1. **CDK5 influences the organization of the Circadian Machinery in peripheral clocks.**

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**Abstract**

Circadian rhythms are self-sustained physiological changes that drive rhythmicity within the 24-hours cycles. Posttranslational modifications (PMTs), such as protein phosphorylation, acetylation, sumoylation, and ubiquitination, are biochemical processes that modify protein structure and functions, ensuring circadian rhythm precision. For example, phosphorylation is considered the most important hallmark of rhythmicity from cyanobacteria to mammals. Cyclin-dependent kinase 5 (CDK5) has been shown to regulate the mammalian SCN's circadian clock via phosphorylation of PER2. Here, we show that CDK5 influences the clock machinery assembling, using immortalized mouse embryonic fibroblast as an in *vitro* model for studying the peripheral clock. In fact, the circadian period at the cellular level is lengthened. Furthermore, the clock-controlled gene's expression amplitude is dampened in *Cdk5* ko cell lines, while the phase is delayed about 4 hours.

Taken together, we show *in vitro* that CDK5 is critically involved in regulating the peripheral clocks, influencing their temporal and spatial dynamics.

**Introduction**

Circadian rhythms consist of interconnected transcriptional/translational and posttranslational feedback loops that dictate the rhythmicity of physiological responses within 24-hours (Schibler and Sassone-Corsi, 2002; Brenna and Albrecht 2020). These rhythms can be synchronized by stimuli called zeitgebers (ZT). For instance, in mammals, the light can reset the rhythmicity in the suprachiasmatic nuclei (SCN), a bipartite brain region belonging to the hypothalamus, placed above the optic chiasm. Thus, the SCN maintains the internal rhythms synchronously with the dark-light cycles. In addition, the SCN coordinates the synchronization of the independent circadian oscillators situated in peripheral tissues (i.e., liver, kidney, heart) through nervous and hormonal signaling pathways, regulating a wide range of physiological responses (Dibner et al., 2010). The rhythmic transcription is driven by the core factors of the positive loop, the heterodimer CLOCK: BMAL1. This heterodimer drives the expression of many clock-

42  controlled genes (CCGs), such as *Periods* (Per 1, 2, 3) and *Cryptochromes* (Cry 1, 2) by

43  binding the E-box element (CACGTG) within their promoter (Partch et al., 2014). On the other

44  hand, PERs and CRYs protein products are involved in the negative feedback loop. After

45  protein translation, PER: CRY homo/heterodimers translocate into the nucleus and inhibit

46  CLOCK: BMAL1–mediated transcription through direct protein-protein interaction (Buhr and

47  Takahashi, 2013). Thereby, the cellular positive and negative feedback loop promotes the

48  oscillation of about 10–20% of all genes expressed (Panda et al., 2002a; Storch et al., 2002).

49  Additionally, the fine-tuned period of the circadian oscillators is ensured by many additional

50  levels of regulation, such as epigenetic and posttranslational modifications (PMTs) ( Bellet and

51  Sassone-Corsi 2010; Hirano et al., 2016). Within the PMTs, phosphorylation is the most

52  important hallmark of rhythmicity (Roblest et al., 2017). In some organisms, such as

53  cyanobacteria, the circadian rhythmicity is driven only by phosphorylation, without the addition

54  of transcriptional/translational loops (Iwasaki et al., 2002).

55  In mammals, many kinases are involved in regulating circadian rhythms (Brenna and Albrecht,

56  2020). Cyclin Dependent-Kinase 5 is a serine/threonine kinase belonging to the  Cdc2/Cdk1

57  family activated by specific cofactors such as p35 and p39 (Tang et al., 1995; Tsai et al., 1994)

58  and cyclin I (Brinkkoetter et al.,2009). CDK5 plays many roles in the brain, such as

59  neurogenesis, neuronal migration, axon guidance, and aberrant activity leads to many

60  neurodegenerative diseases (Kawauchi, 2014). CDK5 can phosphorylate both CLOCK and

61  BMAL1 (Kwak et al., 2013; Brenna et al., 2019). This kinase influences in the SCN the PER2

62  protein stability and interaction with CRY1, which are the core elements of the negative loop,

63  ensuring the gene expression rhythmicity.  Lack of CDK5 shortens the circadian period length

64  in mice (Brenna et al.,2019). Therefore, this kinase has been proposed as one of the main

65  regulators of circadian rhythmicity in mice (Brenna and Albrecht 2020). In addition, Although it

66  was identified for the first time as one o the main kinases playing a role in the brain, many

67  recent observations led to the discovery of the involvement of CDK5 pathways outside the

68    brain. CDK5 regulates vesicular transport, apoptosis, cell adhesion, and migration ((Contreras-

69    Vallejos et al., 2012). Dysfunction of CDK5 in peripheral tissues is associated with many forms

70    of cancer also connected to the aberration of the circadian clock (Pozo and Bibb 2016; Sulli et

71    al., 2019).

