TCEP) supplemented with 100μM ATP,
761 0.2μM recombinant CK1, and indicated LIN-42 proteins. Reactions were held at room
762 temperature for 1 hour and then 5 μL aliquots were taken and quenched with ADP-Glo
763 reagents. Luminescent measurements were measured at room temperature with a
764 SYNERGY2 microplate reader in 384-well microplates. Data analysis was performed
765 using Excel (Microsoft) and Prism (GraphPad).

766

767 **^{32}P-ATP kinase assays**

768 1 μM CK1δ ΔC was incubated with 10 μM LIN-42 (CK1BD + Tail/CK1BD WT and mutant
769 proteins) in 1x kinase buffer (25 mM Tris pH 7.5, 100 mM NaCl, 10 mM MgCl2, 2 mM
770 TCEP). Reactions were started by the addition of ^{32}P-ATP (final concentration 2 mM) and
771 samples were collected at indicated time points and quenched in an equivalent volume
772 of 2x SDS-PAGE loading buffer. Proteins labeled with ^{32}P were separated and analyzed
773 via SDS-PAGE and the gels were dried at 80ºC for 2 hours before overnight exposure in
774 a phosphor screen (Amersham Biosciences). A Typhoon Trio (Amersham Biosciences)
775 phosphorimager was used to visualize exposed gels and ^{32}P-labeled protein bands were
776 quantified via densitometry using ImageJ (NIH), Excel (Microsoft) and Prism (GraphPad).

777

778 ***In vitro* Mass Spectrometry**

779 Sample preparation:

780 Kinase reactions were performed in 1x kinase buffer (25 mM Tris pH 7.5, 100 mM NaCl,
781 10 mM MgCl2, 2 mM TCEP) for 60 minutes and then quenched with 20mM EDTA.
782 Samples were denatured, reduced, alkylated, and digested according to the In-solution
783 Protein Digestion (Promega) protocol. In brief, phosphorylated samples were
784 denatured/reduced in 8M urea/50mM ammonium bicarbonate/5mM Dithiothreitol (DTT)
785 for 1hr at 37°C. Samples were incubated for 30 minutes in the dark with 15mM
786 iodoacetamide and then digested with X Grade Modified Trypsin according to the Trypsin
787 Digestion Protocol (Promega). Digested Samples were phospho-enriched using the High-
788 Select Fe-NTA Phosphopeptide Enrichment Kit (Thermo Scientific) and sent for analysis
789 at the University of California, Davis Proteomic Core Facility.

790 LC-MS:

791 For each sample, equal volumes were loaded onto a disposable Evotip C18 trap column
792 (Evosep Biosytems, Denmark) as per the manufacturer's instructions. Briefly, Evotips
793 were wetted with 2-propanol, equilibrated with 0.1% formic acid, and then loaded using
794 centrifugal force at 1200g. Evotips were subsequently washed with 0.1% formic acid, and
795 then 200 μL of 0.1% formic acid was added to each tip to prevent drying. The tipped
796 samples were subjected to nanoLC on a Evosep One instrument (Evosep Biosystems).

797 Tips were eluted directly onto a PepSep analytical column, dimensions: 15 cm x 75 um
798 C18 column (PepSep, Denmark) with 1.5 µm particle size (100 Å pores) (Bruker
799 Daltonics), and a ZDV spray emmiter (Bruker Daltronics). Mobile phases A and B were
800 water with 0.1% formic acid (v/v) and 80/20/0.1% ACN/water/formic acid (v/v/vol),
801 respectively. The standard pre-set method of 40 samples-per-day whisper method was
802 used, which is a 31-minute run.
803 Mass Spectrometry – Performed on a hybrid trapped ion mobility spectrometry-
804 quadrupole time of flight mass spectrometer (timsTOF Pro, (Bruker Daltonics, Bremen,
805 Germany) with a modified nano-electrospray ion source (CaptiveSpray, Bruker
806 Daltonics). In the experiments described here, the mass spectrometer was operated in
807 diaPASEF mode. Desolvated ions entered the vacuum region through the glass capillary
808 and deflected into the TIMS tunnel which is electrically separated into two parts (dual
809 TIMS). Here, the first region is operated as an ion accumulation trap that primarily stores
810 all ions entering the mass spectrometer, while the second part performs trapped ion
811 mobility analysis.
812 DIA PASEF:
813 The dual TIMS analyzer was operated at a fixed duty cycle close to 100% using equal
814 accumulation and ramp times of 85 ms each.
815 Data-independent analysis (DIA) scheme consisted of one MS scan followed by MSMS
816 scans taken with 19 precursor windows at width of 50Th per 0.57s cycle over the mass
817 range 300-1200 Dalton. The TIMS scans layer the doubly and triply charged peptides
818 over a ion mobility -1/k0- range of 0.7-1.3 V*sec/cm2. The collision energy was ramped
819 linearly as a function of the mobility from 59 eV at 1/K0=1.4 to 20 eV at 1/K0=0.6.
820 Data Analysis:
821 DIA
822 LCMS files were processed with Spectronaut version 16.1 (Biognosys, Zurich,
823 Switzerland) using DirectDIA analysis mode. Mass tolerance/accuracy for precursor and
824 fragment identification was set to default settings. The unreviewed FASTA for *C. elegans*
825 was downloaded from Uniprot and a database of common laboratory contaminants were
826 used.[73] A maximum of two missing cleavages were allowed, the required minimum
827 peptide sequence length was 7 amino acids, and the peptide mass was limited to a

