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Distinct roles of DBHS family members in the circadian transcriptional feedback loop.

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Factors interacting with core circadian clock components are essential to achieve transcriptional feedback necessary for metazoan clocks. Here we show that all three members of the *Drosophila* Behavior Human Splicing (DBHS) family of RNA-binding proteins play a role in the mammalian circadian oscillator, abrogating or altering clock function when overexpressed or depleted in cells. Although these proteins are members of so-called nuclear paraspeckles, depletion of paraspeckles themselves via silencing of the structural non-coding RNA (ncRNA) *Neat1* did not affect overall clock function, suggesting that paraspeckles are not required for DBHS-mediated circadian effects. Instead, we show that the proteins bound to circadian promoter DNA in a fashion that required the PERIOD (PER) proteins, and potently repressed E box-mediated transcription but not CMV promoter-mediated transcription when exogenously

recruited. Nevertheless, mice with one or both copies of these genes deleted show only small changes in period length or clock gene expression *in vivo*. Data from transient transfections show that each of these proteins can either repress or activate depending on the context. Taken together, our data suggest that all of the DBHS family members serve overlapping or redundant roles as transcriptional cofactors at circadian clock-regulated genes.

INTRODUCTION

The circadian oscillator governs diurnal timing for most aspects of mammalian physiology (7). Its mechanism is cell-autonomous, and consists of interlocked feedback loops of circadian transcription, translation, and protein modification. In one loop, the CLOCK/NPAS2 and BMAL1/ARNTL transcriptional activators drive expression of the Period (*Per1* and *Per2*) and Cryptochrome (*Cry1* and *Cry2*) gene families, whose products subsequently multimerize and repress their own transcription. In a second loop, the transcriptional repressor REV-ERBα, whose transcription is also driven by CLOCK and BMAL1, represses the expression of *Bmal1* itself (7). Beyond these "dedicated" clock genes, a large number of other factors are necessary to the circadian clock or for its regulation of physiology – kinases and phosphatases, chromatin modifying factors, and other proteins (25, 33). We have shown previously that the NONO protein in mammalian cells (or its ortholog NON-A in flies) plays such a role by modulating PER-mediated transcriptional repression via unknown mechanisms (4).

NONO (also known as p54nrb in humans) has two RNA-binding (RRM) domains and has been shown to regulate a variety of processes outside the circadian clock (38). These include transcriptional activation and repression (16, 26), pre-mRNA processing (19), and RNA transport in neurons (18). For example, NONO has been shown to regulate the transcriptional activation of the TORC family of growth and metabolic factors via recruitment of the RNA polymerase II (1). In an apparently unrelated nuclear function, it also mediates the nuclear retention of edited RNAs in nuclear paraspeckles, which are thought to be RNA holding structures (30). These structures contain the NONO, SFPQ, and PSPC1 proteins, as well as the scaffolding ncRNA *Neat1* (3). Both SFPQ and PSPC1 share significant structural and functional similarity to NONO, and for this reason all three proteins have been grouped into the DBHS (*Drosophila* Behavior Human Splicing) family of nuclear factors. Nevertheless, to date only NONO (4) and SFPQ (10) have been implicated in the circadian clock mechanism.

Herein, we show that all three DBHS factors play important roles in the circadian clock by binding directly to the promoter of the *Rev-erbα* clock gene in circadian and PER protein-dependent fashion. In addition, although overexpression or silencing of any one of them influences clock period and amplitude in cells, depletion of paraspeckles themselves has no effect on the circadian oscillator. Mice deficient for two of these proteins show circadian phenotypes, albeit less prominent than *in vitro*. We therefore suggest that all three proteins play redundant roles in circadian transcriptional modulation.

METHODS

Animal husbandry

Chimeric mice were obtained from *Nono* genetrapped (*Nono*^{gt}) embryonic stem (ES) cells (C57/BL6J genotype) via standard blastocyst injection of the ES clone YHA266 into SV129 mice by the University of California, Davis. Individual chimeric mice were back-crossed 4-10 generations against C57/BL6J. The same procedure was chosen to obtain *Pspc1*$^{gt/gt}$ and *Sfpq*$^{gt/+}$ mutant mice, using ES clones RRS358 and BC0256, respectively. Individual chimeric mice were back-crossed 2-4 generations against C57/BL6J. All experiments were performed by comparing wildtype and mutant littermates. Animal housing and experimental procedures are in agreement with veterinary law of the canton of Zurich.

Animal activity measurements

For period measurements of *Nono*^{gt} mice, 24 mice of each genotype were habituated to a controlled 12:12 light-dark (LD) cycle in the presence of running wheels for 2 weeks, and then kept in constant dim red light for an additional two weeks. Data recording and period analysis was performed using the Clocklab software package (Actimetrics). Period measurements of *Pspc1*$^{gt/gt}$ and *Sfpq*$^{gt/+}$ mice were performed identically except that 6 mice of each genotype were used, and measurements were performed twice on each mouse. For skeleton photoperiod measurements, the same mice were given 1 hour of normal room light at each LD transition of a normal day, and otherwise kept in constant dim red light. Running wheel activity was measured as in period experiments,

but plotted as the sum of activities of all the mice over a 24-hour day using the Clocklab software.

Plasmids

The bioluminescence reporter construct *pBmal1-Luciferase* has been described previously (27). Overexpression of NONO, SFPQ, and PSPC1 (tagged with the myc epitope) were achieved using the plasmids described in (21). Plasmids expressing PER1 and PER2 proteins tagged with the FLAG epitope were a gift of T. Wallach (Kramer lab, Charite Universitätsmedizin, Berlin). To create GAL4 fusion constructs, the same constructs were obtained as Entry™ vectors from NITE (the Japanese Bioresource Information Center), and recombined into a Destination™ vector (Invitrogen) containing the GAL4 DNA-binding domain (aa 1-93). This vector was made by cloning PCRed recombination sites from pEF-DEST51 (Invitrogen) into pSCT-GALVP80 (gift of W. Schaffner, University of Zurich). The *Neat1* overexpression vector is described in (6). RNAi vectors against NONO have been described previously in (4). Vectors targeting SFPQ and PSPC1 were purchased from Open Biosystems (clone numbers RRM3981 – 98064499 TRCN0000102241 and RMM3981 – 98064691 TRCN0000102470, respectively). p4xEbox-*luc* is described in (4). pGAL4-Ebox-luc was made by inserting a multimerized 5xGAL4 site (cut from pFR-luc, Invitrogen) upstream of the E boxes in p4xEbox-luc. pGAL4-CMV-luc was made by inserting the same fragment the same distance upstream relative to the transcription start site of the CMV promoter.

Primary cell isolation and culture

Primary adult dermal fibroblasts (ADFs) were taken from a 0.5cm piece of mouse tail that was cut into several small pieces by using a razor blade. Digestion occurred in 1.8ml DMEM containing 20% FBS, 1% penicillin/streptomycin and 1% amphotericin B supplemented with 0.7 units liberase blendzyme (Roche), at 37°C and 5% CO2 for eight hours. After centrifugation in 1x PBS the pellet was resuspended in DMEM containing 20% FBS, 100U/ml penicillin, 100ug/ml streptomycin and 2.5ug/ml amphotericin B and kept at 37°C and 5% CO2. The day after, medium was exchanged and remaining tail pieces were removed. Another medium exchange was done three days later. After a week the medium was exchanged for medium without amphotericin B. ADFs were cultured at 37°C and 5% CO2 in DMEM supplemented with 20% FBS and 1% penicillin/streptomycin.

Transient transfections

For *p4xE-box* luciferase reporter transfection studies in NIH3T3 cells, lipofectamine LTX with PLUS reagent (Invitrogen) was used according to the manufacturer's instructions, cultivating cells in 24-well plates and transfecting them with a total of 850ng DNA of which 50ng were the promoter luciferase reporter construct. Varying amounts of plasmid were “balanced” by the addition of pcDNA3.1 to a total of 800ng. Cells were harvested after 60 hours by washing once with 1x PBS and extracting luciferase with a luciferase assay kit (Promega) and normalizing against amount of total protein in each extract (measured by Coomassie staining compared to a bovine serum albumin standard curve). Transfections in primary cells were performed identically, except that twice the amount of cells was used for each reaction.

Lentiviral infections

Measurements were conducted in U2OS cells stably transfected with a circadian *Bmal1-luciferase* reporter, and then infected with Open Biosystems RNAi lentivectors (pGIPZ), as described previously (24).

Measurement of circadian bioluminescence in cultured cells

After transfection or infection as described above, circadian rhythms in cell populations were synchronized with dexamethasone, and then measured for 3-5 days via real-time luminometry in normal culture medium lacking phenol red but supplemented with 0.2mM luciferin and 25mM HEPES, as described previously (e.g. (27)). Data were analyzed using the Lumicyle Analysis program (Actimetrics).

cDNA production and quantitative real-time PCR

RNA was extracted as described in (42). 500ng of total RNA was transcribed to cDNA with SuperScript II (Invitrogen) using random hexamer primers according to manufacturer's instructions. For quantitative real-time PCR 20ng of cDNA was used and single transcript levels of genes were detected by Taqman probes used with the Taqman PCR mix protocol (Roche) using the AB7900 thermocycler. Primers used for detection of NOPS transcripts are: *Nono* sense TGC GCT TCG CCT GTC A, antisense GCA GTT CGT TCG ACA GTA CTG, probe FAM-AGT GCA CCC TTA CAG TCC GCA ACC TT-TAMRA; *Pspc1* sense GAA CTA TAC CTG GCC CAC CAA T, antisense ACT GCG CC ATTA TCT GGT ATC A, probe FAM-ATA TTT GCA GCT CCT TCT GGT CCC ATG –TAMRA; *Sfpq* sense TTT GAA AGA TGC AGT GAA GGT GTT; antisense CCT GCT TCA CCA CCT TCT TGA, probe FAM-TCC TAC TGA CAA CGA CTC CTC GCC CA-TAMRA. Primers for detection of circadian genes and GAPDH can be found in (31).