72    Here we show for the first time the involvement of CDK5 in the regulations of the peripheral

73    clock, using immortalized mice embryonic fibroblasts as a model. Our results show that the

74    kinase activity of CDK5 is circadian, and deletion of this enzyme leads to a prolonged period

75    length. Furthermore, this phenotype is associated with an aberrant shuttling into the nucleus

76    of the clock factors. Thus, the BMAL recruitment to the E-box is shifted, and gene expression

77    is altered. Altogether our results show that CDK5 is essential for the correct assembly of the

78    clock machinery.

79    **Results**

80    **The kinase activity of CDK5 in immortalized mouse embryonic fibroblasts is**

81    **circadian.**

82    CDK5 kinase activity in the SCN is diurnal, with an off/on the state under light: dark

83    (12:12) conditions (Brenna et al.,2019). To understand whether the enzymatic activity can

84    be circadian, we challenged the hypothesis using the NIH3T3 (immortalized mouse

85    embryonic fibroblasts) cell lines as a model system. We synchronized cells with forskolin, a

86    powerful zeitgeber for circadian rhythmicity (Yagita and Okamura 2000), and collected

87    samples at different circadian time (CT) points. We validated the circadian entrainment of

88    cells by immunoblotting, using antibodies against CRY1 and BMAL1 (Fig. 1A). CDK5 was

89    subsequently immunoprecipitated at each time point, followed by *in vitro* kinase assay using

90    histone H1 as substrate in the presence of radiolabeled ATP. The autoradiography signal

91    showed that CDK5 kinase activity is circadian with an "on" state at CT 12-16 and 28-32 (Fig.

92    1B, C). These CTs correspond to the subjective nights. The specificity of the reaction was

93    verified by repeating the assay by immunoprecipitating the kinase from *Cdk5 KO* cell lines

94    or wt cells pretreated with Roscovitine 34 uM ( a CDKs inhibitor) (Tomov et al., 2019),

95    followed by *in vitro* kinase assay. The phosphorylation of H1 did occur only in the wt samples

96    (Fig. 1D).

97        Altogether these observations support the thesis that CDK5 phosphorylates the histone

98    H1 in a circadian fashion.

99

100   **Lack of CDK5 lengthens period in peripheral clock**

101       To understand whether CDK5 can affect the circadian period in the peripheral clock, we

102   transfected NIH3T3 with *Bmal^luc* promoter and treated cells either with DMSO (vehicle) or

103   Roscovitine (CDKs inhibitor) at different concentrations for 12 hours. This treatment was

104   followed by synchronizing the cells with forskolin and recording the real-time luciferase

105   bioluminescence over days (Fig 2A).  NIH3T3 cell lines treated with DMS0 show a regular

106   period length, which settles around 23 hours while increasing amount of the drug

107   corresponded to an increased period length (Fig 2B). To confirm the involvement of CDK5,

108   we subsequently modulated the Cdk5 gene expression transfecting the *Bmal^luc* promoter in

109   NIH3T3 cell lines with different sh RNA against *Cdk5* mRNA (Brenna et al., 2019 Fig. S1A).

110   The increasing effect of the sh RNA against *Cdk5* mRNA mirrored the lengthening of the

111   circadian period in cells (Fig 2C, D). Thus, we finally confirmed that lack of CDK5 lengthens

112   the period, comparing NIH3T3 vs. *Cdk5* KO cell lines (Fig. 2E, 2F). Altogether, these results

113   suggest that lack of CDK5 has a tissue-specific effect in the circadian period of the peripheral

114   clocks

115

116   **CDK5 affects the clock factors nuclear localization.**

117   The lack of CDK5 slows the clock down in the peripheral clocks (Fig.2). Thus, we aimed to

118   define the effect of CDK5 on the clock at the molecular level. So far, only PER2 and CLOCK

119   are directly phosphorylated by CDK5 (Brenna et al., 2019; Kwak et al., 2013). Therefore, we

5

120    investigated whether both PER2 and CLOCK accumulation and distribution in the NIH 3T3

121    could be affected in the absence of the kinase.  Cells were synchronized with forskolin and

122    collected at different CTs. This samples collection was followed by nucleo/cytoplasm

123    fractionation to observe whether the cellular distribution of CLOCK and PER2 was affected

124    by CDK5. In control cell lines, we could observe PER2 at CT 12,16, CT 28, and 32 in both

125    nuclear and cellular extracts with a peak between CT28-32 (Fig. 3A, B, wt).

126    On the other hand, PER2 was observable, in a reduced amount only at CT 12 in *Cdk5* KO

127    cell lines both in cytoplasmic and nuclear extract (Fig. 3A, B, ko cells). As expected, the

128    protein accumulation did not last longer, confirming that CDK5 is important for PER2 protein

129    stability (Brenna et al., 2019). Subsequently, we analyzed CLOCK cellular distribution. The

130    cytoplasmic protein accumulation showed a flat profile, while rhythmic in the nuclei in wt cell

131    lines peaking between CT 24-32 (Fig. 3A, B wt). By contrast, cytoplasmic accumulation of

132    CLOCK was dampened in *Cdk5* KO cells lines compared to WT while in the nuclei lost the

133    rhythmicity and was way more abundant (Fig. 3A, B, ko cells).  Altogether, these results

134    show CDK5 has an opposite effect on CLOCK and PER2 in murine embryonic fibroblasts.

