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The circadian clock regulates rhythmic erythropoietin expression in the murine kidney

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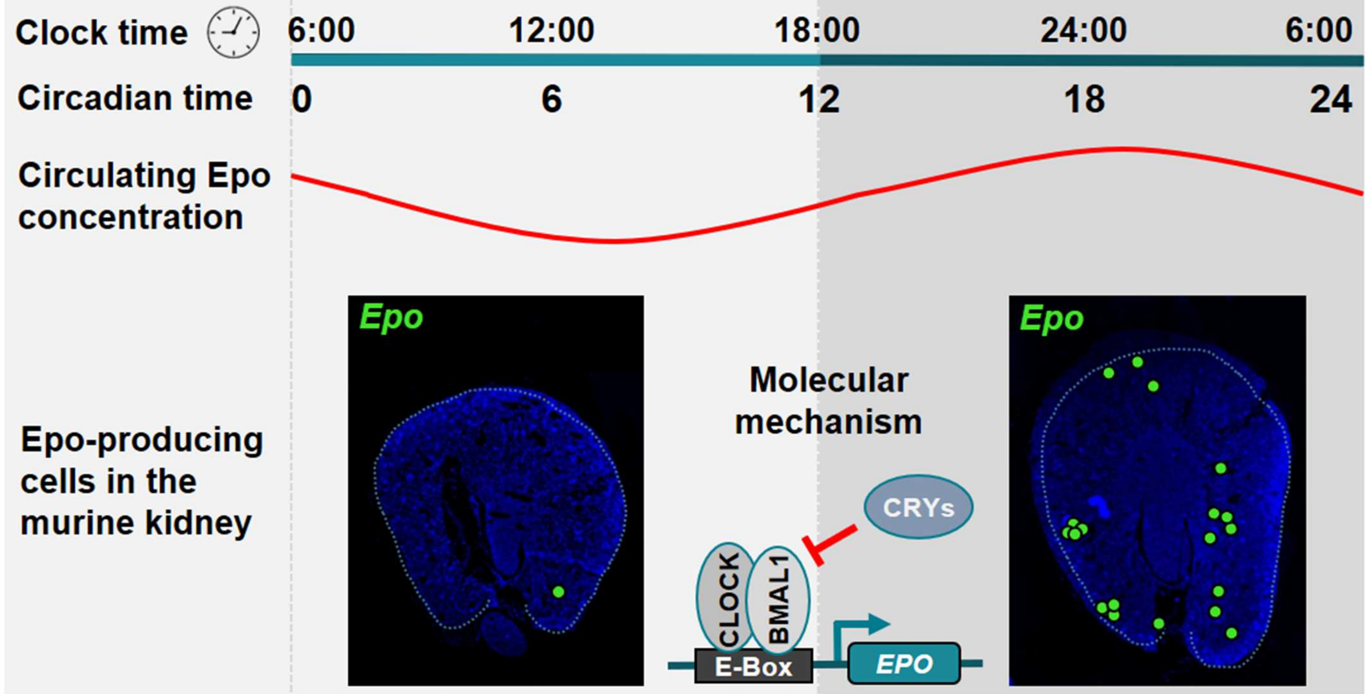
Materials and Methods

- Rhythmic wild-type vs. arrhythmic *Cry*-null mice
- ELISA
- Live-cell bioluminescence recording
- Fluorescent RNAscope *in situ* hybridization
- Arterial blood gas analysis
- Blood cell count examination

Results



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CONCLUSION In normoxia, diurnal Epo expression in the kidney is transcriptionally controlled by the circadian clock, activating additional *Epo*-expressing cells. This knowledge may impact on nephrology, hemotherapy and laboratory diagnostics.

Abstract

The molecular mechanism and hemopoietic implication of circadian erythropoietin (Epo) expression is the focus of this study. Mutant mice with homozygous deletion of the core circadian clock genes *cryptochromes 1* and *2* (*Cry*-null) were used to elucidate circadian Epo regulation. Wild-type controls exhibited a significant difference in renal *Epo* mRNA expression between circadian times (CT) CT06 and CT18. In parallel, a significantly higher number of *Epo*-producing cells in the kidney (REPCs, by RNAscope®) and of circulating Epo protein (by ELISA) was detected. Such changes were abolished in *Cry*-null mice and were independent from oxygen tension, oxygen saturation, or expression of *hypoxia-inducible factor 2 alpha (Hif2a)*, indicating that circadian Epo expression is transcriptionally regulated by Cry1 and Cry2. Reporter gene assays showed that the CLOCK/BMAL1 heterodimer activated an E-box element in the 5' *EPO* promoter. RNAscope® *in situ* hybridization confirmed the presence of *Bmal1* in REPCs. In *Cry*-null mice, a significantly reduced number of reticulocytes was found while erythrocyte numbers and hematocrit were unchanged. In conclusion, circadian Epo regulation is transcriptionally controlled by CLOCK/BMAL1, master circadian activators and repressors *Cry1*/*Cry2*. Circadian Epo regulation may have implications for nephrology, hemotherapy and laboratory diagnostics.

Key words

Chronobiology – Circadian Rhythm – Clock – Cryptochrome – Erythropoietin – Hematopoiesis – Hypoxia-inducible Factor

Translational Statement

Molecular clocks in nearly all cell types drive gene transcription in collaboration with tissue-specific factors. So far, circadian oscillatory mechanisms in the kidney have not been linked to the biology of erythropoietin (Epo). Herein, it has been elucidated that circadian Epo expression is regulated by master clock proteins (*cryptochromes 1* and *2*, *Clock*, *Bmal1*). As Epo acts distinctively within the complex regulatory network of erythropoiesis, optimal use of recombinant human Epo (rhEpo) in patients with renal insufficiency may include its application just preceding its physiological circadian maximum around midnight.