173

174 **Protein Extraction and western blotting**

175 For *in vitro* immunoprecipitation a 10cm culture dish of HEK 293T cells were co-
176 transfected with each 5ug of NONO-myc, SPFQ-myc or PSCP-myc together with 5ug
177 PER1-FLAG or PER2-FLAG, via polyethyleneimine transfection (JetPEI, Polyplus)
178 following manufacturer's instructions. The cells were harvested 24h later by rinsing with
179 PBS and resuspending in a total of 100ul of lysis buffer as described previously for liver
180 nuclei in (23). Extracts were stored in 500ul aliquots in -80°C until usage. Liver nuclei
181 were prepared by sucrose cushion centrifugation as described in (23), then extracted
182 exactly as for cells. Western blotting was performed using standard procedures
183 (*Current Protocols in Molecular Biology*, Wiley). Equal loading and size detection using
184 protein ladder was verified by Ponceau-S staining of membranes prior to probing.

185 **Immunohistochemistry**

186 Immunohistochemistry was performed according to the protocols described at
187 http://www.pharma.uzh.ch/research/neuromorphology/researchareas/neuromorphology/Protocol
188 s/protocol_immuno.pdf. Substrates were either brains collected in isopentane at -20°C
189 and cryostatically sliced, or cells grown on glass coverslips, rinsed with PBS, and fixed
190 5' at room temperature in PBS/4%paraformaldehyde.

191 **Antibodies**

192 Polyclonal antibodies against NONO, SFPQ, PSPC1, and PER2 were produced from
193 rabbits by Charles River Laboratories using bacterially-overexpressed proteins.
194 Antibody from each serum was immunopurified over a column whose resin consisted of
195 the relevant antigen covalently coupled to Affygel 10 (BioRad). Anti-PSPC1 is

196 described in (12). For detection in Co-IP experiments primary anti-MYC antibody
197 (Roche, Cat N°11667149001) was diluted at 1:2000, primary anti-FLAG antibody
198 (Sigma, F3167) 1:2000, primary anti-NONO antibody at 1:2000, primary anti-PSPC1 at
199 1:1000, primary anti-SPFQ antibody at 1:2000, primary anti-PER2 antibody at 1:1000.
200 The probing of the secondary antibody was done at 1:10´000 for IRDye 680 Goat Anti-
201 Mouse IgG (Licor, 926-32220) and 1:10´000 for IRDye 800 Goat Anti-Rabbit IgG (Licor,
202 926-33210). For immunoprecipitations, primary anti-cMYC antibody was diluted at
203 1:500, primary anti-FLAG antibody at 1:500, primary anti-NONO antibody for IP at
204 1:100, primary anti-SFPQ antibody for IP at 1:100, primary anti-PSCP1 antibody for IP
205 at 1:100 and primary anti-PER2 antibody for IP at 1:100.

206 **Immunoprecipitation**

207 Immunoprecipitation was performed using standard procedures with the below
208 mentioned adjustments (*Current Protocols in Molecular Biology*, Wiley). Extracts were
209 pre-cleared by incubation the crude extracts with protein-A beads (Calbiochem, Cat. N°
210 IP06) and 0.1%BSA for 1h at 4°C. 500ug of pre-cleared extract were bound for 2h to
211 antibody with Co-IP buffer. The Antibody-protein complex was then incubated for 1h
212 with protein-A beads. The beads were washed gently with Co-IP buffer (without
213 protease inhibitor mix) and denatured for 15min at 65°C with 2xSDS sample buffer
214 containing beta-mercaptoethanol. Equal amounts of IP reactions were loaded on a 7%
215 (overexpression in cells IP) or 9% (liver nuclei extracts IP) SDS PAGE gel together with
216 1/10 of the IP amounts of pre-cleared extract as input. The protein gel and blotting was
217 performed as described in the *Western blotting and immunohistochemistry* section
218 above.

219

220 **Chromatin Immunoprecipitation**

221 Chromatin from mouse liver and tissue culture cells was obtained as described
222 previously (34). Equal amounts of precleared chromatin were incubated overnight at 4°C
223 with 1 ul of anti-NONO antibody or anti-PER2 antibody. The capture of the DNA:protein
224 complexes, the washing conditions and the purifiction of the DNA fragments prior to
225 qPCR as well the control antibodies have been described previously (36). The region-
226 specific primer/probe pairs are listed in Supplementary Methods.

227 **Paraspeckle Quantification**

228 For paraspeckle detection, after immunodetection of PSPC1 as described above, cells
229 were analyzed with a LSM710 Zeiss confocal microscope. Pictures taken were with 40x
230 (NA1.3), and the pinhole was kept at 1AU or 0.8 to 0.9um. Nuclei were manually
231 detected using ImageJ software routines (http://rsbweb.nih.gov/ij/index.html). Speckles
232 were determined by subtracting background nucleoplasmic PSPC1 protein staining, and
233 thereafter counting remaining pixel clusters in nuclei. The total amount of paraspeckles
234 per cell was estimated by counting all pixels brighter than 140 (arbitrary units) with spot
235 sizes between 0.25-10 squaremicrometers. Nuclei smaller than 200 pixels or 100
236 suqaremicrometers as well as dividing cells were excluded. The averaged number of
237 speckles was normalized to mean area and compared to the control transfected cells
238 (hairpin NEAT-S).

239

240

241

RESULTS

NONO-deficient mice show significant changes in circadian period

To better understand the function of NONO in the circadian clock and in mammalian physiology, we obtained NONO-deficient mice from ES cells bearing a genetrap in the intron preceding the *Nono* translational start site (Fig S1A, B). In wildtype mice, NONO is expressed in most tissues including the suprachiasmatic nuclei in the brain. *Nono*^{gt} mice showed no expression of *Nono* mRNA or protein in all tissues examined (Fig 1A, B). These mice showed a twenty-minute reduction in circadian behavioral period when placed into constant dark conditions (Fig 1C). This reduction in period length was highly significant, but it was nevertheless far less dramatic compared to a *Drosophila* hypomorphic *nonA* strain that we observed previously to become arrhythmic (4). Hence, we suspected that in mammals the lack of *Nono* may be compensated by other factors.

NONO binds to the circadian promoter of the Rev-erbα gene

Next, we verified the relevance of NONO *in vivo* by looking for its presence at the promoters of clock genes. Since we showed previously that NONO interacted with PER proteins (4), we guessed that it ought to be found at PER-regulated clock genes. Chromatin immunoprecipitation experiments confirmed that this was indeed the case: NONO interacted with the promoter of the *Rev-Erb*α gene in circadian fashion, sharing the same kinetics as the PER1 protein (Fig 1D, top). This interaction was considerably reduced but surprisingly not absent in *Nono*^{gt} mice, which completely lack NONO transcript and protein (Fig 1D, bottom). Equivalent results were seen for the *Dbp*

promoter (Fig 2A), and no binding was observed at the promoter of the antiphasic *Bmal1* gene (Fig 2B). Based upon the residual binding observed at the *Rev-Erbα* and *Dbp* promoters, we considered the possibility that NONO might be redundant with homologous DBHS-family factors with which our antibody might weakly cross-react. Conserved domain analysis (with the Conserved Domain Architecture Tool CDART, (14)) showed that the other two known DBHS proteins, PSPC1 and SFPQ, shared both high homology with NONO and a similar domain architecture (Fig S1C, D). In addition, SFPQ was recently shown to play a role in circadian transcriptional repression (10). Therefore, we speculated that all three proteins might have similar functions in the circadian oscillator.

Overexpression or silencing of DBHS proteins interferes with circadian function

To test this idea, we transfected vectors expressing each of the three proteins into cultured cells together with a luciferase reporter under control of the circadian *Rev-Erbα* gene promoter. After synchronizing circadian clocks in these transfected cells with dexamethasone (2), we monitored reporter bioluminescence in real time. Overexpression of any of the three proteins in human U2OS fibroblasts perturbed circadian rhythmicity (Fig 3A).

We next undertook loss-of-function experiments based upon RNA interference (RNAi), in which U2OS human osteosarcoma cells containing an integrated *Bmal1-luciferase* reporter were infected with lentiviruses expressing shRNAs targeting *Pspc1* or *Sfpq.* RNAi hairpins against SFPQ dampened circadian oscillations dramatically (Fig 3B)

similar to what was observed previously for NONO (4), but those against PSPC1 lengthened it and somewhat dampened amplitude (Fig 3C). Measurement of *Sfpq* and *Pspc1* RNA levels in these cells showed that these hairpins reduced expression of *Sfpq* 7-fold, and *Pspc1* 2.5-fold (Fig 3D,E).

To insure that the effects that we observed were not cell type-specific, identical experiments were conducted using NIH-3T3 mouse fibroblasts. Again, overexpression of any of the three proteins perturbed circadian rhythmicity (Fig 4A). Suppression of circadian rhythmicity was also seen in NIH3T3 cells transiently transfected with the circadian *Rev-Erbα* promoter reporter together with RNAi hairpins targeting *Pspc1* or *Sfpq* (Fig 4B,C). In this case, immunofluorescence experiments showed that these hairpins reduced expression of SFPQ 2-fold, and PSPC1 10-fold (Fig 4D).