135    Both CLOCK and PER2 are main members of the molecular clock machinery (Arya et al.).

136    Thus, We sought to investigate how the lack of CDK5 could affect the nuclear accumulation

137    of the other proteins part of the machinery, such as BMAL, CRY1, and PER1. Thus, we

138    subsequently investigated whether BMAL, PER1, CRY1 nuclear localization was affected

139    as well. Our immunoblotting showed that BMAL was accumulated in the wt nuclei at CT20,

140    while the protein accumulation was shifted about 4 hours in the *Cdk5* ko cells (Fig. 3C).

141    Furthermore, Immunoblottings using antibodies against PER1 and CRY1 showed that in wt

142    cell lines, the nuclear accumulation of these factors was higher at the subjective night. By

143    contrast, the nuclear accumulation of these proteins in the *Cdk5* ko cell lines was much

144    higher than wt, and we could detect PER1 and CRY1 during the subjective day (FIG. 3D).

145    Taken together, our results show that CDK5 affect the assembly of the clock machinery in

146    mouse embryonic fibroblast. Due to the severe impact on the clock machinery caused by

147    the lack of CDK5, we wondered whether this enzyme could be part of the clock machinery.

148    There are many examples of kinases being part of the complex in central and peripheral

149    clocks (Brenna et Albrecht 2020). Therefore, we immunoprecipitated CDK5 at different CT,

150    every 4-hours, starting from CT12 to CT32, followed by wb immunodetecting CLOCK,

151    BMAL1, PER1/2, CRY1. Our results showed that CDK5 could interact with PER1, PER2,

152    and CRY1 in the subjective night. CDK5:CRY was observable at CT12-16, while

153    CDK5:PER1 was detected at CT16-20.

154    Interestingly, both CLOCK and BMAL1 interact with CDK5 during the subjective day, with

155    an identical profile. This observation confirms that CLOCK and BMAL work as a heterodimer

156    (Fig. 3D). Altogether our results show that CDK5 is part of the clock machinery.

157

158    **Lack of CDK5 shifts BMAL recruitments to the chromatin and, as a consequence, the**

159    **mRNA rhythmicity.**

160    Our results show that CDK5 is part of the clock machinery, and the lack of this enzyme is

161    responsible for a spatial and temporal redistribution of the complex. We subsequently

162    wondered how this event could affect the expression of the CCGs. To investigate that

163    aspect, we synchronized both wt and *Cdk5* ko cells with forskolin and collected samples

164    every 4 hours starting from CT 12 up to CT40. We subsequently extracted total mRNA and

165    performed RT-qPCR to detect the accumulation of clock factors pre-mRNA. We investigated

166    *Bmal1* gene expression, which is under control of ROR alpha, and, *Per1* which is under the

167    control of CLOCK: BMAL1, and *Clock,* which should not show any oscillation. Our results

168    show interesting molecular behaviors. *Bmal1* gene expression in wt cells peaked around 24

169    hours after the stimulus. In *Cdk5* ko cells, the peak of pre-mRNA accumulation was shifted

170    4 hours ahead (Fig. 4A). Interestingly, *Bmal1* expression mirrored the nuclear protein

171    accumulation, suggesting a control mediated by CDK5 on BMAL at the transcriptional level.

172    Furthermore, *Per1* gene expression in both wt and Cdk5 ko cells mirrored exactly the *Bmal1*

173    profile (Fig. 4B). Surprisingly, Clock pre-mRNA, which did not show any oscillation in wt

174    cells, gained ex Novo rhythmicity in the *Cdk5* ko (Fig 4C). Altogether our results show that

175    CDK5 plays a role in the regulation of the clock-controlled genes.

176    Additionally, our previous results showed that CDK5 affects the nuclear localization of the

177    heterodimer CLOC: BMAL1. Particularly, BMAL1 nuclear accumulation in *Cdk5* ko cells is

178    delayed by 4-hours. BMAL1 is the main transcriptional factor promoting the expression of

179    genes whose promoter contains E-boxes (Ripperger and Schibler 2006). Thus, these

180    observations raised whether the different nuclear localization of BMAL would also mirror

181    different temporal recruitment of the transcriptional factor on the E-box of CCGs. We

182    performed chromatin immunoprecipitation (ChiP) to challenge the hypothesis.    We

183    immunoprecipitated the transcriptional factor at the same time we analyzed the pre-mRNA

184    profiles before. Thus, we performed RT-qPCR to amplify the E-box within the promoter of

185    the *Per1* gene. Interestingly, our results show that the recruitment of BMAL1 on the *Per1*_E-

186    box  was rhythmic in both cell lines. However, while in wt cells, the peak of rhythmicity was

187    at CT24 in the Cdk5 ko, at CT28 (Fig. 4D). Thus, our results showed that CDK5 influences

188    the circadian period in the peripheral via influencing nuclear accumulation and, therefore,

189    temporal promoter occupancy of BMAL1 on the CCGs.