maximum of 4600 Da. Carbamidomethylation of cysteine residues was set as a fixed modification, and methionine oxidation and acetylation of protein N termini as variable modifications. A decoy false discovery rate (FDR) at less than 1% for peptide spectrum matches and protein group identifications was used for spectra filtering (Spectronaut default). Decoy database hits, proteins identified as potential contaminants, and proteins identified exclusively by one site modification were excluded from further analysis.

Data availability:

Data is available from the massive online repository https://massive.ucsd.edu/ ID number MSV000096641 and proteome Exchange ID number PXD058784

Author contributions

Rebecca K. Spangler: Conceptualization; formal analysis; investigation; writing – original draft. **Guinevere E. Ashley:** Conceptualization; formal analysis; investigation; writing – original draft. **Kathrin Braun:** Conceptualization; Formal analysis; investigation; writing – review and editing. **Marit van der Does:** Formal analysis; investigation; writing – review and editing. **Daniel Wruck:** Formal analysis; investigation; writing – review and editing. **Andrea Ramos-Coronado:** Formal analysis; investigation; writing – review and editing. **James Matthew Ragle:** Formal analysis; investigation; writing – review and editing. **Vytautas Iesmantavicius:** Formal analysis; investigation; writing – review and editing. **Lucas J Morales Moya:** Formal analysis; writing – review and editing. **Keya Jonnalagadda:** Formal analysis; writing – review and editing. **Carrie L. Partch:** Conceptualization; supervision; funding acquisition; writing – review and editing; project administration. **Helge Großhans:** Conceptualization; supervision; funding acquisition; writing – original draft; project administration. **Jordan D. Ward:** Conceptualization; supervision; funding acquisition; writing – original draft; project administration.

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Data availability

All relevant data can be found within the article and its supplementary information.

Figure legends

Figure 1. *lin-42ΔPAS* mutations cause mild heterochronic phenotypes while *lin-42ΔCK1BD* mutations cause asynchronous molting (A) Schematic depiction of the *lin-42* genomic locus. The gene structure of the *lin-42 a, b*, and *c* isoforms are shown. The location of sequences encoding putative PAS-A, PAS-B, SYQ, and LT sequence motifs are indicated. Published *lin-42* deletion alleles *n1089* and *ok2385* are indicated in dark and light grey, respectively, newly generated targeted Δ*PAS* and Δ*CK1BD* deletions in navy and teal, respectively. **(B)** Heatmaps showing trend-corrected luminescence traces from the indicated genotype. Each horizontal line represents one animal. Traces