Depletion of paraspeckles does not perturb overall circadian clock function

Since the three NONO-related proteins are also the three known members of nuclear paraspeckles, we speculated that the paraspeckle itself might serve a circadian role. This subnuclear domain requires the nuclear noncoding RNA *Neat1*, probably as a scaffold, and depletion of *Neat1* has been shown to eliminate paraspeckles themselves (5, 6). By transiently transfecting shRNAs complementary to *Neat1* into U2OS cells, we were able to reduce *Neat1* levels (Fig 4E) and thereby deplete paraspeckles, measured by counting the number of punctate PSPC1 foci (Fig 5A,B). However, cotransfection of the circadian *Bmal1-luc* reporter showed that the circadian clock retained normal period length in these paraspeckle-depleted cells (Fig 5C), making it unlikely that paraspeckles

311 *per se* play a significant role in the circadian oscillator. Therefore, it is probable that
312 nucleoplasmic, non-paraspeckle-associated pools of NONO, SFPQ, and PSPC1
313 proteins were responsible for the circadian effects that we have documented.
314
315 *DBHS proteins bind to clock promoter DNA and repress clock gene transcription*
316 Since NONO can bind to circadian clock gene promotors *in vivo* (Fig 1, 2), it was logical
317 to imagine that the other DBHS factors might do the same. Indeed, similarly to NONO,
318 SFPQ and PSPC1 could also be immunoprecipitated at the *Rev-Erb*α promoter in a
319 circadian fashion in liver nuclear extracts (Fig 6A).
320 All three proteins are present at clock gene promoters with the same temporal profile as
321 PER proteins, suggesting a co-repressor function. Moreover, both NONO and SFPQ
322 were identified as PER-interacting proteins, and show interactions with PER1 and PER2
323 in various contexts ((4), (10), and Fig 6B-D). However, actual functions of these
324 proteins are less clear. For example, we have shown previously that NONO can
325 *antagonize* PER-mediated transcriptional repression when transfected into immortalized
326 cells (4). In fact, the same is seen with SFPQ and to a lesser extent with PSPC1 (Fig
327 S2A-B), whereas overexpression of NEAT does not influence BMAL1/CLOCK mediated
328 transcriptional activation (Fig S2C). In other reports, NONO and SFPQ have been
329 reported by different investigators as either transcriptional coactivators or corepressors
330 (16, 26), and SFPQ has been shown to act as a transcriptional repressor in the
331 circadian clock (10).
332

Within the circadian clock, we favor a repressive role of these factors because of the following experiments. First, when we transfected primary mouse fibroblasts with an E-box-driven luciferase reporter, together with the transcriptional activators CLOCK and BMAL1 and either NONO, PSPC1, or SFPQ, both NONO and SFPQ repressed CLOCK-BMAL-mediated transcription from the reporter, and to a lesser extent PSPC1, which was initially activating and then repressing at higher concentrations (Fig 7A). When equivalent transfections were performed using fibroblasts from *Per1*$^{brdm/brdm}$ / *Per2*$^{brdm/brdm}$ mice that lack functional PER proteins and circadian clocks (44), repression was no longer observed, but instead weak activation (Fig 7A). Similarly, in *Per1*$^{brdm/brdm}$ / *Per2*$^{brdm/brdm}$ mice no circadian immunoprecipitation of NONO was observed at the *Rev-Erb*α promoter (Fig 7B). Therefore, PER recruits NONO and presumably the other family members too.

Secondly, to confirm that DBHS factors are repressors at circadian promoters, we designed a hybrid GAL4 DNA-binding domain-E-box-luciferase reporter and fusions of NONO, PSPC1, and SFPQ with the GAL4 DNA-binding domain in order to enable their direct recruitment to DNA independently of PER proteins. When the GAL4DBD-Ebox-luciferase construct was transfected into primary mouse fibroblasts together with the GAL4-VP16 transcriptional activator, strong activation was observed, demonstrating the functionality of the construct. When GAL4-NONO, -PSPC1, and -SFPQ were cotransfected into primary mouse fibroblasts together with the GAL4 DNA-binding domain-E-box-luciferase reporter, all three proteins strongly repressed CLOCK-BMAL-

355 mediated transcription (Fig 7C), though they had no statistically significant effect when
356 similarly recruited to the constitutively active CMV promoter (Fig 7D).
357
358 *Importance of DBHS proteins to circadian behavior*
359 Finally, in order to verify the relevance of these factors to the circadian clock *in vivo*, we
360 obtained mice with genetrap-based inactivations of *Pspc1* and *Sfpq*, to match the
361 *Nono*^{gt} mouse described earlier in this paper. Homozygous *Pspc1*-genetrapped mice
362 showed fivefold reduction in *Pspc1* transcript levels in multiple tissues (Fig 8A), and no
363 detectable levels of PSPC1 protein in liver nuclear extracts (Fig 8C). Although the *Sfpq*
364 genetrap was homozygous lethal, heterozygous mice showed up to twofold reduction in
365 both RNA and protein (Fig 8B, D). When tested for circadian wheel-running behavior,
366 these *Sfpq*$^{gt/+}$ mice also showed a trend toward shortening of period similar to that of
367 *Nono*^{gt} in some animals (Fig 9A, B), as well as altered entrainment in a minimal-light
368 "skeleton" photoperiod in all animals (Fig S3A-C). *Pspc1*$^{gt/gt}$ mice showed no
369 abnormalities (Fig 9A-B, Fig S3).
370
371 Consistent with the proposed repressive role of these factors, at the gene expression
372 level, *Rev-Erb*α RNA showed modestly increased expression in liver extracts from all
373 three knockouts at the time (CT8-12) that coincides with binding of NONO and PER2
374 (Fig 9C). Interestingly, its timing coincides with the peak of *Rev-Erb*α expression levels
375 and the beginning of their decline, but not with maximum repression. Hence, it is
376 possible that these factors are associated with the establishment of repression but not
377 its maintenance. Similar but smaller gene expression effects were seen upon *Per2*

transcript levels, but the expression of other clock genes remained mostly unchanged (Fig S4).

DISCUSSION

Because of their homologies, shared functions, and abilities to interact with one another, the three factors NONO, PSPC1, and SFPQ have recently been classified by multiple authors as a family of proteins: the NOPS family (for NOno and PSpc1, (40)) or DBHS family (for Drosophila Behavior Human Splicing, (3)). Our data and that of others point to another important role of these proteins within the circadian oscillator. We initially isolated NONO as a PER-interacting protein (4), and Duong *et al.* recently isolated SFPQ in the same way (10). Here we present data that all three DBHS proteins likely play overlapping roles within the circadian clock.

Nuclear paraspeckles and the circadian clock

All three DBHS proteins are part of nuclear paraspeckles (13), subnuclear bodies probably involved in splicing and RNA storage. Nevertheless, our data suggests that the paraspeckle *per se* is not important for circadian function: depletion of these nuclear bodies by targeting the structural ncRNA *Neat1* (6) has no effect upon the circadian clock, nor does transfection of this ncRNA into cells alter E-box-mediated transcription. Instead, our results imply that DBHS proteins likely exists in at least two nuclear pools. One of these pools is present in paraspeckles, and appears to play no role so far in the circadian clock, though it may be important for nuclear retention of

edited RNAs as reported by others (5, 30, 43). A second pool is nucleoplasmic, and could be in part responsible for the transcriptional roles reported for DBHS proteins.

DBHS proteins as transcription factors

Besides their roles in nuclear paraspeckles, the previously reported functions of DBHS proteins have ranged widely. They have been implicated in splicing (17, 29) and axonal transport of RNA (18). They are players in the regulation of pre-mRNA processing and transcription termination (19), and in the DNA damage response (32, 35). NONO has also been characterized as a nonclassical carbonic anhydrase (20). In addition, however, all have been implicated in transcription. In some cases they have been implicated as activators (1, 16, 21), and in other cases as repressors (8, 26, 39), even for the same gene (37). Interestingly, a mechanism has been proposed in both cases: whereas NONO and SFPQ can interact directly with the RNA polymerase II CTD in a way that might explain transcriptional activation (11), SFPQ has been proposed to recruit the mSIN3A histone deacetylase to promote repression (10, 26). For the circadian clock we originally identified NONO as an antagonist of PER-mediated repression (4), and others demonstrated PER-mediated repression by SFPQ (10). Apparently the roles of DBHS protein depend on the cellular context. In this paper alone, we show that transient transfection of SFPQ into U2OS cells can activate transcription from E box reporters, but its transfection into mouse primary fibroblasts represses it. To try to resolve the role of these factors within the circadian clock, we therefore created GAL4 fusion proteins to unambiguously recruit these factors to promoters. All three had no effect on the CMV promoter, but strongly repressed

transcription mediated by the circadian transcription factors CLOCK and BMAL1 at a circadian E-box promoter. With this experiment, we show a) that the effects of these factors upon transcription are context-specific, and b) that they are likely to be repressors in the circadian context.

Overlapping functions of DBHS proteins in the circadian clock

One possible explanation for our results and those of others suggesting activation or repression is overlapping function of related genes: if two repressors have different repressive potentials, for example, then titrating increasing exogenous amounts of the weaker results in an increase in transcription as the endogenous stronger one is displaced. In this paper, we present considerable evidence for overlapping functions of the three DBHS proteins within the circadian clock. Depletion or overexpression of all three unambiguously affects circadian function in cells and in cellular transcription assays, but depletion of any one in mice results in only small circadian phenotypes, and generation of double DBHS mutants -- or even complete knockouts of *Sfpq* -- is complicated by embryonic lethality. Moreover, we and others have shown by chromatin immunoprecipitation that all three DBHS proteins can bind directly to clock promoters or clock-controlled promoters in circadian fashion *in vivo* and in cells (10, 15, 26). At least for circadian function, it is likely that this binding requires PER proteins. SFPQ and NONO were identified as PER-interacting proteins, and clearly immunoprecipitate with them as shown here and elsewhere (4, 10). Moreover, we show here that in PER-deficient mice, binding of NONO to circadian promoters is no longer observed. Thus, we think it is likely that PER proteins recruit DBHS proteins to clock-controlled genes to

control and orchestrate PER-mediated transcriptional repression. The degree of this repression could be precisely controlled by the mix of these factors recruited. Functional redundany of this family of proteins is also highlighted by the recently published crystal structure of a NONO-PSPC1 complex (28). Not only do these proteins probably form obligate heterodimers, which would suggest a role for multiple DBHS family members within the circadian clock, but their structure also allows for possible higher-order oligomers, which might provide an ideal platform for the recruitment of other factors that have been found associated with these factors in various contexts.