190    **Discussion**

191    It has been recently described that phosphorylation is the stronger mark of rhythmicity in

192    circadian mammal models (Robles et al., 2017). Furthermore, many kinases were described

193    to influence the circadian clock in livings in the past decades, such as CKI, CDK1, GSK3 B

194    (Brenna et Albrecht 2020).

195   It was recently showed the impact of Cyclin-Dependent Kinase 5 (CDK5) on the circadian

196   clock in the SCN, which is the peacemaker of circadian rhythmicity (Brenna et al., 2019).

197   Indeed in mice, when CDK5 is silenced in the SCN, it affects PER2 protein accumulation

198   and nuclear translocation. Consequently, mice show a shortening in the period length when

199   their behavior is measured in constant darkness. However, it was described in the recent

200   past that CDK5 could influence many pathways in peripheral tissues, such as regulating

201   vesicular transport, apoptosis, cell adhesion, and migration ((Contreras-Vallejos et al., 2012)

202   Dysregulation of CDK5 can alter these pathways leading to many diseases connected to

203   circadian dysfunctions ((Pozo and Bibb 2016; Sulli et al., 2019).

204   Since CDK5 has a profound impact on the central clock, we questioned whether it could

205   also affect the clock in the periphery. For this reason, we used mouse embryonic fibroblast

206   cell lines as *in vitro* model system.  After synchronization with forskolin, CDK5 shows a

207   higher kinase activity during the subjective night than the subjective day  (Fig. 1B, C). Our

208   data align with the diurnal activity of CDK5 in the SCN, which was also peaking at night

209   (Brenna et al., 2019).  We further questioned the impact of CDK5 on the circadian rhythmicity

210   in NIH3T3. We performed a Real-Time Bioluminescence measurement using the *Bmal^{luc}*

211   promoter as a reporter.  We tested the rhythmicity in different conditions: 1) we used

212   Roscovitine which is a potent inhibitor of CDK5 kinase activity (Ref); 2) we used different sh

213   RNA targeting *Cdk5* gene; 3) we used CRISPR/CAS9 Cdk5 KO cell lines.

214   All the different approaches showed that the lack of CDK5 causes a lengthening of the

215   circadian period in cell lines (Fig 2). This observation came as a surprise since it looks quite

216   the opposite of what was observed before in the SCN of mice (Brenna et al., 2019). This

217   difference shows that CDK5 might have a tissue-specific function. However, a systemic

218   comparison should be performed between the SCN and the MEFs to understand what

219   factor(s) regulated by CDK5 can switch from shorter to longer period length. CLOCK and

220   PER2 are the only described target for CDK5 kinase activity (Brenna et al., 2019; Kwak et

221    al., 2013). Therefore, we performed nuclear/cytoplasm fractionation on synchronized cells

222    to detect the circadian subcellular localization of both CLOCK and PER2 (Fig. 3A, B).  We

223    observed less CLOCK in the cytoplasm in the *Cdk5* KO compared to wt.

224    Moreover, the nuclear accumulation in wt cells showed a circadian profile that overlapped

225    the profile of clock-controlled gene expression such as *Per1* (Fig. 4B). On the other hand, in

226    *Cdk5* ko cells, the profile of protein accumulation was flat, and there was generally more

227    CLOCK at each time point than wt.  These results were aligned with previous observations

228    showing that CDK5 influences the nuclear localization of CLOCK (Kwak et al., 2013). These

229    results are interesting since the wider accumulation of CLOCK can be related to the

230    prolonged period length observed in cell culture (Fig.2). Furthermore, since CLOCK is

231    observed as a heterodimer with BMAL1, we also investigated the nuclear accumulation of

232    the transcriptional factor. Our results show that BMAL1 nuclear accumulation in wt cells

233    peaks at CT 20 while Cdk5 ko at CT24, 4-hours later (Fig. 3C).

234    Interestingly, CRY1 and PER1,  members of the repressive loop of the circadian clock, were

235    accumulated in the nuclei of the wt cells with a coherent profile, peaking at the subjective

236    night. However, in the ko cell lines, these factors were highly accumulated also during the

237    subjective day (Fig. 3C). Since CDK5 affected so strongly the cellular localization of all the

238    clock factors described above, we wondered whether the kinase per se could be part of the

239    clock machinery. Therefore we performed an immunoprecipitation around the clock to define

240    the temporal profile of interaction between CDK5 and the other clock factors. Surprisingly,

241    CDK5 interacts with CRY1 at the early night, while with PER1 at the late night. CDK5 also

242    interacts with both CLOCK and BMAL simultaneously during the subjective day, confirming

243    that CLOCK and BMAL1 work as heterodimers (Wang et al., 2013). Thus, CDK5 seems to

244    be part of the clock machinery. Other kinases are part of the complex, such as Casein

245    Kinase I (Aryal et al., 2017), which directly phosphorylates both PER1 and PER2 (Camacho

246    et al., 2001).