Introduction

Circadian rhythms in nephrology are largely unexplored but highly relevant since their disruption by shift-work, lifestyle choices and senescence is associated with an increased risk of various diseases, including cardio-vascular disorders.[1-4] Animal models of disturbed circadian clocks provide first evidence of the negative impact of circadian dysregulation on the hematopoietic system, e.g. by increased numbers of aged erythrocytes.[5, 6]

Concerning erythropoiesis, not only cellular effects, but also rhythmicity of its primary regulator erythropoietin (Epo) is of particular interest since athletes have been repetitively accused of blood doping with recombinant human Epo (rhEpo) due to time-of-day dependent differences in circulating Epo concentrations and hematological parameters.[7, 8] Diurnal variations in human serum Epo (S-Epo) levels were first described in 1981 in patients with chronic lung and coal workers respiratory disease,[9] and subsequently in healthy subjects.[10, 11] To date, several reports indicate high S-Epo levels in the night (08:00 pm to 04:00 am) and low S-Epo levels in the early morning (04:00 am to 08:00 am). The phase and amplitude of S-Epo oscillations, if present at all, are variable between human individuals.[9, 10, 12, 13] On average, S-Epo levels changed approximately 1.5-fold relative to its minimum.[14-22] This diurnal rhythm was shown to be unaffected by aging,[20] training,[16] or altitude exposure.[17] In contrast, the diurnal oscillation of S-Epo is abolished in patients with chronic obstructive pulmonary disease (COPD) complicated by daytime hypoxemia, in myeloma with renal failure, and in myelodysplasia.[15, 21, 23]

In 2009, we have described circadian *Epo* mRNA expression in the murine kidney in a large-scale analysis of the promoters of clock-controlled genes (CCGs), but were unable to identify neither a distinct genetic element in the *EPO* gene nor a trans-activating factor responsible for circadian oscillation.[24] More recently, a rat model of hemorrhagic shock suggested, according to parallel expression patterns, that the clock genes (*Bmal1, Per2*) were involved in the regulation of Epo secretion during hypoxia/ischemia.[25]

Generation of circadian rhythms is cell-autonomous and relies on a transcription-translation feedback loop controlled by a family of circadian clock transcription factors including CLOCK, BMAL1, PER1, PER2, CRY1 and CRY2.[26] The CLOCK/BMAL1 heterodimer activates the transcription of circadian clock genes *PER1/2* and *CRY1/2* via binding to E-box elements in their promoters. PER and CRY proteins, however, provide negative feedback by inhibiting CLOCK/BMAL1 activity, thereby reducing their own expression. The net results lead to the oscillation patterns of circadian gene expression and rhythmic changes in cellular and organ physiology.[27]

To understand the implications of the biological clock, various types of mutant mice with disrupted or ablated single core clock transcription factors have been studied so far.[28, 29] Here,

we took advantage of *Cry1*$^{-/-}$/*Cry2*$^{-/-}$ double mutant mice (*Cry*-null), which lack the ability to express endogenous circadian rhythms.[26, 30] Combined *in vivo* and *in vitro* data demonstrate that Cry1/2 regulate circadian Epo expression via CLOCK/BMAL1-induced transcription in the normoxic kidney.

Methods

Animal experiments

Homozygous *Cry1*$^{-/-}$/*Cry2*$^{-/-}$ animals (*Cry*-null, male and female, C57BL/6J-based) [31] and wild-type (WT) controls were bred (Forschungseinrichtungen für Experimentelle Medizin Charité, Berlin, Germany) and raised for 5 to 7 months. The wild-type mice came from the breeding of the *Cry*-null colony.

The *Cry*-null genotype was confirmed by PCR (Supplementary Table S1). For entrainment, mice were group-housedand had food and water *ad libitum* at a 12h:12h light/dark cycle for 14 days. On day 2 after release into constant darkness, animals were sacrificed at circadian times (CT) 06 or CT18 (n=13-15 for each group and time point). Tissues (liver, kidney) were quickly obtained and snap-frozen in liquid nitrogen. A subgroup of male and female WT and *Cry*-null animals was analyzed in detail for body weight and differential hemogram.

For blood gas analysis, animals were anesthetized (Fentanyl 0.075 mg/kg, Midazolam 1.5 mg/kg, Medetomedin 0.75 mg/kg), tracheotomized, intubated and ventilated (tidal volume: 9 ml/kg, respiratory rate: 160 min^{-1}, positive end-expiratory pressure: 2 cm H_2O) as described [32]. A polyethylene catheter was surgically introduced into the left carotid artery. After 5 minutes of stabilization, the experiment was terminated through rapid exsanguination via the carotid catheter, blood gases were analyzed (ABL-800; Radiometer, temperature-controlled) and kidneys were excised for *post hoc* analyses.

All procedures were authorized by the Local Animal Care Committee (T0307/08, G0100/17 with addendum from 01/2021) and performed in accordance with the guidelines and regulations of the German animal protection law.

Preparation of RNA and quantitative PCR analysis

Total RNA was extracted as described.[33] 1000 ng total RNA were reverse-transcribed with SuperScript^{TM} III reverse transcriptase (Thermo Fisher, #18080085) and random hexamers (Thermo Fisher, #SO142) according to the manufacturer's instructions. Quantitative PCRs were run on a StepOnePlus cycler (Life Technologies) with intron-spanning primers or TaqMan assays (Supplementary Table S2) Absolute mRNA quantification was achieved by comparison with a standard curve from serial dilutions of PCR template.

Detection of *Epo* mRNA expression in the kidney by RNAscope® technique

RNAscope® assay was performed according to the manufacturer's protocols (ACD, Technical note 320536). 10 µm mid-kidney transverse cryosections were stained with a C1-probe against *DapB* (negative control, ACD, #310043) or *Epo* (ACD, #315501), respectively. Hybridization steps using Amp 4-6 and detection of the red signal were omitted. Two independent, blinded researchers counted *Epo*-positive cells at 200x magnification, on 3-8 cryosections per animal at an Axioplan 2 Imaging system (Zeiss).

Representative *Epo* quantification images and double fluorescent staining were performed using the RNAscope Multiplex Fluorescent Detection Reagent V2 Kit (ACD, #323110) according to the manufacturer's protocols. 1.5 µm mid-kidney transverse paraffine sections were stained with a C1-probe against *Bmal1* (ACD, #438741) and a C2-probe against *Epo* (ACD, #315501-C2), respectively. Opal dye 520 (Akoya BioSciences, #FP1487001KT) was used with the C1- and 650 (Akoya BioSciences, #FP1496001KT) with the C2-probe. At 400x magnification, *Epo*-positive cells were imaged for *Bmal1* co-localization at an Eclipse Ti2 imaging system (Nikon). DAPI was used as counterstaining.

Epo serum concentrations

Blood samples were allowed to clot for 1 h at room temperature before centrifuging for 20 min at 2000 x g. Serum was removed and immediately frozen at -80°C until performing the enzyme-linked immunosorbent assay for Epo (Quantikine, R&D Systems, #MEP006) with undiluted samples. Absorbance was read with an iMARK Microplate Absorbance Reader (Bio-Rad) at 450 nm with wavelength correction at 570 nm and a 4-parameter fit standard curve as described previously.[33]

Blood cell counts

Total and differentiated cells counts from EDTA-anticoagulated blood were measured by Synlab.vet Berlin with an ADVIA2120i (Siemens) automated cell counter for murine blood.