890 are sorted by entry into the first molt. Darker color indicates low luminescence signal and
891 corresponds to the molts. **(C)** Bar plot quantifying the percentage of animals of the
892 indicated genotype with precocious complete or partial alae at the L3-L4 molt. **(D)** Bar
893 plot quantifying the percentage of animals of the indicated genotype that exhibited egg-
894 laying defects as determined by the presence of hatched larvae in the animal. **(E)** Bar plot
895 depicting the average number of live progeny from hermaphrodites of the indicated
896 genotype. **(F)** Bar plot quantifying the percentage of animals of the indicated genotype
897 that arrested or died as larvae. (C-F) Statistical significance was determined using an
898 ordinary one-way ANOVA. $p<0.05$ was considered statistically significant. **** indicates
899 < 0.0001. Error bars in C-F represent standard deviation.
900
901 **Figure 2 LIN-42 and KIN-20 interact *in vivo* (A)** Volcano blot comparing protein
902 enrichments in 3xFLAG::LIN-42b+c and 3xFLAG::SART-3 (control)
903 immunoprecipitations, determined by mass spectrometry. **(B)** mRNA expression profile
904 for *lin-42* (dark blue) and *kin-20* (light blue) mRNAs throughout larval development. Data
905 Source: Meeuse et al., 2020. **(C)** Western blot with extracts from *3xflag::lin-42b+c; 3xflag-*
906 *ha::kin-20* (HW3479) larvae collected hourly for 11 hours, starting at 13 h after plating
907 synchronized L1 stage animals on food at 25°C. Top part probed with anti-FLAG-HRP
908 (1:1,000), lower part with anti-actin-1 (1:7,500). Arrows indicate bands for LIN-42b, KIN-
909 20 and ACT-1 (see Fig. S5A). **(D)** Anti-HA pulldown from *3xflag::lin-42b+c; 3xflag-ha::kin-*
910 *20* animals. Blot probed with anti-FLAG (1:1,000).
911
912 **Figure 3 The LIN-42 SYQ and LT domains constitute a functional CK1-binding**
913 **domain (A)** Schematic representing PER2 and LIN-42 protein domains. Protein
914 constructs used in this study, CK1BD + Tail and CK1BD, are indicated i. CK1BD, Casein
915 Kinase 1-Binding Domain; CBD, CRY-binding domain. **(B)** Representative pulldown
916 assay of human CK1 and biotinylated LIN-42 CK1BD + Tail proteins using the indicated
917 protein variants. **(C)** Values for K_D from kinetic analysis of BLI data (D-H) based on a 2:1
918 heterogeneous ligand binding model and global analysis (Octet). Mean ± SEM, ordinary
919 one-way ANOVA. $p<0.05$ was considered statistically significant. **** indicates $p< 0.0001$.
920 **(D-H)** Bio-layer interferometry (BLI) data for indicated LIN-42 protein binding to

921 immobilized, biotinylated CK1. Inset values represent the concentrations of LIN-42 for
922 individual binding reactions. Model fit to association and dissociation over time is
923 represented by black lines. Data shown from one representative experiment of n ≥ 3
924 assays.
925
926 **Figure 4 The extended C-terminus of LIN-42 is phosphorylated by CK1 (A)**
927 Representative ^{32}P-radiolabeled ATP enzymatic assay of CK1 activity on LIN-42 CK1BD
928 + Tail and CK1BD wild-type and indicated domain deletion mutants. **(B)** Quantification of
929 band intensity depicted in (A) and replicates. Model fit to Michaelis- Menten, n = 3. **(C)**
930 Fold-change quantification of band intensity from panels A & B compared to CK1
931 autophosphorylation at 120 minutes. Ordinary one-way ANOVA. $p<0.05$ was considered
932 statistically significant. ****$p < 0.0001$, n ≥ 3, mean ± SEM. (D) Representative ADP-Glo
933 enzymatic assay comparing activity of CK1 on LIN-42 CK1BD + Tail wild-type (maroon),
934 CK1BD-ΔA (orange), CK1BD-ΔB (yellow), and CK1BD-ΔA/B (green). **(E)** Values of k_{cat}/K_m
935 calculated from (D). n=3, mean ± SEM, Ordinary one-way ANOVA. $p<0.05$ was
936 considered statistically significant. * indicates $p<0.03$ and ** indicates $p<0.002$. **(F)** LIN-
937 42 phosphorylation sites identified via mass spectrometry from *in vivo* samples from
938 whole worm lysate collected hourly from synchronized L1 animals from 14 hrs (early L2)
939 to 25 hrs (late L3) after plating at 25°C (top arrows) and *in vitro* kinase reactions of CK1
940 activity on LIN-42 CK1BD + Tail (bottom arrows). Serine, threonine, and tyrosine residues
941 are indicated via vertical blue, purple, and pink ticks, respectively, along the CK1BD +
942 Tail construct schematic.
943
944 **Figure 5 *kin-20* null and catalytically dead alleles exhibit larval arrest and**
945 **asynchronous molting similar to *lin-42(ΔCK1BD)* mutants (A)** Heatmaps showing
946 trend-corrected luminescence traces from the indicated genotype. Each horizontal line
947 represents one animal. Traces are sorted by entry into the first molt. Darker color indicates
948 low luminescence signal and corresponds to the molts. **(B)** Bar plot depicting the average
949 number of live progeny from hermaphrodites of the indicated genotype. **(C)** Bar plot
950 quantifying the percentage of animals of the indicated genotype that exhibited egg-laying
951 defects as determined by the presence of hatched larvae in the animal. **(D)** Bar plot