247   As a consequence, the expression of the clock-controlled genes is also affected. For

248   example, *Bmal1* gene expression shows a 4-hours delayed peak in *Cdk5* ko cells. Since

249   *Bmal1* gene expression is regulated by the stabilizing loop formed by Rora and Reverb alpha

250   (Akashi et al., 2005), this result suggests that CDK5 might be part also of the stabilizing

251   complex. *Per1* expression also shows 4-hours of delay in the peak in Cdk5 ko cells, which

252   mirrored the profile of BMAL1 nuclear accumulation. Thus, together we show that the shift

253   of BMAL1 accumulation in Cdk5 Ko cells overlaps the shift in the expression of target genes.

254   To finally confirm that the shift in the gene expression of *Per1* is related to delayed

255   recruitment of BMAL1 onto its E-box in Cdk5 ko cells, we performed a ChIP assay using an

256   antibody against BMAL1. Our ChIP assay showed that while BMAL1 is recruited onto the E-

257   box in wt cells with a circadian profile peaking at CT24, the peak was observed at CT28 in

258   the ko cell lines. Thus, The recruitment of BMAL was shifted by 4 hours (Fig. 4D). Finally,

259   we acquired another interesting observation. Clock gene expression, which showed a flat

260   profile in wt cells, gained an ex Novo oscillation in the *Cdk5* ko cells. Altogether, our evidence

261   shows that CDK5 plays an important role in the peripheral clock using the cell culture model

262   system. However, it is not clear how the lack of this enzyme can provoke a shortening of the

263   period length in the SCN, while the opposite in the cells. It would also be interesting how

264   this mechanism of regulation described in this paper would influence specific organs such

265   as the liver, gut, muscles, testis, which show all independent oscillations (Dibner et al., 2010)

266

267   **MATERIALS and METHODS**

268   **Cell culture**

269   Mouse embryonic fibroblasts (MEFs) were obtained from mice without functional CDK5

270   using the CRISPR/CAS9 approach. The details are explained in Brenna et al., 2019. All lines

271   were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/l glucose,

272    stable glutamine, sodium pyruvate, and 3.7 g/l $NaHCO_3$ (PAN Biotech), supplemented with

273    10% fetal calf serum (FCS) and 100 U/mL penicillin-streptomycin at 37°C in a humidified

274    atmosphere containing 5% $CO_2$. Forskolin stimulation (100 μM) was used to synchronized

275    the cellular circadian rhythm. Samples were collected at specific time points mentioned in

276    the text.

277    ***In vitro* kinase assay immunoprecipitating CDK5 from cells.**

278    CDK5 was immunoprecipitated from cells samples at different circadian times (CTs) (circa

279    200 μg of protein extracts. After immunoprecipitation at 4°C overnight with 2x Protein A

280    agarose (Sigma-Aldrich), samples were diluted in washing buffer and split in two halves.

281    One half of the IP was used for an in vitro kinase assay. Briefly, 1 μg of histone H1 (Sigma-

282    Aldrich) was added to the immunoprecipitated CDK5 and assays were carried out in reaction

283    buffer (30 mM Hepes, pH 7.2, 10 mM $MgCl_2$, and 1 mM DTT) containing [γ-$^{32}$P] ATP (10 Ci)

284    at room temperature for 1 hr and then terminated by adding SDS sample buffer and boiling

285    for 5 min. Samples were subjected to 15% SDS-PAGE, stained by Coomassie Brilliant Blue,

286    and dried, and then phosphorylated histone H1 was detected by autoradiography. The other

287    half of the IP was used for Western blotting to determine the total amount of CDK5

288    immunoprecipitated from the SCN samples. To quantify the kinase activity at each time

289    point, the following formula was used: ([$^{32}$P] H1/total H1 for each reaction)/CDK5 IP protein.

290    **RNA extraction from cells**

291    Cells were grown to confluency on 6 cm Petri dishes and induced with 10 μM forskolin (50

292    mM stock in dimethyl sulfoxide) for the indicated time. Total RNA was extracted using the

293    Nucleospin RNA II kit (Macherey & Nagel) and adjusted to 1 μg/ μl with water. An amount

294    of 1 μg was reverse-transcribed using Superscript II with random hexamer primers (Thermo

295    Fisher). Real-time PCR was performed using the KAPA probe fast universal master mix and

296    the indicated primers on a Rotorgene 6000 machine. The relative expression was calculated

297    compared to the geometric mean of expression of the inert genes Nono, SirT2, Atp5h, and

298    Gsk3b [69]. For a complete list of primers used in the paper, please see Table 1.

299

300    **Chromatin Immunoprecipitation**

301    Chromatin immunoprecipitation from cells was performed as described before [70]. Briefly, the

302    cells were grown to confluency on 15 cm Petri dishes, induced with 10 µM forskolin (50 mM

303    stock in dimethyl sulfoxide), and fixed at the indicated time with 1% formaldehyde/ 1x