Reporter gene assays

Human embryonic kidney cells HEK293 (DSMZ, Germany, #ACC305, passage numbers 3-10, mycoplasma-negative) were grown in DMEM/Ham's F12 (Biochrom, #FG4815) supplemented with 10% fetal bovine serum (Merck, #F7524). Cell transfection was performed in 12-well plates containing $1.67 \cdot 10^5$ cells/well. Each well was transfected with 333 ng plasmid DNA (1/10 of which was renilla construct) and 1 µl Fugene 6 transfection reagent (Promega, #E2691) as described.[34] All constructs used are listed in Table S3. Cells were lysed 48 hrs after transfection with Passive Lysis buffer (Promega, #E1941). Luciferase activity was

determined with the Beetle-Juice and Renilla-Juice kits (both pjk GmbH, Germany, #102511/102531), respectively, at a Lumat LB9501 luminometer (Berthold, Germany). Each experiment was performed in technical duplicates and mean values were used for calculations.

Statistical analysis

In all animals, the circadian gene expression was analyzed; two animals were excluded as they showed outlier values (>1.5-fold the interquartile range) in 4 out of 6 circadian expressed genes. Data were analyzed using IBM SPSS Statistics 27 and are presented as means ± standard deviation or as median with the 25th and 75th percentile. Mann-Whitney-U test or Kruskal-Wallis with Bonferroni as post-hoc test were performed.

Results

Ablation of circadian Epo expression in Cry-null mice

To elucidate the molecular mechanism of circadian Epo regulation, we analyzed Epo mRNA and protein expression in wild-type (WT) and *Cry*-null mutant mice. Whereas in WT kidney, canonical clock genes showed a time-of-day dependent expression, this was abolished in *Cry*-null mice, as expected (Supplementary Figure S1). We previously reported circadian oscillation of *Epo* mRNA expression over 24 hrs for adult WT murine kidneys.[24] Focusing now on the minimal and maximal values, we observed a significant, ~9-fold difference between circadian time 06 (CT06; mouse sleeping period; expected minimum) and CT18 (activity phase; expected maximum; Figure 1A). In the kidneys of *Cry*-null mice, however, no significant difference of renal *Epo* mRNA expression was detected. Notably, in *Cry*-null mice, the absolute amounts of renal *Epo* transcript levels at both times were in-between the median WT *Epo* mRNA levels at CT06 and CT18 in (Figure 1A). *Epo* mRNA in corresponding livers was below the detection limit (not shown).

Diurnal changes in Epo serum concentrations

To estimate the translation of circadian *Epo* mRNA expression into circulating Epo protein, we analyzed serum samples by ELISA. Blood samples were taken prior to organ specimens. Serum Epo increased ~2.3-fold between CT06 and CT18 in WT mice, but this difference was abolished in *Cry*-null mice (Figure 1B). Notably, the median S-Epo concentrations averaged over time (CT06 and CT18) were similar in WT and *Cry*-null mice (22 mU/ml, range 3-59 mU/ml vs. 21 mU/ml, range 7-50 mU/ml).

Circadian Epo expression in relation to arterial blood gas parameters and pulse oximetry

To investigate whether potential diurnal changes in blood and tissue oxygen levels could cause circadian oscillation of Epo expression, arterial blood specimens were obtained and blood gas analyses were performed in anesthesized, tracheotomized and mechanically ventilated *Cry*-null and WT mice at CT06 or CT18, which correspond to the lowest and highest circadian *Epo* mRNA levels (Figure 1A). We detected neither significant differences in the arterial pH, $paCO_2$ or paO_2 nor in the standard base excess or lactate concentrations (Figure 2A-E) between CT06 and CT18 or WT and *Cry*-null mice. Oxygen saturation levels measured by pulse oximetry also did not show any differences (Figure 2F). Gene expression of the EPO master regulator Hif2a did not differ between CT06 and CT18, neither in WT nor *Cry*-null mice (Figure 3). Thus, under normoxic conditions, circadian *Epo* regulation is not caused by changes in oxygen tension.

Circadian ON-OFF switch of Epo expression in renal Epo producing cells (REPCs)

To study whether circadian *Epo* mRNA expression is mediated by switching on additional renal Epo-expressing cells (REPCs) or solely by increasing *Epo* expression per cell, RNAscope® *in situ* hybridization was used on mid-kidney transverse sections (example in Supplementary Figure S2). In WT mice, the number of REPCs significantly increased between CT06 (median 5, range 0-25) and CT18 (median 22, range 5-82, 4.4-fold; p=0.010). In contrast, *Cry*-null kidneys displayed similar numbers of REPCs at CT06 (median 18, range 2-33) and CT18 (median 15, range 4-87; n.s., Figure 1C). We considered that *Cry*-null mice exhibit growth restriction due to impaired signaling of insulin-like growth factor 1 (IGF1), which results in a continuously increasing difference in body weight and organ size between *Cry*-null and WT mice.[30] As we used relatively young animals, the absolute kidney weight of *Cry*-null mice was only slightly lower than in WT mice (-12% in *Cry*-null), but the relative kidney-to-body weight ratio did not differ (Supplementary Figure S3). Thus, the circadian increase in *Epo* mRNA expression seems to be regulated by an ON switch of additional REPCs.

Activation of the minimal EPO promoter by CLOCK/BMAL1

To identify the regulatory sequences responsible for circadian *Epo* expression (Figure 4A), luciferase reporter gene assays were performed in human embryonic kidney cells (HEK293). The HEK293 cell line was chosen not for its renal origin but for its lack of an endogenous circadian clock. Thus, the stimulatory effect of CLOCK/BMAL1 could be tested in a low, non-oscillating CLOCK/BMAL1 background. Overexpression of CLOCK/BMAL1 significantly stimulated the activity of the minimal human *EPO* promoter (Figure 4B,-I vs. -II).

If the E-box motif (-36 to -31 bp relative to the TSS) is mutated,[35] this effect is blunted (Figure 4B-III).

To study whether the observed circadian regulation was cell type-dependent, we screened several *EPO*-expressing cell lines for endogenous clock activity by monitoring *Bmal1* promoter-mediated oscillations of a luciferase reporter. Those with an endogenous rhythm included human hepatoma-derived HEP3B cells, human neuroblastoma-derived KELLY cells, and PDGFRβ$^+$ mouse kidney cells (formerly EPO-expressing mouse cell line FAIK1-10).[36, 37] Among the three cell lines, only KELLY cells exhibited *EPO* promoter-driven luciferase oscillations (Supplementary Figure S4A). Although PDGFRβ$^+$ cells exhibited a strong circadian rhythm of *Bmal1* promoter activity, we did not detect *EPO* promoter-mediated oscillations suggesting a lower amplitude of the *EPO* promoter-driven reporter construct (Supplementary Figure S4B).