DBHS proteins as orchestrators of circadian physiology

Although we have shown clear roles of DBHS proteins in a cellular context, the circadian behavioral phenotypes of DBHS prtein-deficient mice were relatively minor. As discussed above, functional redundancy could account for this lack of phenotype. In addition, however, the uniquely coupling of SCN cells into a network renders them more resilient to the effects of mutation (22). Therefore, it is also possible that more severe circadian effects of DBHS proteins might occur in peripheral tissues.

Indeed, it is likely that considerable further circadian physiology directed by DBHS proteins remains to be elucidated. Mice deficient in these factors show a spectrum of unique phenotypes, ranging from embryonic lethality (*Sfpq)* to neurological phenotypes (*Nono). Pspc1* protein is strongly regulated in circadian fashion although the other two factors are not (Fig 8 and data not shown). Moreover, the E-box is a standard motif for orchestrating clock-controlled physiology (34), and directs circadian transcription at

thousands of promoters (41). DBHS factor binding has been observed at multiple clock-regulated promoters containing this motif, including prolactin (15), progesterone (9), *Rev-Erbα* (Fig 1B), and androgen receptor (8). Through their interaction with PER proteins, we show here that DBHS factors play an important role directly in the circadian oscillator. Binding to clock gene promoters and modulating transcriptional repression, they regulate a portion of the transcriptional feedback which is the hallmark of metazoan circadian clocks.

ACKNOWLEDGEMENTS

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625

626 **FIGURE LEGENDS**

627

628 **Fig 1. (A)** NONO RNA expression measured by qPCR in various tissues taken from
629 wildtype (black) and *Nono*^{gt} animals (grey, not detectable). Inset, NONO protein
630 measured in liver nuclear extract from the same animals, as well as in unrelated C57-
631 Bl6J mice (Bl6/J). **(B)** NONO protein expression in brain coronal sections from wildtype
632 and *Nono*^{gt} animals, visualized by immunohistochemistry using a polyclonal anti-NONO
633 antibody. Arrows from left to right show principal areas of NONO expression in wildtype
634 mouse brain: suprachiasmatic nuclei, hippocampus, and neocortex. **(C)** Wheel-running
635 activity of wildtype and *Nono*^{gt} mice in 12:12 light-dark cycles (LD, arrow) and in
636 constant darkness (DD, arrow). Darkness is indicated by grey shading. N=23. **(D)**
637 Chromatin immunoprecipitation of NONO (black bars) and PER1 (striped bars) at the
638 *Rev-Erb*α promoter in liver nuclei harvested at different circadian times (CT) of day in
639 constant darkness. CT0 = beginning of subjective day. Top panel, wildtype mice.
640 Bottom panel, *Nono*^{gt} mice. (N=3 experiments, shown +/- standard deviation, **
641 corresponds to a p-Value of ≤0.01).

642

643 **Fig 2. (A)** Chromatin immunoprecipitation of NONO (black bars) and PER2 (striped
644 bars) at the *Dbp* promoter in liver nuclei harvested from wildtype mice (top panel) and
645 *Nono*^{gt} mice (bottom panel) at different times of day in constant darkness. N=3
646 experiments, +/-SD. **(B)** Identical experiments for the *Bmal1* promoter.

647

Fig 3. (A) Bioluminescence from U2OS cells transiently transfected with the *Rev-Erbα-luciferase* circadian reporter and constructs expressing either NONO, SFPQ, or PSPC1. Data shown is detrended and expressed in arbitrary units relative to mean expression. Solid black line, cells transfected with the empty vector. Dashed line, cells overexpressing NONO. Dashed and dotted line, cells overexpressing PSPC1. Dotted line, cells overexpressing SFPQ. **(B)** Bioluminescence from U2OS cells containing an integrated *Bmal1-luciferase* circadian reporter, infected with viruses expressing two different RNAi hairpins targeting the *Sfpq* gene, and then clock-synchronized with dexamethasone. Data shown is detrended and expressed in arbitrary units relative to mean expression. Solid black line, scrambled-sequence shRNA. Grey line, shRNA3. Dashed line, shRNA5. **(C)** Similar experiment with RNAi constructs targeting *Pspc1.* Dashed line, shRNA3. Grey line, shRNA4. **(D,E)** Transcript levels of *Pspc1* (D) and *Sfpq* (E) in U2OS cells infected with lentiviruses expressing the indicated RNAi targeting vectors used in Figure 2. N=3 +/- SE.

Fig 4. (A) Bioluminescence from 3T3 cells transiently transfected with the *Rev-Erbα-luciferase* circadian reporter and constructs expressing either NONO, SFPQ, or PSPC1. Data shown is detrended and expressed in arbitrary units relative to mean expression. Solid black line, wildtype cells. Dashed line, cells overexpressing NONO. Dashed and dotted line, cells overexpressing PSPC1. Dotted line, cells overexpressing SFPQ. **(B,C)** Bioluminescence from 3T3 cells transiently transfected with the *Rev-Erbα-luciferase* circadian reporter and RNAi constructs targeting either *Pspc1* (B) or *Sfpq* (C). After synchronization with dexamethasone, cultures were measured 3 days. Data are shown

671 detrended and expressed in arbitrary units relative to mean expression. Solid black
672 line, wildtype cells. Dashed lines, duplicate plates of cells expressing an *Sfpq*- or
673 *Pspc1*-targeting vector. **(D)** Quantification of depletion of SFPQ and PSPC1 protein
674 from experiments above. Relative repression from 3T3 cells cotransfected with a GFP-
675 expressing plasmid and a plasmid expressing an RNAi interference construct targeting
676 *Sfpq* or *Pspc1*. Averages shown are from 10 cells each, +/- SE. Mean fluorescence is
677 expressed in arbitrary units. **(E)** Quantification (+/- SE; n=2 independent experiments,
678 performed in triplicate) of *Neat1* levels for two different RNAi constructs (R and B), as
679 well as a scrambled hairpin (S) used in Fig 4, quantified from RNA of bulk-transfected
680 cells (black bars), or from cells cotransfected with a GFP-expressing plasmid and then
681 FACS-sorted to isolate GFP-expressing cells (grey bars).
682
683 **Fig 5 (A)** Immunofluorescence from cells transfected with a plasmid expressing GFP
684 and an RNAi interference construct targeting *Neat1 (Neat-R)*. Top panel, red filter
685 (PSPC1 protein). Bottom panel, same cells, green filter (GFP). White arrow,
686 paraspeckle in transfected cell; yellow arrow, paraspeckle in untransfected cell. Size
687 bar, 10μm. **(B)** Quantification (+/- SD) of paraspeckles per cell for two different RNAi
688 constructs (R and B), as well as a scrambled hairpin (S), quantified by immunostaining
689 similarly to (A), N= 12 cells (*Neat1-R)*, 24 (*Neat1- B)*, 18 (*Neat1-S).* Significance from
690 Student t-test, *<0.05, **<0.01. **(C)** Period length of circadian reporter expression for
691 U2oS cells cotransfected with the hairpins described in (A) and the *Bmal-luciferase*
692 circadian reporter. (N=6 per sample, no significant differences as determined by
693 Student t-test).