304    phosphate-buffered saline buffer (PBS) for 10 min at 37°C. Then the cells were washed

305    twice with ice-cold 1x PBS and scraped in 1 ml of 10 mM dithiothreitol/ 100 mM

306    tris(hydroxymethyl)aminomethane (Tris) HCl, pH 8.8. Cells were lysed in 10 mM

307    ethylenediaminetetraacetic acid (EDTA), 1 mM ethylene glycol-bis(2-aminoethyl ether)-N,

308    N, N', N'-tetraacetic acid (EGTA), 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic

309    acid (HEPES), pH 7.6, 0.2% Triton X-100 for 5 min on ice and the obtained nuclei washed

310    with 1 mM EDTA, 0.5 mM EGTA, 200 mM NaCl, 10 mM HEPES, pH 7.6. The purified nuclei

311    were sonicated in 2 mM EDTA, 150 mM NaCl, 20 mM Tris, pH 7.5, 1% SDS using a

312    BRANSON SLPe sonicator with a 4C15 tip for 6 cycles of 10 s each kept on ice, and diluted

313    1:10 with 2 mM EDTA, 150 mM NaCl, 20 mM Tris, pH 7.5, 1.1% Triton X-100. Equal amounts

314    of chromatin were incubated with the indicated antibodies for 1h at RT. (Table 2) and the

315    immune complexes were captured with protein A agarose fast-flow beads (Sigma-Aldrich)

316    for 1h at RT. The beads were washed with 2 mM EDTA, 150 mM, 20 mM Tris, pH 7.5, 0.1%

317    SDS, 1% Triton X-100, then 2 mM EDTA, 500 mM, 20 mM Tris, pH 7.5, 0.1% SDS, 1%

318    Triton X-100, then 2 mM, 250 mM LiCl, 20 mM Tris, pH 7.5, 0.5% Na-deoxycholate, 0.5%

319    NP40 substitute, and finally 2 mM EDTA, 150 mM, 20 mM Tris, pH 7.5. DNA fragments were

320    eluted, and the crosslinks reversed in 2 mM EDTA, 150 mM NaCl, 20 mM Tris, pH 7.5, 1%

321    SDS at 65°C overnight. The DNA fragments were purified using a MinElute PCR fragment

322 purification kit (Qiagen). Real-time PCR reactions were performed using the KAPA probe

323 fast universal master mix and the indicated primers on a Rotorgene 6'000 machine. The

324 efficiency of the precipitations was calculated by comparing the amount of precipitated

325 material to 1% of the starting material.

326

327 **Protein extraction from cells.**

328 Total confluent cells plated in 10 cm dishes were washed two times with 1x PBS (137 mM

329 NaCl, 7.97 mM $Na_2HPO_4 \times 12\ H_2O$, 2.68 mM KCl, 1.47 mM $KH_2PO_4$). Then PBS was added

330 again, and plates were kept for 5 min at 37°C. Cells were detached and collected in tubes

331 and frozen in liquid $N_2$. They were subsequently resuspended in Ripa buffer (50 mM Tris-

332 HCl pH7.4, 1% NP-40, 0.5% Na-deoxycholate, 0.1% SDS, 150 mM NaCl, 2 mM EDTA, 50

333 mM NaF) with freshly added protease or phosphatase inhibitors, and homogenized by using

334 a pellet pestle. Homogenates were kept on ice for 15 min followed by sonication (10 s, 15%

335 amplitude). Right after, the samples were centrifuged for 15 min at 16,100 g at 4°C. The

336 supernatant was collected in new tubes and pellet discarded.

337

338 **Immunoprecipitation**

339 Circa 400 µg of protein extract was diluted with the appropriate protein lysis buffer in a final

340 volume of 250 µl and immunoprecipitated using the indicated antibody (ratio 1:50) at 4°C

341 overnight on a rotary shaker. The day after, samples were captured with 50 µl of 50% (w/v)

342 protein-A agarose beads (Roche) for 3 hr at 4°C on a rotary shaker. Before use, beads were

343 washed three times with the appropriate protein buffer and resuspended in the same buffer

344 (50% w/v). The beads were collected by centrifugation and washed three times with NP-40

345 buffer (100 mM Tris-HCl pH7.5, 150 mM NaCl, 2 mM EDTA, 0.1% NP-40). After the final

346 wash, beads were resuspended in 2% SDS 10%, glycerol, 63 mM Trish-HCL pH 6.8, and

347  proteins were eluted for 15 min at RT. Laemmli buffer was finally added, samples were

348  boiled 5 min at 95° C and stored at -20° C.