Co-localization of Bmal1 and Epo in the kidneys of Cry-null vs. WT mice

To further elucidate (i) the circadian regulation of renal Epo production by recruiting renal Epo producing cells and (ii) the co-localization of *Bmal1* and *Epo* expression, we performed RNAscope® *in situ* hybridization. *Epo* and *Bmal1* co-localized, and microscopy on low magnification represents differences in the recruitment of Epo producing REPCs (Figure 5) as quantified in Figure 1C.

Hematologic findings in Cry1/Cry2 deficiency

To assess (i) the hematopoietic effects of the lack of circadian Epo expression and (ii) possible other hematologic abnormalities in mice without a functional clock, blood cell counts were analyzed. The time-of-day differences of *Epo* mRNA expression and S-Epo concentration were not mirrored by significant differences in peripheral reticulocyte counts at CT06 vs. CT18 in WT mice. Reticulocyte counts, however, were significantly lower at both CT6 and CT18 in *Cry*-null animals compared to WT mice (Figure 6A). There was no significant difference in the erythrocyte numbers or hematocrit values in WT vs. *Cry*-null mice. Both parameters, however, did not vary between CT06 and CT18 in both strains (Figure 6B/C) and did not correspond to the lower peripheral reticulocyte number in *Cry*-null mice (Figure 6A). To test whether nutritional deficiencies (e.g. iron, folate etc.) due to impaired IGF1 signaling are involved in the discrepancy of reduced reticulocyte but normal erythrocyte numbers in *Cry*-null mice, red blood cell size and shape were analyzed, but did not show any significant differences between *Cry*-null and WT mice (Supplementary Figure S5).

Notably, median platelet counts in *Cry*-null mice tended to be higher than in WT mice, but platelet numbers were not significantly different between CT6 and CT18 in both strains (Figure 6D). In contrast, white blood cell (WBC) counts differed significantly between CT06

and CT18 in WT but not in *Cry*-null mice. The median WBC number in *Cry*-null mice was similarly high as in WT mice at CT06 (Figure 6E).

Discussion

Herein, we demonstrate that circadian Epo expression is regulated at the transcriptional level. The analysis of *Epo* mRNA and S-Epo concentrations in arrhythmic *Cry1* and *Cry2* deficient mice redirected our search for "Epo's clock" to their downstream target transcription factors CLOCK and BMAL1, which are repressed by CRY1 and CRY2.[26] Ablation of *Cry1*/*Cry2* leads to the loss of rhythmic repression of CLOCK/BMAL1, resulting in constant *Epo* transcript levels that were in-between the median levels at CT06 and CT18 in WT mice (Figure 1A). *In situ* hybridization by RNAscope® indicates that the circadian oscillation is achieved by switching on *Epo* mRNA expression in interstitial cells (Figures 1C), and *in situ* hybridization by RNAscope® also revealed the expression of *Bmal1* in REPCs (Figure 5).

Importantly, the significantly higher S-Epo levels at CT18 confirm that the circadian, transcriptional *Epo* regulation indeed translates into circadian oscillations of circulating Epo protein under normoxic conditions (Figure 1B). The real maximum of S-Epo levels is expected slightly later than CT18 since *de novo* synthesis of Epo protein requires ~80-120 min,[38] and circadian times were chosen based on maximal *Epo* mRNA levels.[24] We found that circadian Epo regulation is likely mediated by transcriptional activation of an E-box motif in the 5' *EPO* promoter by CLOCK/BMAL1 (Figure 4, Supplementary Figure S4), which is consistent with the described positive correlation between BMAL1 protein and S-Epo levels in a rat model of acute hemorrhage.[25]

There is evidence for a bidirectional regulation between the CLOCK/BMAL1 and the HIF pathways through direct protein-protein interaction.[39, 40] Upregulation of HIF2α, the major activator of the *EPO* promoter,[41, 42] resulted in altered expression levels of clock genes in human hepatoma cells.[43] Furthermore, BMAL1 dimerizes with HIF1α and HIF2α proteins,[44, 45] and the circadian clock control of HIF activity is regulated in a tissue-specific manner.[39] In mice, exposed to acute hypoxia (4 hrs 6% vs. 21% O_2), *Epo* mRNA was excessively increased but did no longer show circadian differences.[46] Thus, normoxic conditions are probably most appropriate for dissecting the molecular mechanism of circadian Epo regulation. In rodents, however, diurnal changes in blood and tissue oxygen levels have been reported.[39, 47] In rats, there is a low range of rhythmic daily changes in kidney oxygenation of approx. Δ 3%, with a peak in the dark (= rodent activity).[47] Such differences could not be detected in our experiments, in which only anesthesized, tracheotomized and mechanically ventilated mice could be studied for animal regulatory reasons. However, analysis of arterial pH, $paCO_2$, and

paO_2, standard base excess or lactate as well as oxygen saturation did not indicate major differences between WT and *Cry*-null mice at both CTs (Figure 2). Furthermore, *Hif2a* transcript levels in kidney were also similar in all conditions (Figure 3). Thus, under normoxic conditions, circadian *Epo* regulation seems to be independent from diurnal changes in oxygen tension. However, the question to which extent high altitude or hypoxia (low pO_2) influence the circadian oscillation of Epo production deserves further attention. Human S-Epo levels are generally higher at high altitude while phase and amplitude are unchanged,[17, 22] and in healthy volunteers exposed to normobaric hypoxia, S-Epo concentrations show a pronounced oscillation.[48]

Circadian Epo regulation is probably most relevant for clinical nephrology and hematology, but also regarding laboratory diagnostics such as blood testing for doping with erythropoiesis stimulating agents. For evaluation of circulating Epo concentrations in doping analyses, the time of day (external time) at collection of the blood and the chronotype of the person (internal time) need to be considered.[49, 50]

In erythropoiesis, burst-forming units-erythroid (BFU-Es) are the first lineage-specific cells, followed by colony-forming units-erythroid (CFU-Es) which show abundant expression of Epo receptor. After two days in culture with Epo, murine CFU-Es give rise to erythroblast colonies. Once the stage of orthochromatic erythroblasts is reached after 7 days, the cells extrude their nuclei to become reticulocytes lacking EpoR expression.[51] Considering the time it takes for the CFU-Es to mature to reticulocytes or even fully mature erythrocytes, it is probably not surprising that no differences were observed between CT06 and CT18 in WT mice (Figure 6A-C). Notably, overall reticulocyte numbers were significantly higher in WT than in *Cry*-null mice (Figure 6A), suggesting that differentiation into reticulocytes is impaired in *Cry*-null mice. Previous data indicate that early erythroid progenitor cells also exhibit a circadian pattern of DNA synthesis. *In vivo* administration of rhEpo enhances the circadian rhythms of erythroid colony numbers.[52] Lower reticulocyte numbers despite relatively high overall S-Epo concentrations in the *Cry*-null mice could result from abolishing circadian DNA synthesis on the level of erythroid progenitors. Such constellation of high S-Epo and reduced erythropoiesis has been observed in mice with genetic ablation of pineal melatonin production (*C3H/HeN* mice that lack a rate-limiting N-acetyl transferase),[53] arguing for intrinsic circadian activities on the erythroid progenitor cell level.