694

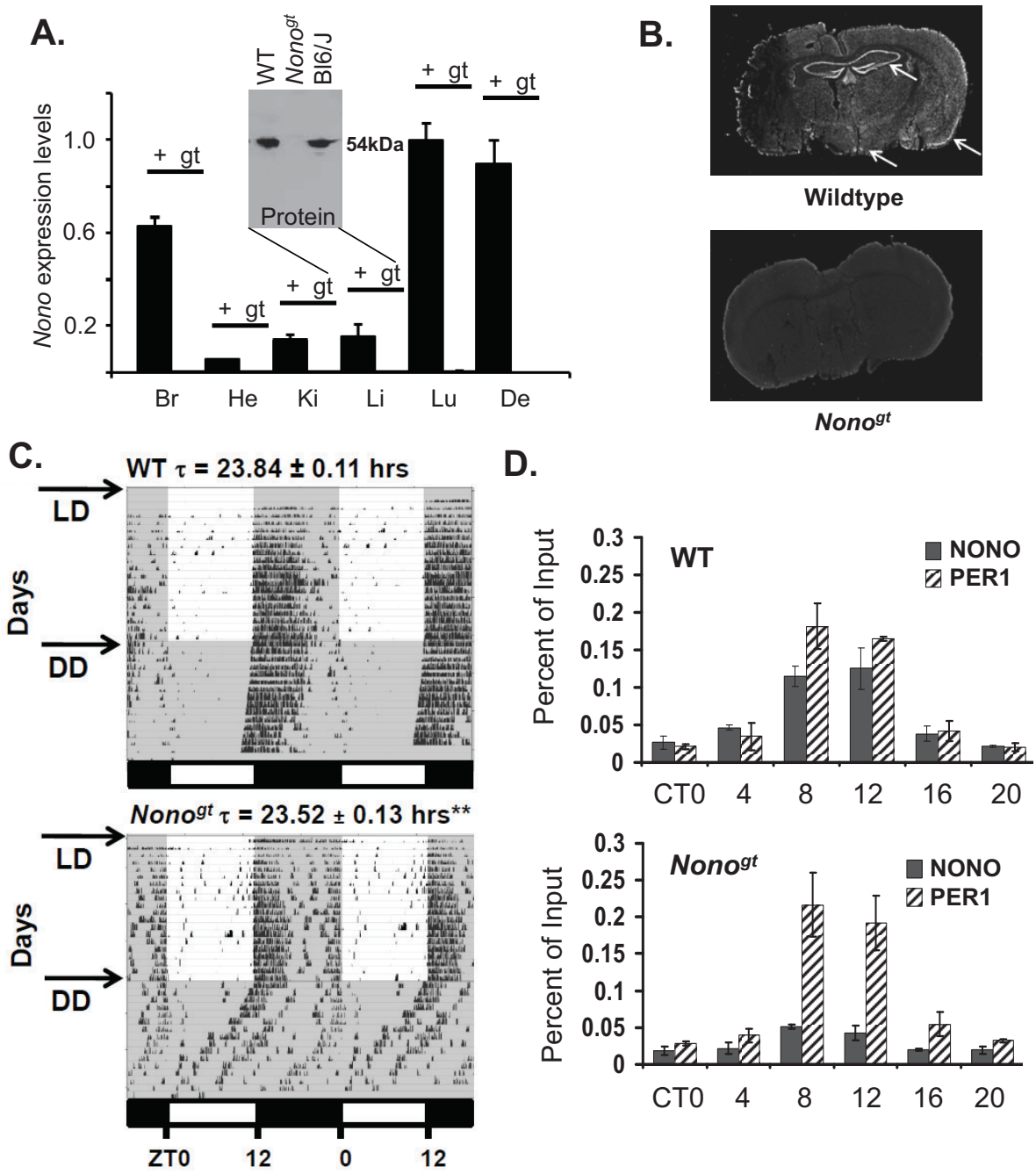
695 **Fig 6. (A)** Chromatin immunoprecipitation of the indicated proteins at the *Rev-*
696 *Erb*α promoter in liver nuclei harvested at different circadian times (CT) of day in
697 constant darkness. N=4, +/- SD, expressed relative to timepoint of minimum binding.
698 Ctrl reactions used an unrelated antibody raised at the same time in the same species
699 (anti-PAR-BZIP). **(B)** Immunoprecipitations from whole-cell extracts from 293T cells
700 cotransfected with myc-tagged NONO and Flag-tagged PER1 (left panel) or PER2 (right
701 panel). For each panel, left lane is 1/10 input, middle is immunoprecipitate with anti-
702 Myc antibody, and right lane is precipitation with IgG. Subsequent to
703 immunoprecipitation, all blots are probed with both anti-myc and anti-FLAG antibodies.
704 **(C)** Identical experiments performed with whole-cell extracts from 293T cells
705 cotransfected with myc-tagged SFPQ and Flag-tagged PER1 (left panel) or PER2 (right
706 panel). **(D)** Mouse liver nuclear extracts from ZT16 were immunoprecipitated with anti-
707 PER2 and probed with anti-NONO or anti-SFPQ. Left lane 1/10 input, right lane IP.

708

709 **Fig 7. (A)** Bioluminescence measured after transient transfection of mouse primary
710 fibroblasts from wildtype (black bars) or *per1*$^{brdm/brdm}$*/per2*$^{brdm/brdm}$ double mutant
711 animals (striped bars) transfected with an *E-box-luciferase* reporter, and vectors
712 expressing CLOCK and BMAL proteins, and NONO, SFPQ, or PSPC1 as indicated.
713 N=3 experiments in duplicate, +/- SE, for all of figure **(B)** Chromatin
714 immunoprecipitation of NONO in wildtype (WT, black bars) or PER-deficient (p1/p2 mut,
715 striped bars) mice at the *Rev-Erb*α promoter in liver nuclei harvested at different
716 circadian times (CT) of day in constant darkness +/- SD . CT0 = beginning of

717 subjective day. N=3 **(C)** Transient transfection of fibroblasts with a *GAL4 DNA-binding*
718 *domain-Ebox-luciferase* reporter and vectors expressing GAL4-NONO, -PSPC1, -
719 SFPQ, or –VP16. Black bars, no exogenous activator added. Striped bars, vectors
720 expressing CLOCK and BMAL1 also added. **(D)** Identical experiments showing no
721 statistically significant effects using a *GAL4-CMV-luciferase* reporter without exogenous
722 activator.
723
724 **Fig 8. (A)** *Pspc1* RNA levels measured by qPCR from different tissues of genetrapped
725 mice (striped bars) and wildtype littermates (black bars). For parts A-B, N=2 mice per
726 measurement, measured 4x in duplicate, +/- SE. **(B)** *Sfpq* RNA levels measured by
727 qPCR from different tissues of genetrapped mice (striped bars) and wildtype littermates
728 (black bars). **(C)** PSPC1 protein levels in liver nuclear extracts harvested at different
729 times of day from wildtype and genetrapped animals kept in darkness. Top panel,
730 western blot probed with anti-PSPC1. Bottom panel, Ponceau-S staining of filter to
731 show equal loading. **(D)** SFPQ protein levels in liver nuclear extracts harvested at
732 different times of day from wildtype and genetrapped animals kept in darkness. Top
733 panel, western blot probed with anti-SFPQ. Bottom panel, Ponceau-S staining of filter
734 to show relative loading.
735
736 **Fig 9. (A)** Left, wheel-running activity of wildtype, *Pspc1*$^{gt/gt}$, and Sfpq$^{gt/+}$ mice in 12:12
737 LD (arrow) and in constant darkness (DD). Darkness is indicated by grey shading. **(B)**
738 Period lengths of twelve mice of each genotype, together with wildtype littermates. No
739 significant differences for either Pspc1 or *Sfpq* using Student t-test. **(C)** *Rev-Erb*α RNA

740 expression from *Nono, Sfpq,* and *Pspc1*-genetrapped mice (striped bars) and wildtype
741 littermates (black bars), measured by quantitative RT-PCR from liver extracts harvested
742 at different circadian times (CT) of day from mice in constant darkness. N=2 mice per
743 time point, RNA measured 4x in technical duplicate. Data shown is +/- SE.
744
745