952 quantifying the percentage of animals of the indicated genotype with precocious complete
953 or partial alae at the L3-L4 molt. **(E)** Bar plot quantifying the percentage of animals of the
954 indicated genotype that arrested or died as larvae. (**B-E)** Statistical significance was
955 determined using ordinary one-way ANOVA. $p<0.05$ was considered statistically
956 significant. ** indicates $p<0.0001$****, indicates $p<0.0001$. Error bars in B-E represent
957 standard deviation.
958
959 **Figure 6. The LIN-42 CKBD is required for KIN-20 nuclear localization. (A)** Confocal
960 images of *splitgfp::kin-20* animals staged according to vulva morphology (Mok et al.,
961 2015) from late L3 to adult. Arrows indicate seam cell nuclear (white) and cytoplasmic
962 (blue) localization; white arrowheads indicate nuclear hyp7 localization. Scale bar=20 µm.
963 **(B)** Confocal images of *gfp::lin-42b+c; wrmscarlet::kin-20* staged according to vulva
964 morphology. An arrow indicates seam cell, an arrowhead hyp7 nuclear localization. Scale
965 bar=20 µm. **(C)** Confocal images of *splitgfp::kin-20* in wild type and *lin-42ΔCK1BD* mutant
966 animals during early - mid L4 stage based on the vulvae morphology. Arrows indicate
967 seam cell nuclear (white) and cytoplasmic (blue) localization, white arrowheads indicate
968 hyp7 nuclear localization. Scale bar=20 µm. **(D)** Quantification of GFP::KIN-20 signal in
969 hyp7 (nucleus), seam cells (nucleus) and hyp7 (cytoplasm) of wild-type (blue) and *lin-*
970 *42ΔCK1BD* (orange) animals. Each condition with n=9 mid-L4 stage worms.
971
972 **Figure S1 PER proteins have a crucial role in mammalian circadian rhythms. (A)**
973 Cartoon schematic of the primary transcription-translation feedback loop which generates
974 ~24-hour rhythms in mammals. CCGs, Clock-controlled genes. **(B)** Cartoon schematic
975 illustrating the mammalian phosphoswitch that dictates CK1-dependent regulation of PER
976 stability. Crystal structure of human CK1δ (gray) bound to phosphorylated PER2 FASP
977 (orange, 4pFASP) peptide, PDB: 8d7o. pD, phosphodegron; FASP, Familial Advanced
978 Sleep Phase.
979
980 **Figure S2 Larval stage durations for *lin-42* mutant animals.** Boxplots showing
981 durations (in hours) for **(A)** larval stages **(B)** molts and **(C)** intermolts from luciferase
982 assay. Wild type in white, *lin-42(n1089)* in dark grey, *lin-42(ok2385)* in light grey, *lin-*