349  **Nuclear/Cytoplasm fractionation (Brenna et al., 2019)**

350  Tissues or cells were resuspended in 100 mM Tris-HCl pH 8.8/10 mM DTT and

351  homogenized with a disposable pellet pestle. After 10 min incubation on ice, the samples

352  were centrifuged at 2500 g for 2 min at 4°C and the supernatant discarded. After adding 90

353  µL of completed cytoplasmic lysis buffer (10 mM EDTA, 1 mM EGTA, 10 mM Hepes pH 6.8,

354  0.2% Triton X-100, protease inhibitor cocktail (Roche), NaF, PMSF, ß-glycerophosphate),

355  the pellet was resuspended by vortexing, followed by centrifugation at 5200 rpm for 2 min

356  at 4°C. The supernatant transferred into a fresh 1.5 mL tube was the CYTOPLASMIC

357  EXTRACT. The pellet was washed three times with cytoplasmic lysis buffer and

358  resuspended in 45 µL 1x NDB (20% glycerol, 20 mM Hepes pH 7.6, 0.2 mM EDTA, 2 mM

359  DTT) containing 2x proteinase and phosphatase inhibitors followed by adding 1 vol of 2x

360  NUN (2 M Urea, 600 mM NaCl, 2% NP-40, 50 mM Hepes pH 7.6). After vortexing the

361  samples were incubated 30 min on ice, centrifuged 30 min at 13,000 rpm at 4°C and the

362  supernatant that was transferred into a fresh tube was the NUCLEAR EXTRACT.

363  **Western blot**

364  The indicated amount of protein was loaded onto 10% SDS-PAGE gel and run at 100 Volt

365  for two hours. Once the migration was completed, we performed a semidry transfer (40 mA,

366  1 hour 30 s) using Hybond® ECL™ nitrocellulose membranes followed by red ponceau

367  staining (0,1 % of Ponceau S dye and 5% acetic acid) to validate the success of the transfer.

368  The membrane was subsequently washed with TBS 1x/Tween 0.1% and blocked with TBS

369  1x/Milk 5%/Tween 0.1% for 1 hour. After washing, the membrane was stained with the

370  appropriate primary antibodies overnight. The antibodies against all the clock factors were

371  homemade (final dilution 1:1000). CDK5 purchased by Cell Signaling Tech (1:1000), while

372  Tubulin by Abcam (1:1000) and Lamin B by Santa Cruz (1:1000). The day after, membranes

373    were washed three times with TBS 1x/Tween 0.1% followed by secondary antibody

374    immunoblotting for 1 hour at room temperature. The densitometric signal was digitally

375    acquired with the Azure Biosystem.

376

## Statistical analysis

378    Statistical analysis of all experiments was performed using GraphPad Prism8 software.

379    Depending on the type of data, either an unpaired t-test with Welch's correction or a paired

380    t-test, when a sample was set to 1, were performed. Two-way ANOVA was performed on

381    time and genotype-dependent experiments. Values considered significantly different are

382    highlighted, $*p<0.05$, $**p<0.01$, or $***p<0.001$.

383

## Acknowledgments

## Author Contributions

388    Conceived and designed the experiments: JR, AB.

389    Performed the experiments: JR, AB.

390    Analyzed the data:, JR, AB.

391    Contributed reagents, materials, analysis tools: UA

392    Wrote the paper: AB

393    Edited the manuscript: AB

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## Competing financial interests

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397    The authors declare no competing financial interests

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**Figure 1. CDK5 kinase activity is circadian.**

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431  A) Western Blot performed on forskolin-synchronized wt cell, collected at different circadian

432  points (CTs). Samples were stained with antibodies against CRY1 and BMAL1 to validate

433  the circadian cellular synchronization. B) CDK5 was immunoprecipitated at different CTs,

434  and subsequently, an *in vitro* kinase assay was performed using histone H1 as substrate in

435  the presence of radiolabelled $^{32}P^-$ATP. C) The radioactive signal was normalized on the

436  immunoblotted signal for CDK5 after the IP. Quantification of three independent experiments

437  (mean ± SEM). One-way ANOVA with Bonferroni's post-test, *: p<0.001. D) the experiment

438  in (B) was performed in the presence of cells previously treated with Roscovitine and a *Cdk5*

439  ko cell line. The experiment confirmed the specificity of the phosphorylated signal due to the

440  CDK5 kinase activity.

441

442  **Figure 2. CDK5 affects the circadian clock.**

443  A) NIH3T3 cells previously treated with an increasing amount of Roscovitine (7, 21, 34 uM)

444  were transfected with *Bmal* $^{luc}$ promoter and synchronized with forskolin. The

445  bioluminescence was recorded over several days. B) The period length was quantified, and

446  statistical analysis was performed. The results showed that an increased amount of forskolin

447  corresponded to a prolonged period of length. Quantification of three independent

448  experiments (mean ± SEM). One-way ANOVA with Bonferroni's post-test, *: p<0.001.