A.
WT
Nonogt
Bl6/J
54kDa
Protein
Nono expression levels
1.0
0.6
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+ gt
Br
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Lu
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B.
Wildtype
Nonogt
C.
WT τ = 23.84 ± 0.11 hrs
LD
DD
Days
Nonogt τ = 23.52 ± 0.13 hrs**
ZT0
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0
12
D.
WT
NONO
PER1
Percent of Input
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0.2
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CT0
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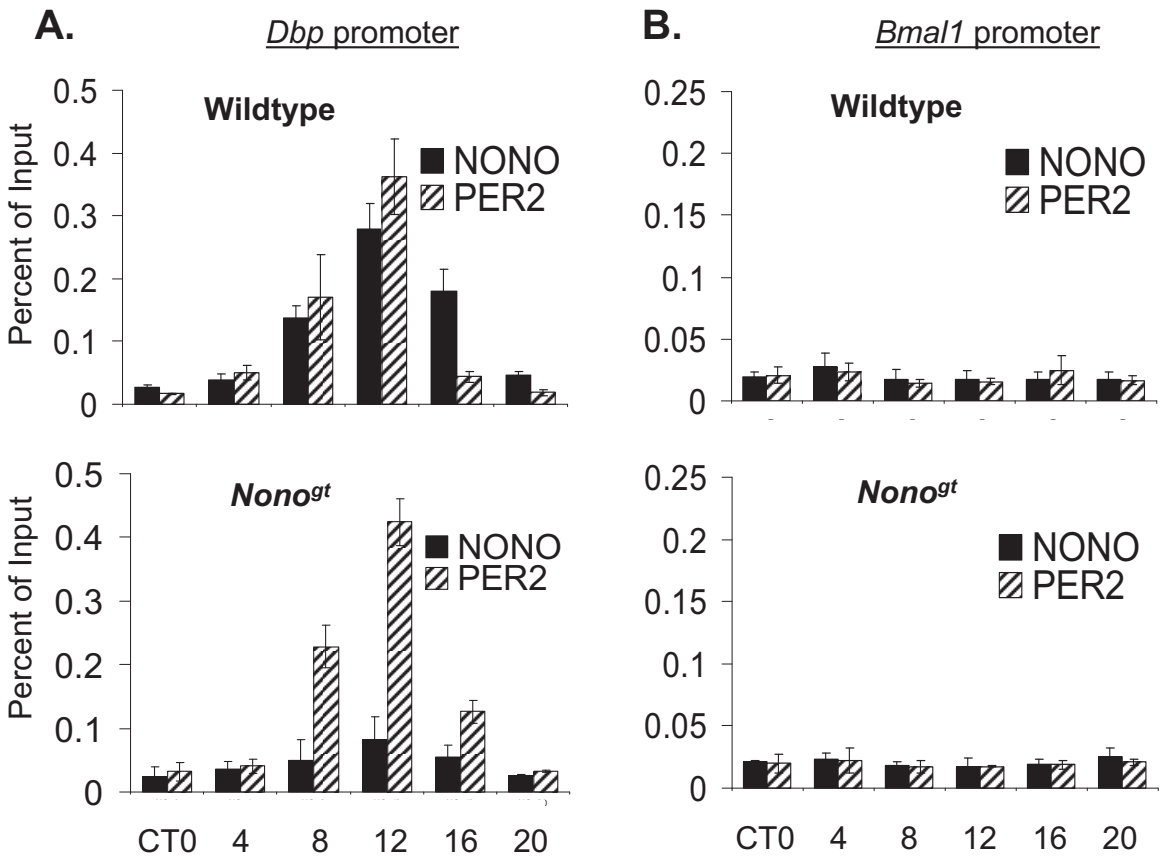
A.
Dbp promoter
B.
Bmal1 promoter
Wildtype
Nonogt
NONO
PER2
Percent of Input
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0.25
0.2
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CT0
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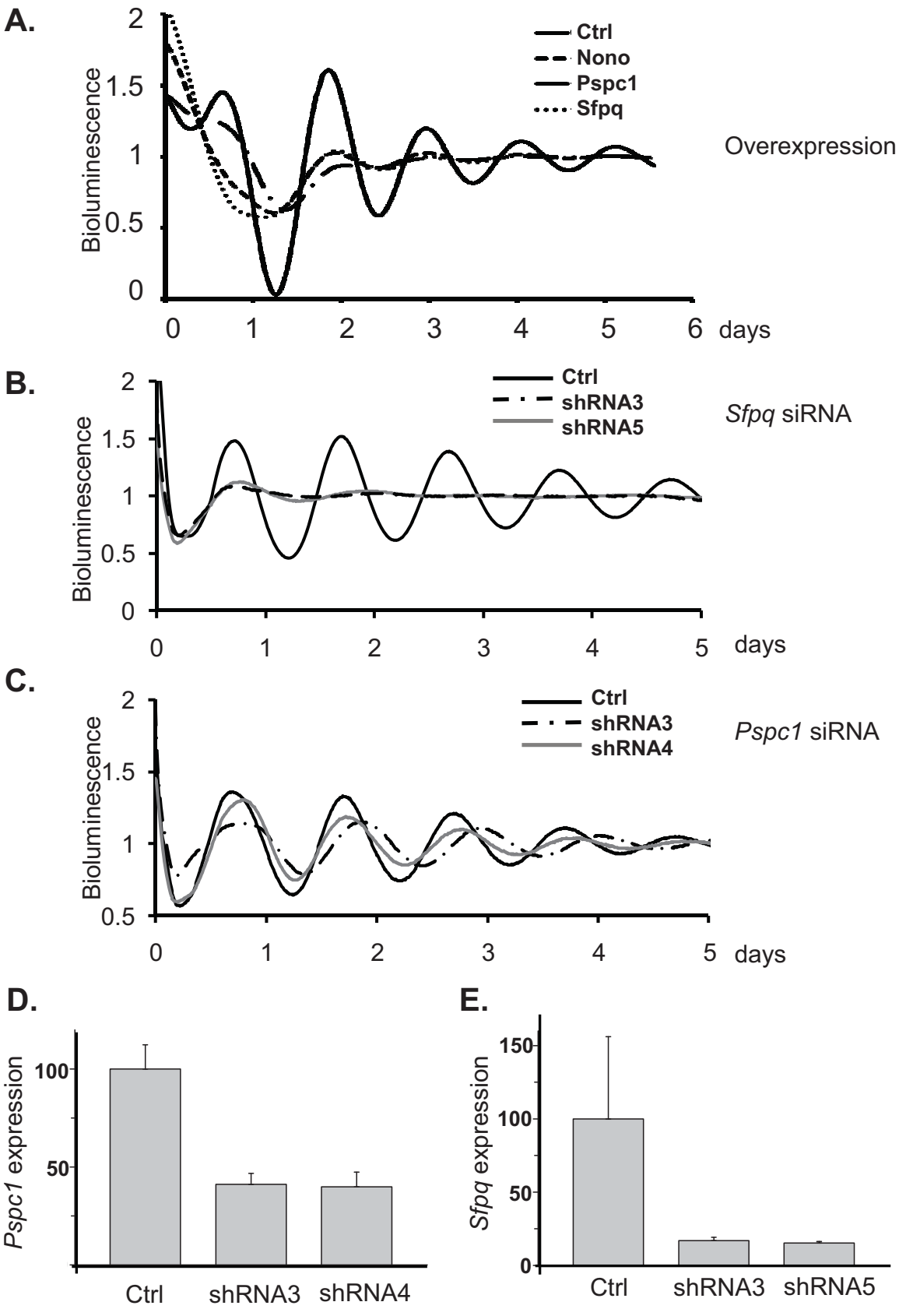
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shRNA3
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Sfpq siRNA
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1.5
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shRNA3
shRNA4
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D.
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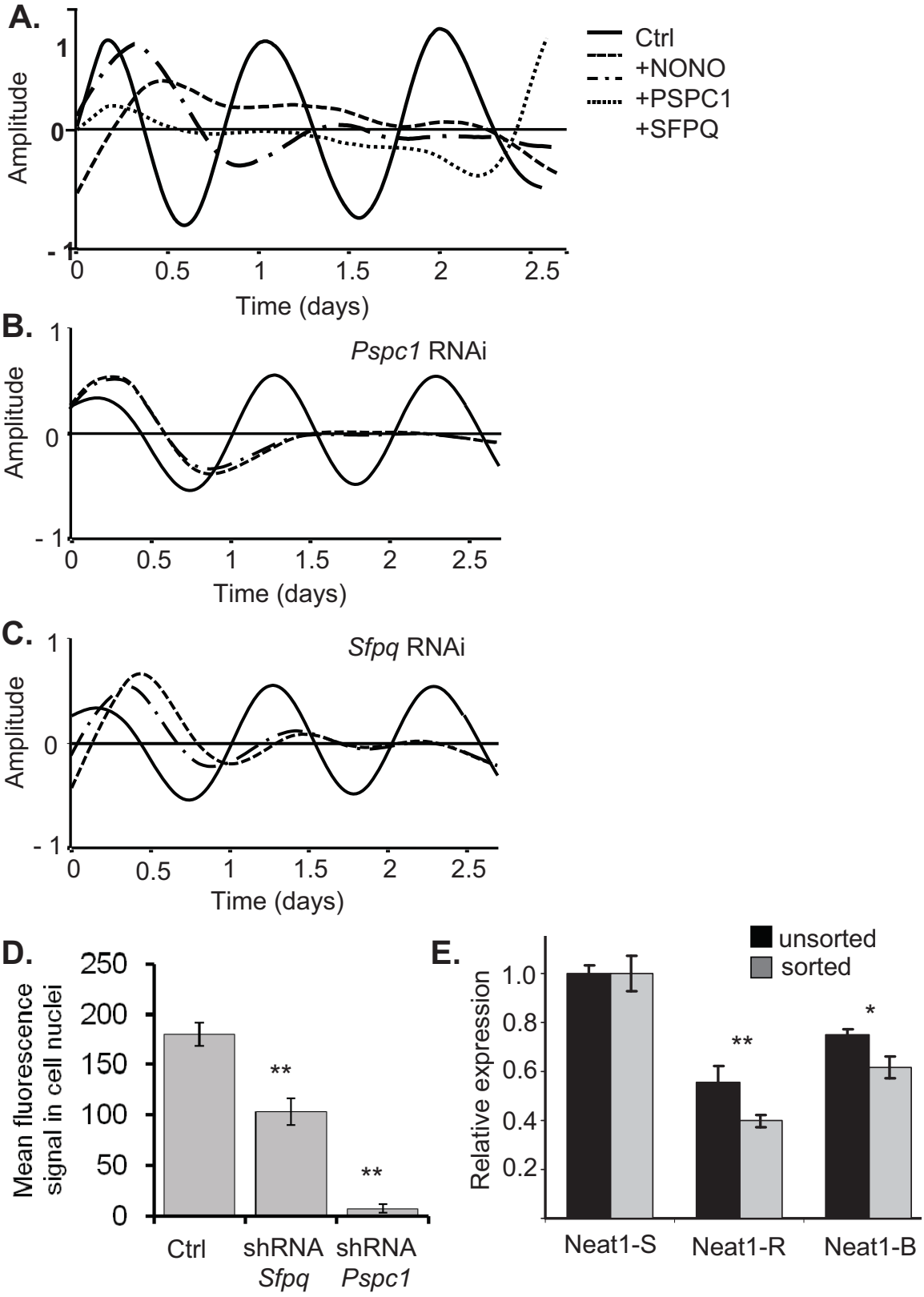
shRNA5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+NONO
+PSPC1
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B.
Pspc1 RNAi
Amplitude
Time (days)
C.
Sfpq RNAi
Amplitude
Time (days)
D.
Mean fluorescence signal in cell nuclei
250
200
150
100
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0
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E.
unsorted
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Relative expression
1.0
0.8
0.6
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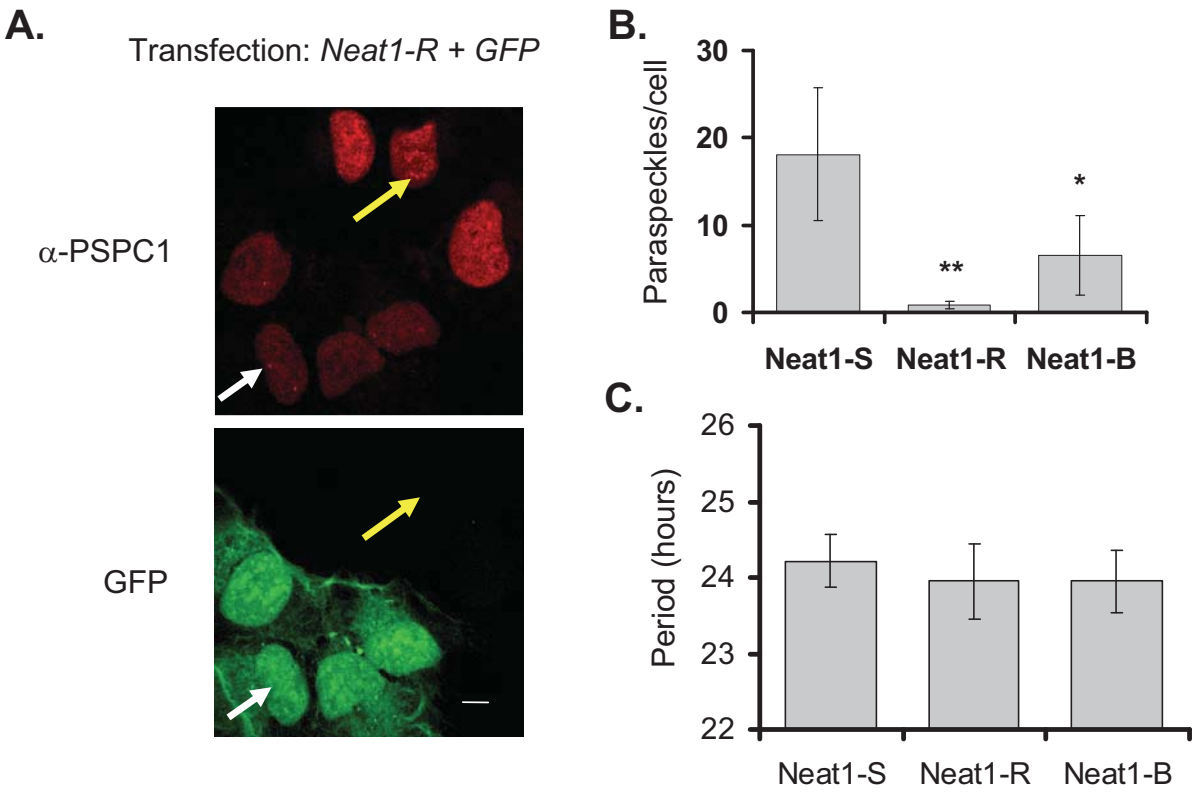
*
Neat1-S
Neat1-R
Neat1-B