Notably, *Cry*-null mice show an approximately 80% reduction in IGF1 levels, leading to reduced IGF1 signaling and a 30% reduction of body weight and organ size compared to WT, an effect that exacerbates over lifetime.[30] Although animals used in our experiments were relatively young (median 21 to 29 weeks), they displayed a moderate effect on total body and absolute kidney weights in *Cry*-null mice but the kidney-to-body weight ratio was normal

(Supplementary Figure S3). The latter fact may be relevant for capacity of Epo protein synthesis (Figure 1B).

However, analysis of red blood cell size or shape (MCV, MCH, MCHC; Supplementary Figure S5) did not suggest nutritional deficiencies (e.g. iron, folate) as explanation for the discrepancy between reduced reticulocyte and normal erythrocyte counts in *Cry*-null mice (Figure 6A-B). Notably, Epo and IGF1 signaling synchronize cell proliferation and differentiation during erythropoiesis via interaction with the GATA-1/FOG-1 transcriptional complex.[54] In this process, Epo activates the cellular AKT pathway and thereby increases the affinity of GATA1, the major transcriptional regulator of erythropoiesis, to its cofactor FOG1 (friend-of-GATA1). This mechanism is inhibited, however, if IGF1 signaling is abolished.[54] Thus, a positive effect of relatively higher Epo in *Cry*-null mice on red cell differentiation may outweigh the reduced IGF1 activity in the relatively young *Cry*-null mice studied here.

The general implication of complete circadian arrhythmicity on hematopoiesis is further elucidated by our analysis of platelet and WBC numbers. Previous results from mice expressing a dominant-negative form of *Clock* (*Clock*$^{\Delta19/\Delta19}$) indicated that the disruption of the expression of thrombopoietin (Thpo, the primary regulator of megakaryopoiesis) and its receptor Mpl results in increased numbers of mature marrow megakaryocytes and circulating platelet numbers.[55] The described significant circadian oscillation of platelet numbers with at peak at zeitgeber time (ZT)20 in WT mice as well as higher platelet numbers at ZT08 in *Clock*$^{\Delta19/\Delta19}$ mice,[55] resulting in a lack of circadian oscillation of platelet numbers, could not be confirmed in our mouse model (Figure 6D). Another important finding is the loss of time-of-day differences of circulating WBCs in *Cry*-null mice in contrast to WT mice (Figure 6E), which is consistent with the reported activity of CLOCK/BMAL1 on mature WBC production.[56, 57]

For the interpretation of our data, differences in day-night activities in mice and humans need to be considered: The time CT06 in humans roughly corresponds to midnight (sleeping phase), while CT18 roughly corresponds to midday (activity phase). The opposite is true for nocturnal mice, but both organisms show the same circadian Epo expression pattern (low at CT/ZT06, high at CT/ZT18). This indicates that additional mechanisms, e.g. metabolic factors, could modify the rhythms of Epo expression in both organisms. The synchronization between Epo peak levels and EpoR expression in early erythroid progenitors (BFU-E and even CFU-E cells)[52] suggests that patients receiving rhEpo treatment (e.g. in end-stage renal anemia or hematological disorders) would benefit from mimicking the normal circadian physiology by applying rhEpo during the night. Hematopoietic disorders have not been reported in humans with *CRY1* (OMIM*601933) or *CRY2* (OMIM*603732) loss-of-functions mutations yet. Clinical reports associate *CRY1* variants primarily with attention deficit/hyperactivity disorders, frequently accompanied by insomnia, anxiety and depression or delayed sleep phase

disorder.[58, 59] This deserves further investigation, since such diseases can be worsened by anemia or hematopoietic disorders.

In conclusion, this study provides the first evidence that circadian *Epo* expression in the normoxic adult murine kidney is regulated at the transcriptional level by CLOCK/BMAL1-mediated activation of an E-box element in the 5' *EPO* promoter. As Epo acts distinctively within the complex regulatory network of erythropoiesis, optimal use of rhEpo in patients with renal insufficiency may include its application just preceding its physiological circadian maximum in the night.

Disclosure of Conflicts of Interest

All the authors declared no competing interests.

Data Sharing Statement

For original data not included in the manuscript or supplements, please contact christof.dame@charite.de.

Authorship Contributions

LKS, AK and CD designed research. LKS, MF, LM, KMK, GL, CLJJ, TW, VL, DL and CD performed research. LKS, MF, LM, KMK, GL, CLJJ, AK, and CD analyzed data. LKS, KMK, AK and CD interpreted data. LKS and CD wrote the manuscript. All authors approved of the final version of the manuscript.

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Supplementary Material

Table S1. Genotyping of *Cry*-null mice (*Cry1*$^{-/-}$/*Cry2*$^{-/-}$).

Genotypes were routinely assessed from ear-marking biopsies' gDNA (prepared with NucleoSpin Tissue kit, Macherey-Nagel, #740952) by conventional PCR with GoTaq G2 polymerase (Promega, #M7845) with the primers listed below (**A**). The *Cry1* and *Cry2* mRNA expression status (Cry1/2$^{+/+}$ vs. Cry1/2$^{-/-}$) was confirmed in the kidney specimens from animals by SYBR Green qPCR with the primers listed below (**B**).