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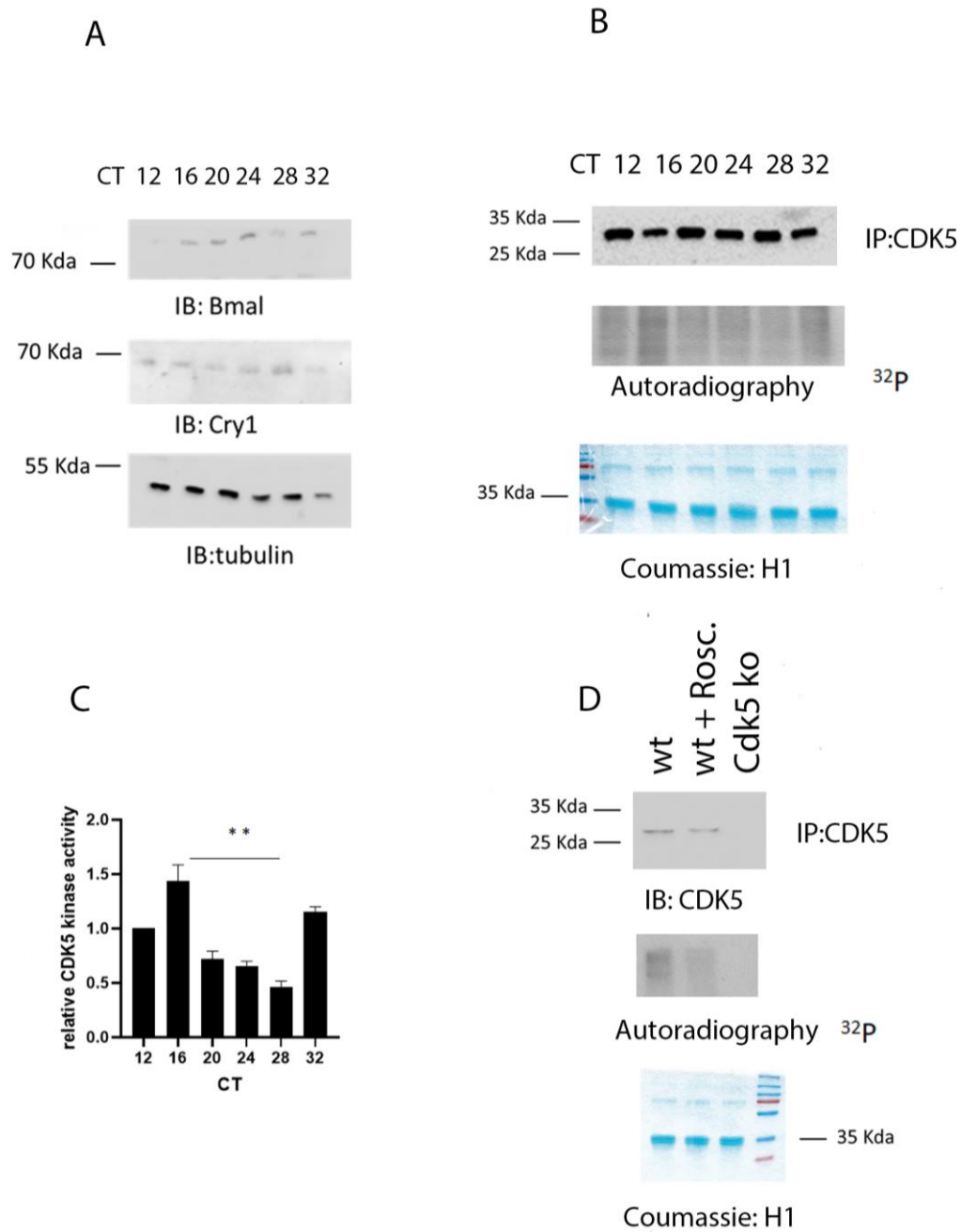450  **Figure 3. CDK5 affects the clock machinery.**

451  A) western blot was performed on cytoplasmic extracts obtained from wt and *Cdk5* ko

452  synchronized cells, showing how the kinase affected the circadian accumulation of CLOCK

453  and PER2. B) western blot performed on nuclear extracts obtained from wt and Cdk5 ko

454  synchronized cells, showing how the kinase affected the circadian accumulation of CLOCK

455  and PER2. C) western blot performed on nuclear extract obtained from wt and Cdk5 ko

456  synchronized cells, showing how the kinase affected the circadian accumulation of BMAL1,

457   CRY1 PER1. D) Immunoprecipitation performed around the clock on wt cells synchronized

458   with forskolin using CDK5 antibody as a bait. CDK5 co-resolved with BMAL1, CLOCK,

459   CRY1, PER1 at specific time points

460   **Figure 4. CDK5 influences the expression of clock-controlled genes**. The temporal

461   profile of pre-mRNA accumulation for *Bmal1* (A), *Per1* (B), *Clock* (C) was analyzed in

462   synchronized wt and Cdk5 ko cells. Quantification of three independent experiments (mean

463   ± SEM). One-way ANOVA with Bonferroni's post-test, *: $p < 0.001$ D) Chromatin

464   immunoprecipitation was performed using BMAL1 antibody as a bait. RT-qPCR was

465   performed using specific probes that amplified the E-box of Per1. Quantification of three

466   independent experiments (mean ± SEM). One-way ANOVA with Bonferroni's post-test, *:

467   $p < 0.001$

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482   **Figures**

483 **Figure 1**

489    **Figure 2**



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## Figure 3

**Figure 4**