A.
Transfection: Neat1-R + GFP
α-PSPC1
GFP
B.
Paraspeckles/cell
30
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**
*
Neat1-S
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Neat1-B
C.
Period (hours)
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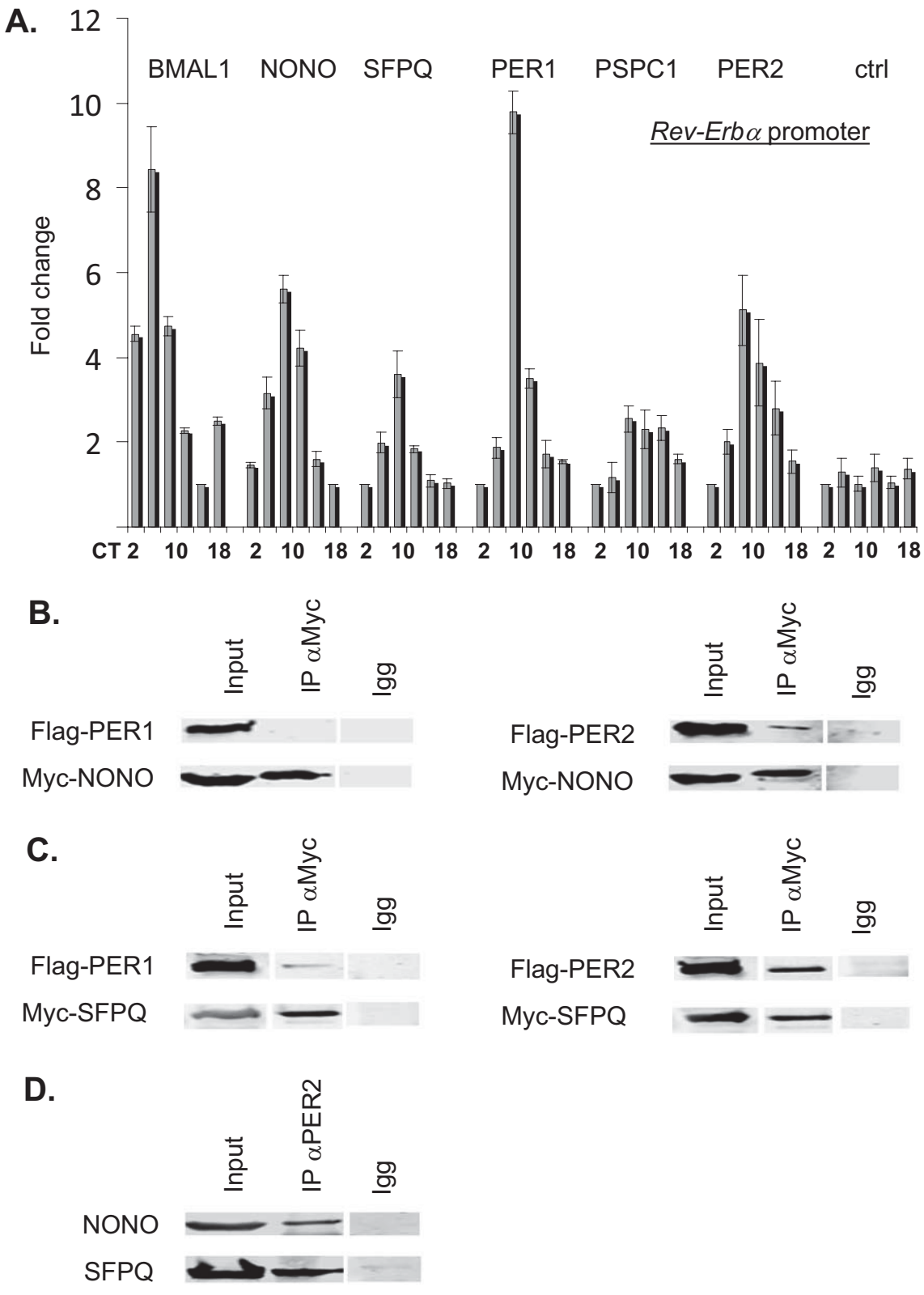
Neat1-B