	Primer sequence (5' → 3')
A	
Cry1_geno_wt_f	TGTGCTTATAGTGCCGCTGA
Cry1_geno_ko_f	TCGCCAATGACAAGACGCTG
Cry1_geno_uni_r	CTGACAAGGAGACACGTCCA
Cry2_geno_uni_f	GCTTCATCCACATCGGTAACTC
Cry2_geno_wt_r	CCAGAGACGGGAAATGTTCTT
Cry2_geno_ko_r	GAGATCAGCAGCCTCTGTTCC
B	
Cry1 geno cDNA-F4	GTGCATGACCCCTCTGTCTG
Cry1 geno cDNA-R4	CTAGCCCTCTGTACCGGGAA
Cry2 geno cDNA-F1	CGTCTGTTTGTAGTCCGGGG
Cry2 geno cDNA-R1	TGATGAGGGCCTGAAAGCG

Table S2. Primer pairs and probes used for qPCR

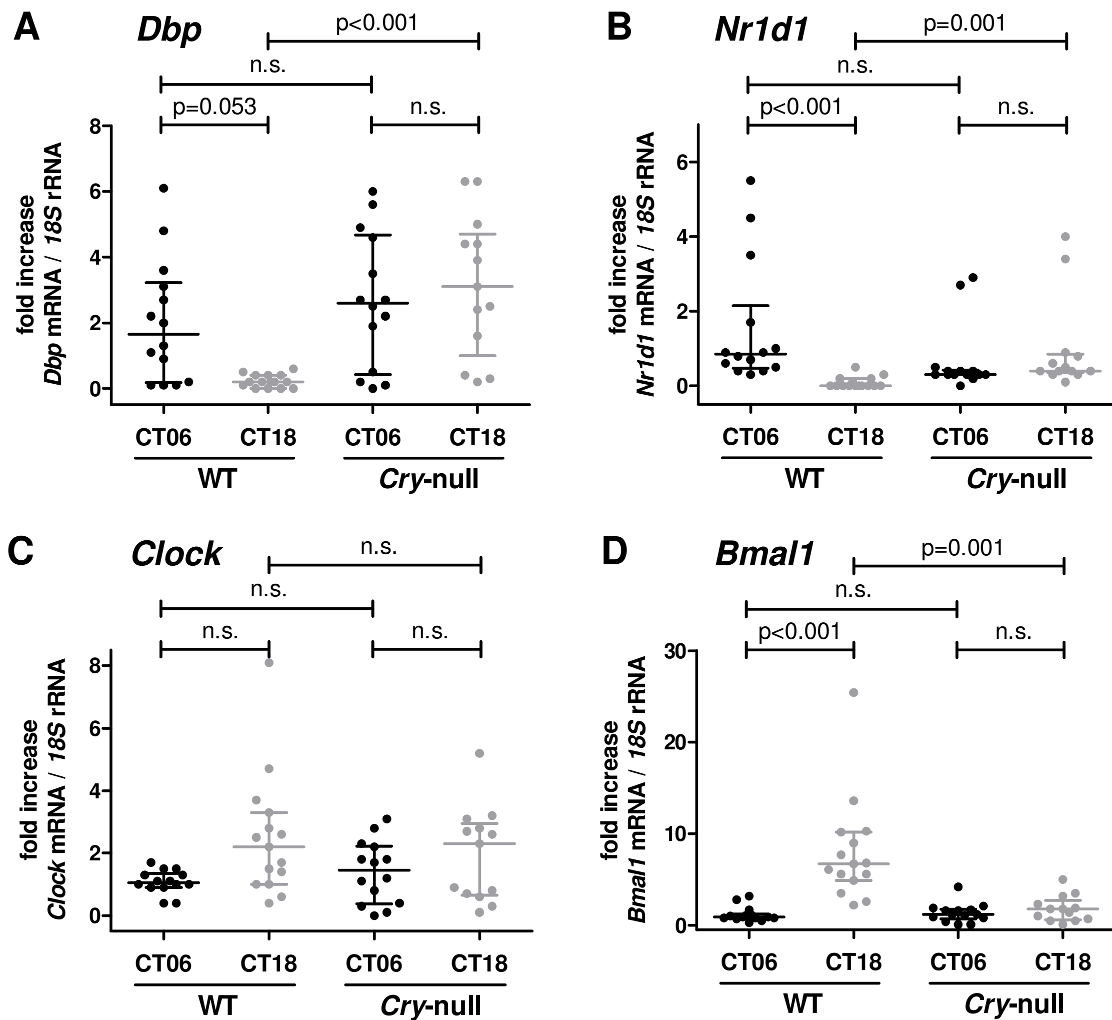
Absolute *Epo, Hif2alpha* mRNA and *18SrRNA* quantification was achieved through comparison with a standard curve from serial dilutions of PCR template (sequences available on request).

Primer name	Primer sequence (5' → 3')	NCBI accession number
mDbp F	TTTGCCTTTCGGGGACGTGG	NM_016974.3
mDbp R	TGGGTCCACAGGACTGGGTG	
mNr1d1 F	ATCCTGATTGCGAACTGCGGG	NM_145434.4
mNr1d1 R	GGACTGGAAGCTGCCATTGGA	
mBmal1 F	GGGACGGAGGTGCCTGTTTA	NM_001243048.1
mBmal1 R	ACCCTCTGGGCCCAAATTCC	
mClock F	ACGCGCTCCCGTGAAAGAAA	NM_001289826.1
mClock R	TGTCCTTGTCATCTTCTTCCACCAA	
m18SrRNA F	GATCAAAACCAACCCGGTGA	NR_003278
m18SrRNA R	CCGTTTCTCAGGCTCCCTCT	
TaqMan assay mEpo	Mm01202755_m1 (Thermo Fisher)	NM_007942.2
TaqMan assay mHif2a	F: 5'- CCATGCCCTGGATTCGGAGAA-3' R: 5'- CGTGTTTGGCTAGCATCCGGTA-3' Probe: 5'-ACTTGTGCACCAAGGGGCAGGTGGT-3'	NM_010137.3
TaqMan assay m18SrRNA	Mm04277571_s1 (Thermo Fisher) with *Epo*) With *Hif2a*: F: 5'-GGTCGCTCGCTCCTCTCCTA-3' R: 5'- GGAGCTCACCGGGTTGGTTT-3' Probe: 5'-TGCCGACGGGCGCTGACCCC-3'	NR_003278.3

Table S3. Plasmids used for reporter gene assays.

Plasmid	**Details**	**Source**
phRL-TK (Renilla)	normalization	Promega
pDEST26	Empty vector	Invitrogen
pDEST26-mBmal1 stop	Bmal1 overexpression	Klemz et al., 2017 [1]
pDEST26-mClock stop	Clock overexpression	
pGL3 basic	Empty vector	Promega
pGL3b-hEPO Prom 119	Wildtype EPO promoter	Orlando et al., 2019 [2]
pGL3b-hEPO Prom 119 E-box mut	EPO promoter with mutated E-box	This study, for details s. Methods section
pGL3promoter	Empty vector	Promega
pGL3prom-E6S	Positive control, 6 E-boxes	Vanselow et al., 2006 [3]