983 *42ΔPAS* in blue and *lin-42ΔCK1BD* in green. **(D)** Bar plot showing the number of molts
984 from the luciferase assay of the indicated genotypes.
985
986 **Figure S3 Conservation and structural prediction of *C. elegans* KIN-20. (A)**
987 Sequence alignment of *C. elegans* KIN-20 and KIN-19, *H. sapiens* CK1δ, *M. musculus*
988 CK1δ, *A. thalia* CK1, *S. cerevisiae* HRR25 (HRR), and *D. melanogaster* Doubletime
989 (DBT). Important enzymatic sequence features are boxed; full kinase domain (blue box -
990 79% identical between KIN-20 and human CK1δ), catalytic lysine (purple - conserved in
991 KIN-20), catalytic loop (pink - 100% conserved between KIN-20 and human CK1δ),
992 Magnesium (Mg)-binding loop (orange - 12 out of 13 residues conserved between KIN-
993 20 and human CK1), anion coordination sites 1 (yellow), 2 (green), and 3 (blue) (all
994 conserved in KIN-20). **(B)** Crystal structure of *H. sapiens* CK1δ (PDB 6pxo). Dark blue
995 indicates residue is conserved in KIN-20; residues that diverge are highlighted in pale
996 blue (similar amino acid) or not conserved (white). **(C)** AlphaFold structural prediction of
997 KIN-20 (https://alphafold.ebi.ac.uk/entry/A8X4B3) colored by the model confidence. **(D)**
998 KIN-20 AlphaFold plot of predicted aligned error.
999
1000 **Figure S4 Larval stage durations for *kin-20 and lin-42* mutant animals.** Boxplots
1001 showing durations (in hours) for **(A)** Larval stages **(B)** Molts **(C)** Intermolts from luciferase
1002 assay. Wild type (*Wt*) in white, *lin-42(ΔTail)* in blue, *kin-20(0)* in light grey, *kin-20 D310A*
1003 in dark grey. Statistics were done using the Mann-Whitney U-test. Stars indicate the
1004 significance of difference between the *Wt* strain and the different *lin-42* and *kin-20* mutant
1005 animals: * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$. **(D)** Bar plot showing the number
1006 of molts detected in the assay in percentage of animals.
1007
1008 **Figure S5 Luciferase replicate of *lin-42(ΔTail).*** Heatmaps showing trend-corrected
1009 luminescence traces from the indicated genotype. Each horizontal line represents one
1010 animal. Traces are sorted to the entry of the first molt. Darker color indicates low
1011 luminescence signal and corresponds to the molts.
1012

1013 **Figure S6 *kin-20(D310A)* affects molt timing. (A)** Western Blot with extracts from
1014 *3xflag::lin-42b+c, 3xflag::kin-20* and *3xflag::lin-42b+c; 3xflag::kin-20* larvae (mid L4
1015 stage). Blot probed with anti-FLAG-HRP (1:1000). Arrows indicate bands for LIN-42b and
1016 KIN-20. **(B)** Western Blot with extracts from *3xflag::kin-20* wild-type, *(xe401[D310A])* and
1017 *(xe400[D310A])* mutant animals. Top panel probed with anti-FLAG-HRP (1:1000). Lower
1018 panel probed with anti-actin-1 (1:7500). Arrows indicate bands for KIN-20. **(C)** Heatmaps
1019 showing trend-corrected luminescence traces from the indicated genotype. Each
1020 horizontal line represents one animal. Traces are sorted by entry into the first molt. Darker
1021 color indicates low luminescence signal and corresponds to the molts. **(D)** Boxplots
1022 showing the duration (in hours) for the larval stage from the luciferase assay.
1023
1024 **Figure S7 KIN-20 dynamics during L2 - L3 stage.** Microscopy images of one
1025 *splitgfp::kin-20* larva from a microfluidics experiment at indicated timepoints. Time
1026 indicated in minutes after Molt 1 (M1) or Molt 2 (M2) exit. Arrows indicate seam cell
1027 nuclear (white) and cytoplasmic (blue) localization; white arrowheads indicate nuclear
1028 hyp7 localization. Scale bar=50 µm.
1029
1030 **Figure S8 Alignment of nematode LIN-42 protein sequences.** LIN-42 homologs from
1031 the indicated nematode species were aligned using Clustal Omega. The length in amino
1032 acids of each homolog follows the species and homolog name. To the left and right of the
1033 alignment are amino acid positions of the end residues for each protein. Blue shading
1034 indicates conserved sequences and the histogram at the bottom depicts the degree of
1035 conservation with a consensus sequence listed below. The positions of the *C. elegans*
1036 CK1BD-A and CK1BD-B motifs are indicated. The location of the phosphosites found in
1037 our *in vivo*, *in vitro* and both datasets are indicated.
1038
1039 **TABLES**
1040 **Table S1 *lin-42* mutant phenotypes**
1041 All animals grew from eggs hatched onto seeded plates at low density after sodium
1042 hypochlorite treatment. n ≥ 20 for all analyses. [a]Percentage of animals that failed to reach
1043 adulthood by day 8 after hatching or died as young larva. [b]Percentage of animals with

alae formation in early L4 (L4.0-L4.2). [c]Percentage of animals that exhibited bag-of-worms phenotype. [d]Average number of progeny from fertile adults; arrested and bagged animals were not included in the calculation. *Broods were not counted in animals with high instance of BOW phenotype.

Table S2 strains

C. elegans strains used in this work.

Table S3 oligos and plasmids

Oligos and plasmids used in this work

Table S4 crRNAs

CRISPR RNAs used in this work

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