A.
BMAL1
NONO
SFPQ
PER1
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ctrl
Rev-Erbα promoter
Fold change
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B.
Input
IP αMyc
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Flag-PER1
Myc-NONO
Flag-PER2
Myc-NONO
C.
Input
IP αMyc
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Flag-PER1
Myc-SFPQ
Flag-PER2
Myc-SFPQ
D.
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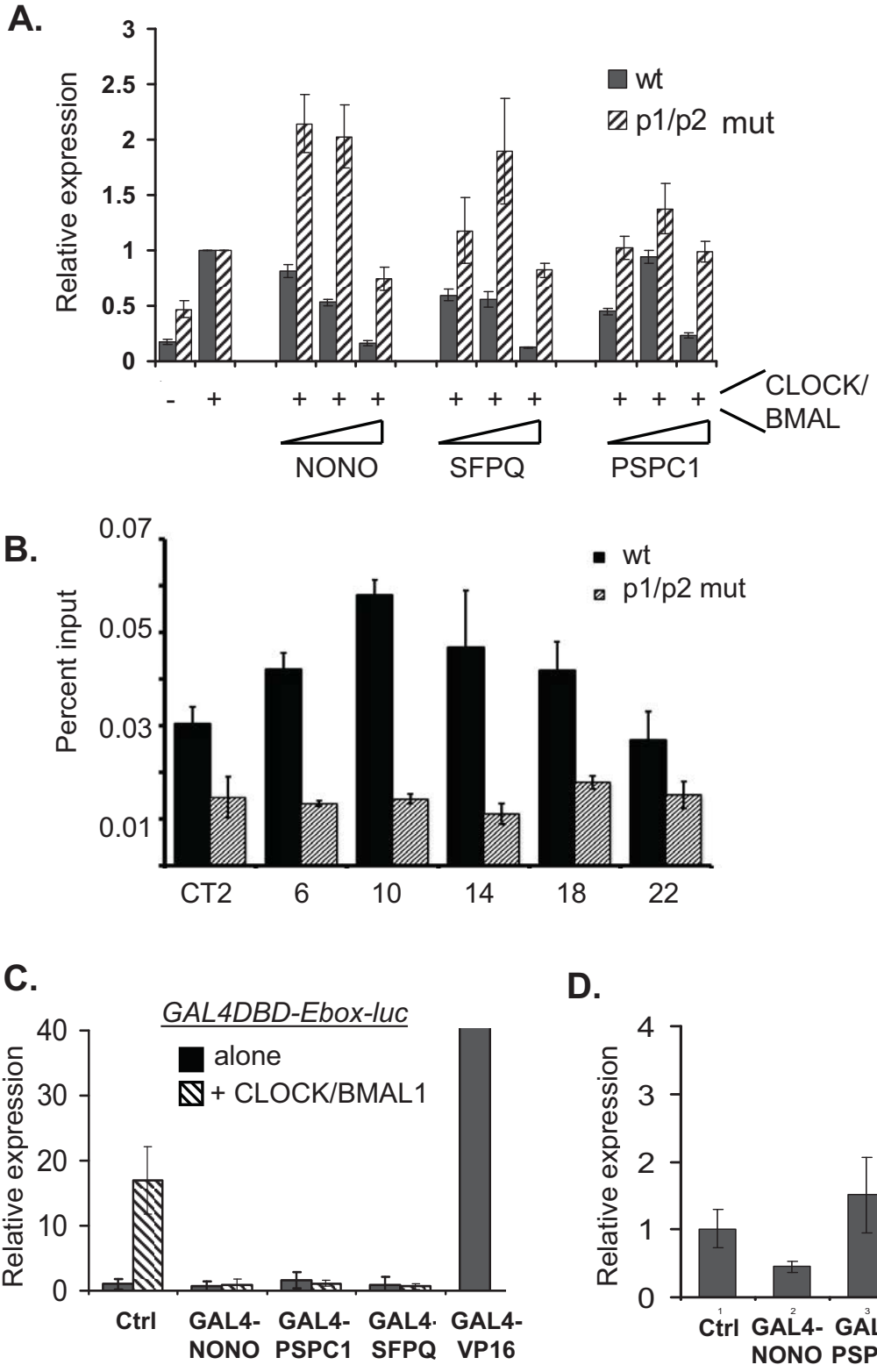
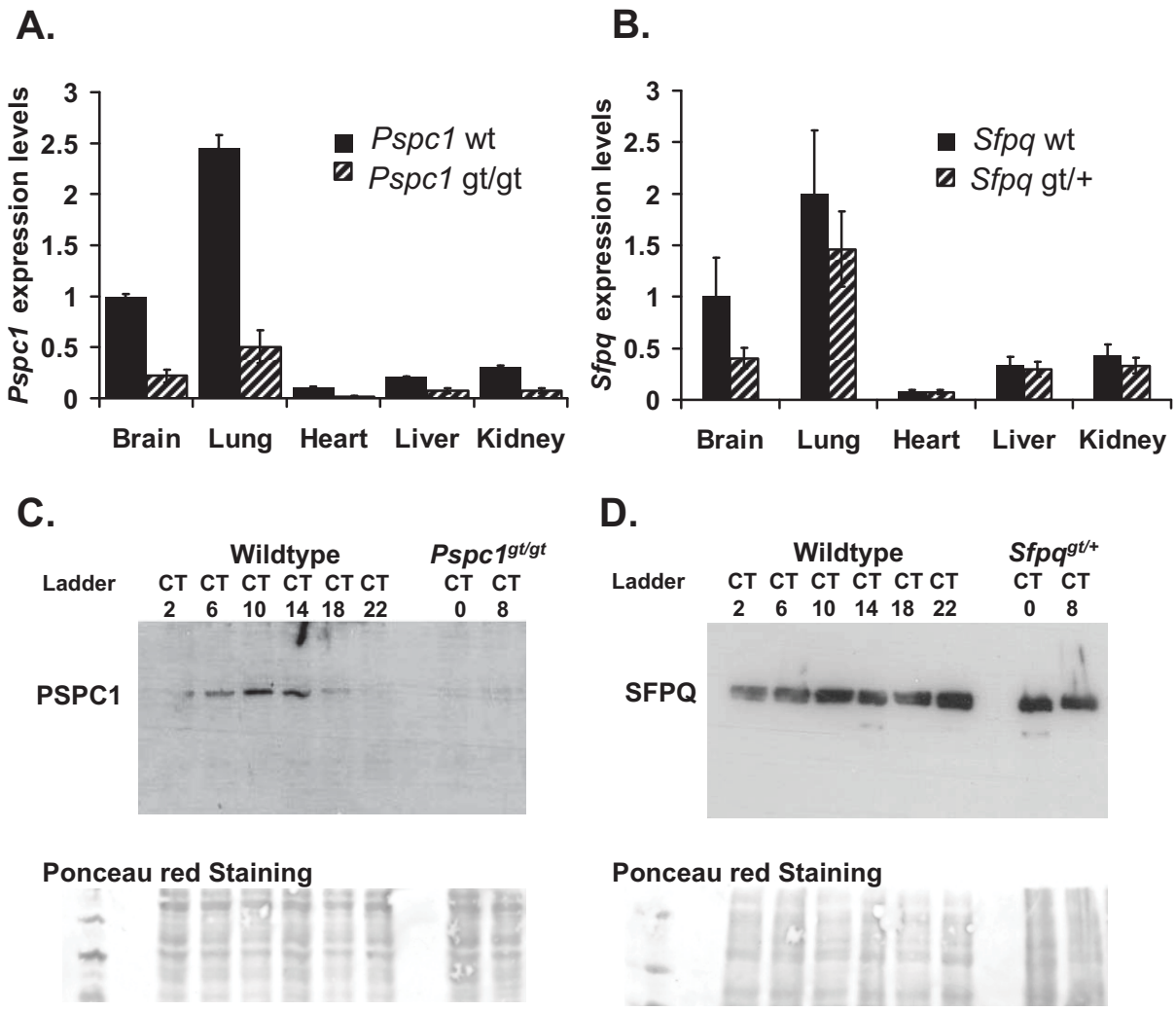
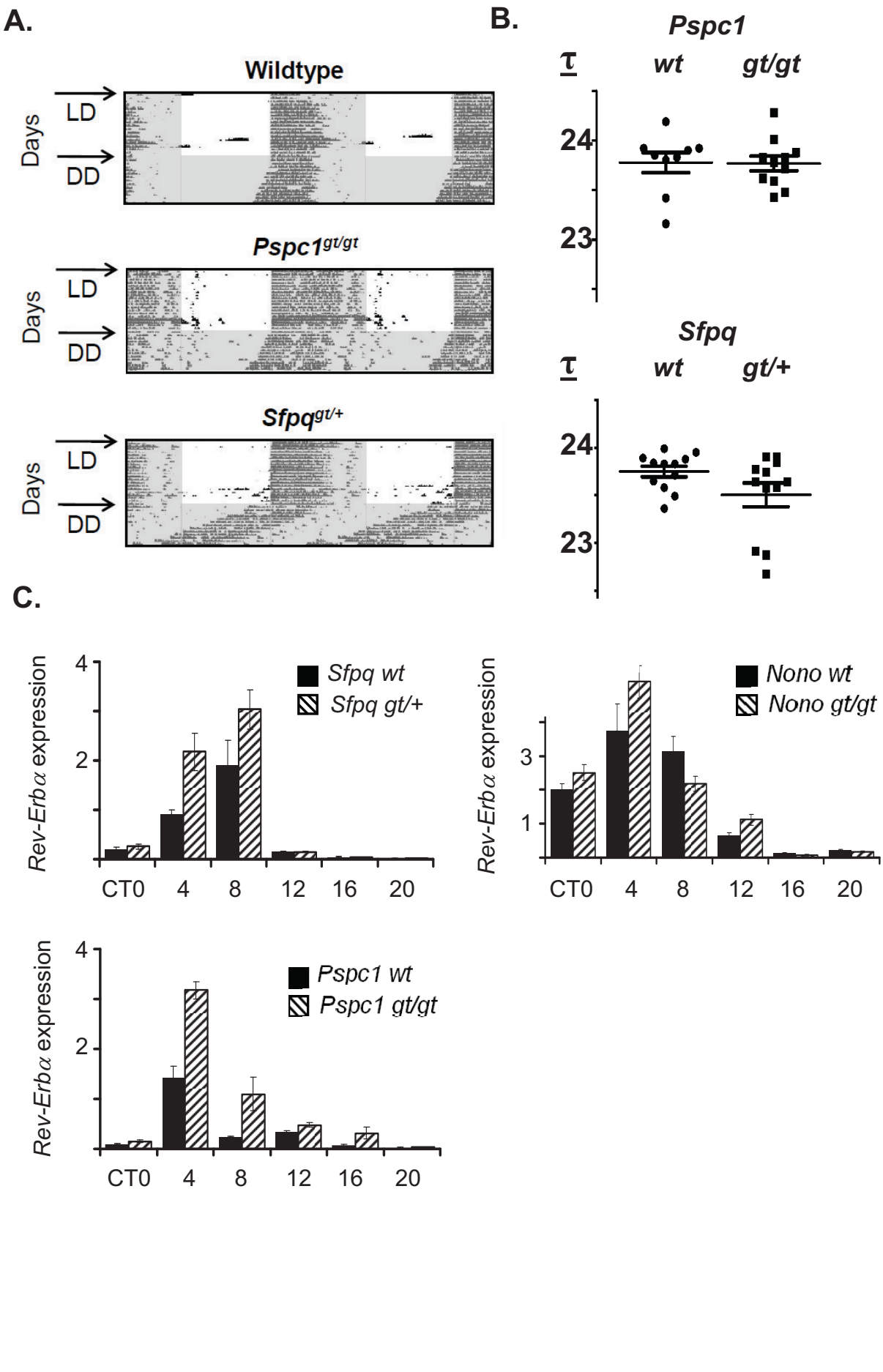
IP αPER2
Igg
NONO
SFPQ


A.
Relative expression
3
2.5
2
1.5
1
0.5
0
wt
p1/p2 mut
- + + + + + + + +
CLOCK/
BMAL
NONO
SFPQ
PSPC1
B.
Percent input
0.07
0.05
0.03
0.01
wt
p1/p2 mut
CT2
6
10
14
18
22
C.
GAL4DBD-Ebox-luc
alone
+ CLOCK/BMAL1
Relative expression
40
30
20
10
0
Ctrl
GAL4-NONO
GAL4-PSPC1
GAL4-SFPQ
GAL4-VP16
D.
Relative expression
4
3
2
1
0
1 Ctrl
2 GAL4-NONO
3 GAL4-PSPC1
4 GAL4-SFPQ


A.
Pspc1 expression levels
3
2.5
2
1.5
1
0.5
0
Pspc1 wt
Pspc1 gt/gt
Brain
Lung
Heart
Liver
Kidney
B.
Sfpq expression levels
3
2.5
2
1.5
1
0.5
0
Sfpq wt
Sfpq gt/+
Brain
Lung
Heart
Liver
Kidney
C.
Wildtype
Pspc1gt/gt
Ladder
CT 2
CT 6
CT 10
CT 14
CT 18
CT 22
CT 0
CT 8
PSPC1
Ponceau red Staining
D.
Wildtype
Sfpqgt/+
Ladder
CT 2
CT 6
CT 10
CT 14
CT 18
CT 22
CT 0
CT 8
SFPQ
Ponceau red Staining


A.
Wildtype
Days
LD
DD
Pspc1gt/gt
Days
LD
DD
Sfpqgt/+
Days
LD
DD
B.
Pspc1
τ
wt
gt/gt
24
23
Sfpq
τ
wt
gt/+
24
23
C.
Rev-Erbα expression
Sfpq wt
Sfpq gt/+
CT0
4
8
12
16
20
Rev-Erbα expression
Nono wt
Nono gt/gt
CT0
4
8
12
16
20
Rev-Erbα expression
Pspc1 wt
Pspc1 gt/gt
CT0
4
8
12
16
20