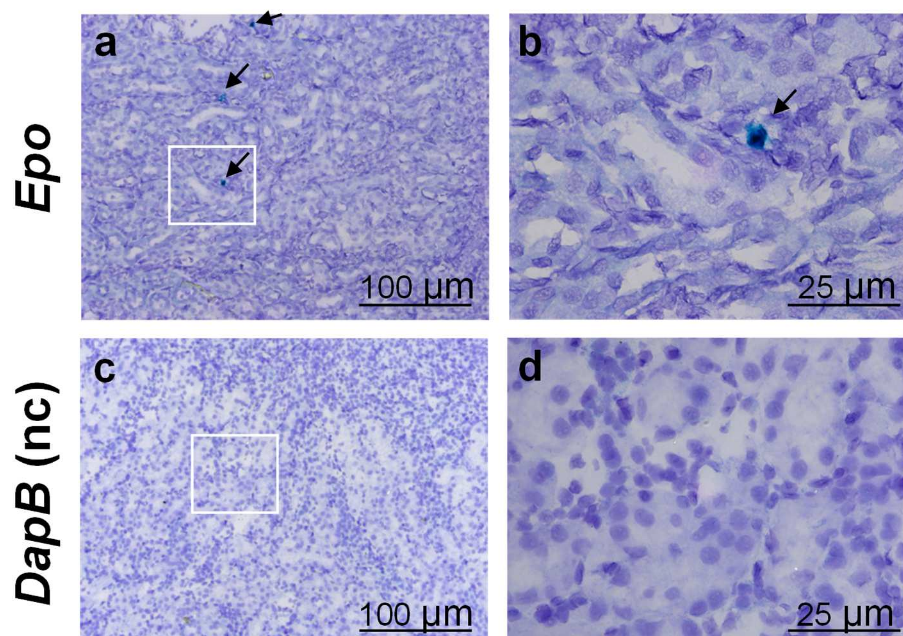
The E-box in the *EPO* promoter construct was mutated by PCR with the following primers:
5'-TATAGATCTCCCCTGCTCTGAC-3' (outer forward),
5'-GCACGCATAGGTACAGATAACAGC-3' (mut forward),
5'-GCTGTTATCTGTACCTATGCGTGC-3' (mut reverse),
5'- TTTAAGCTTGCGGCCGGGGTGG-3' (outer reverse). The mutated PCR fragment was cloned in the pGL3basic vector (Promega, #E1641) via the BglII and HindIII sites. Sequence identity of the resulting plasmid was verified by Sanger sequencing (LGC Genomics, Berlin).



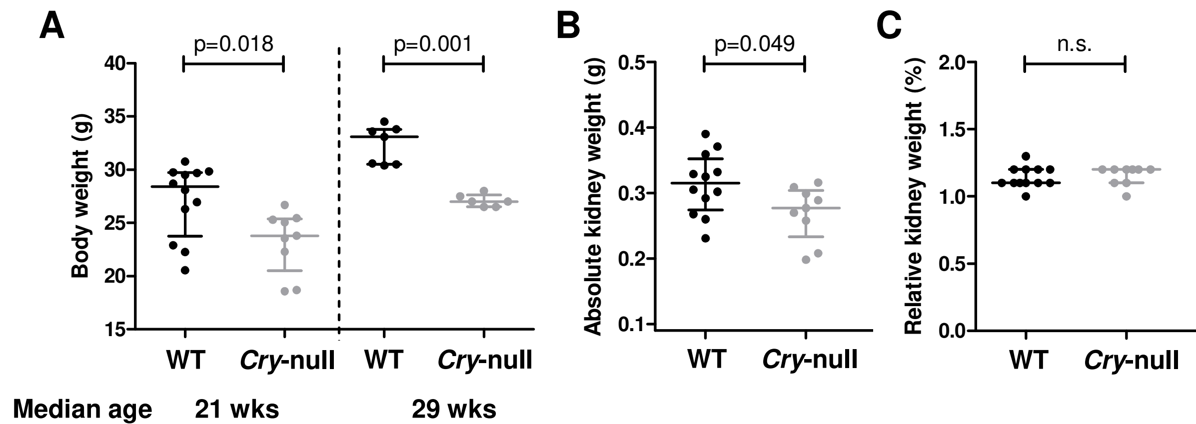
Supplementary Figure S1: Analysis of *Clock/Bmal1* and clock-controlled gene expression. This includes E-box controlled transcriptional regulators (*Dbp:* D site albumin promoter binding protein, *Nr1d1:* nuclear receptor subfamily 1, group D, member 1 *alias* Rev-erb-alpha) as well as positive (*Clock*, *Bmal1*) regulators of the oscillatory system. The known clock-controlled genes *Dbp* (**A**) and *Nr1d1* (**B**) serve as a positive control for the *Cry*-null model by lack of rhythmic mRNA expression at CT06 vs. CT18 which is clearly observable in WT mice. The clock genes *Bmal1* (**C**) and *Clock* (**D**) were measured to determine their basal levels in *Cry*-null in comparison to WT mice. Graphs present fold increase vs. WT_CT06, Kruskal-Wallis test with Bonferroni as post-hoc test, n=13-15 per condition, n.s. not significant.

Please note: The molecular circadian clock is composed of interlocked transcriptional-translational feedback loops. In the core loop, transcriptional activators induce the expression of their own repressors, forming a negative feedback loop that is highly conserved among various species. The core loop consists of basic helix-loop-helix and *Per-Arnt-Sim* (PAS) heterodimeric transcriptional activators (CLOCK) or its paralog, NPAS2, with BMAL1. The

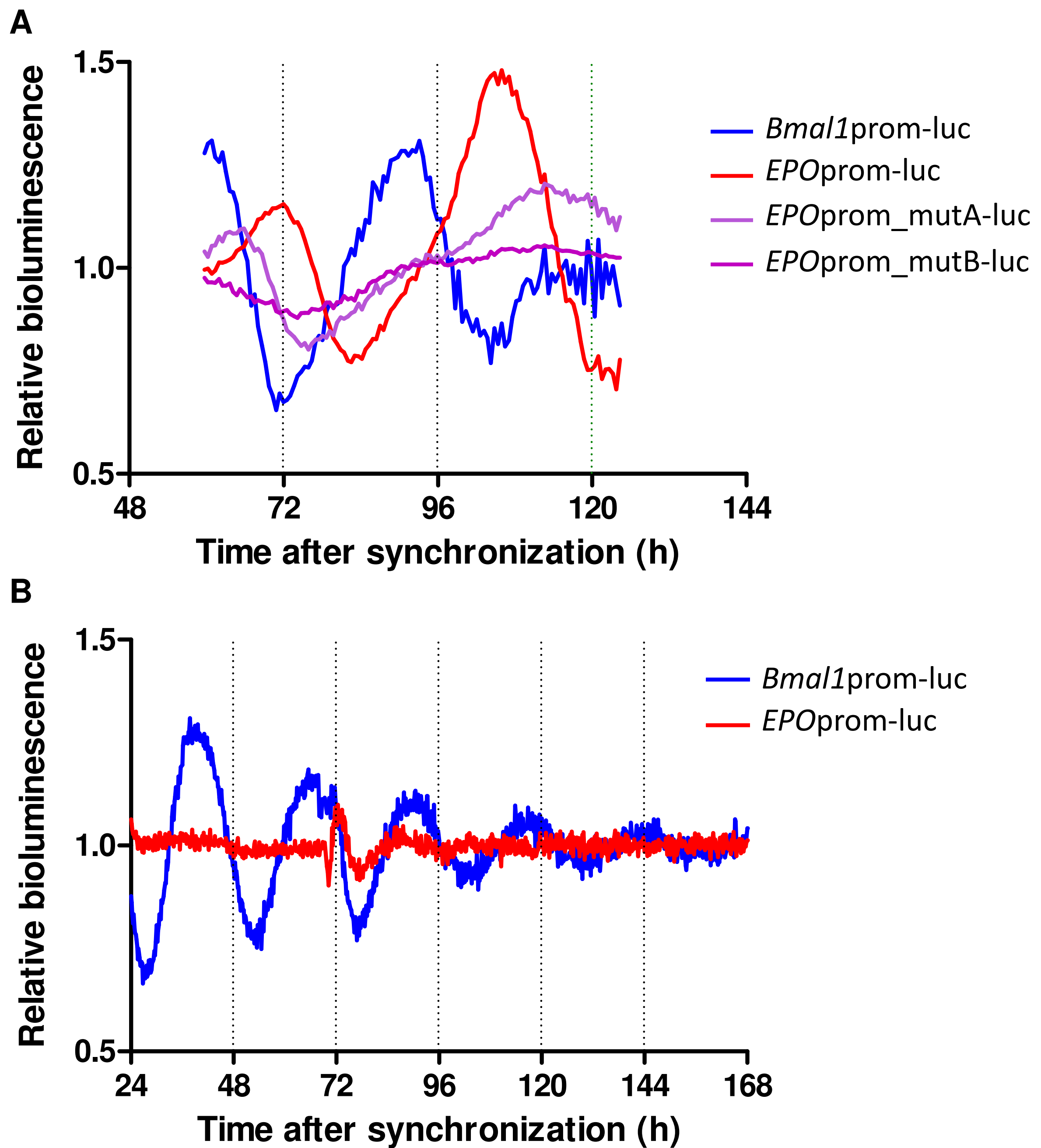
CLOCK/BMAL1 activators bind to E-box elements in the promoters of the repressors Period (*Per1*, *Per2*, or *Per3*) and Cryptochrome (*Cry1* or *Cry2*), which together form a large multi-protein complex that, after a delay of several hours, negatively act on CLOCK/BMAL1 transcriptional activity to control their own transcription. Upon genetic deletion of both *Cry* genes, this negative feedback complex cannot form, and CLOCK/BMAL1 transactivation is not inhibited anymore. As a consequence, direct CLOCK/BMAL1 target gene mRNAs are upregulated at all times, such as *Dbp* and *Nr1d1*. In an interlocked second feedback loop, NR1D1 negatively controls *Bmal1* transcription. Thus, in Cry-null mice the high *Nr1d1* mRNA levels translate in high NR1D1 protein levels, which in turn lead to low *Bmal1* transcript levels. This is exactly observed here.



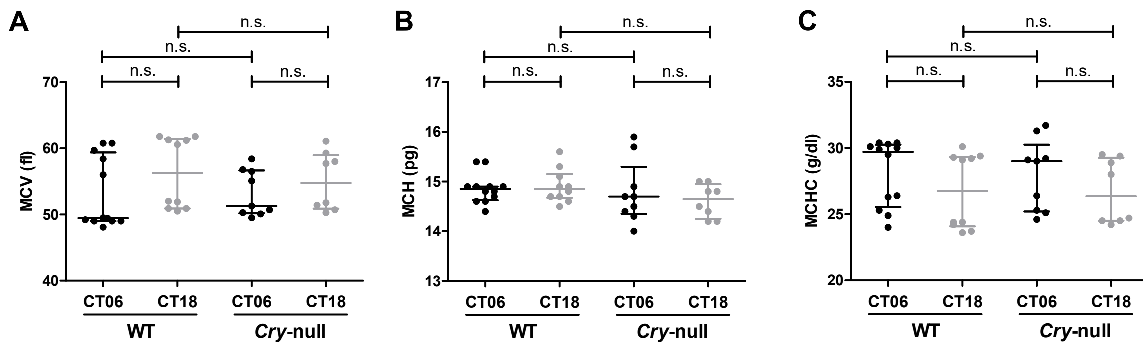
Supplementary Figure S2: Exemplary quantification of renal Epo producing cells (REPCs) by RNAscope®. In order to quantify the amount of REPCs, they were stained by RNAscope (turquoise-blue staining) in mid-kidney transverse sections and counted by two persons blinded for the experimental condition of the samples. REPCs from 3-8 independent 10 µm mid-kidney transverse cryosections were counted at 200x magnification using an Axioplan 2 Imaging system (Zeiss). No staining was observed in sections stained with a negative control probe (*DapB*).



Supplementary Figure S3: Analysis of total body weight, kidney weight and the kidney-to-body weight ratio in 21- vs. 29-week old *Cry*-null and WT mice. During ageing, *Cry*-null mice displayed a continuously increasing significant difference in body weight in comparison to WT animals (**A**), but exhibited only slightly lower absolute kidney weights (**B**) while the relative kidney weight did not differ between both strains (**C**). Mann-Whitney-U test, n=6-12 per condition, n.s. not significant.



Supplementary Figure S4: Oscillations of the *EPO* promoter in different cellular backgrounds. Oscillations of a luciferase reporter in KELLY neuroblastoma cells under the control of the *Bmal1* promoter (blue line), *EPO* promoter (red line) and the *EPO* promoter with two different mutations of the E-box element (purple lines) (A). Oscillations of a luciferase reporter in FAIK1-10 renal, formerly Epo-expressing cells under the control of the *Bmal1* promoter (blue line) and the *EPO* promoter (red line); preliminary data, 4 technical replicates.



Supplementary Figures S5: Analysis of corpuscular parameters of red blood cells obtained in *Cry*-null and WT mice. MCV (mean corpuscular volume) **(A)**, MCH (mean cellular hemoglobin) **(B)** and MCHC (mean corpuscular hemoglobin concentration) **(C)** were not different between *Cry*-null and WT mice. Kruskal-Wallis test with Bonferroni as post-hoc test, n=8-12 per condition, n.s. not significant.

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Figures

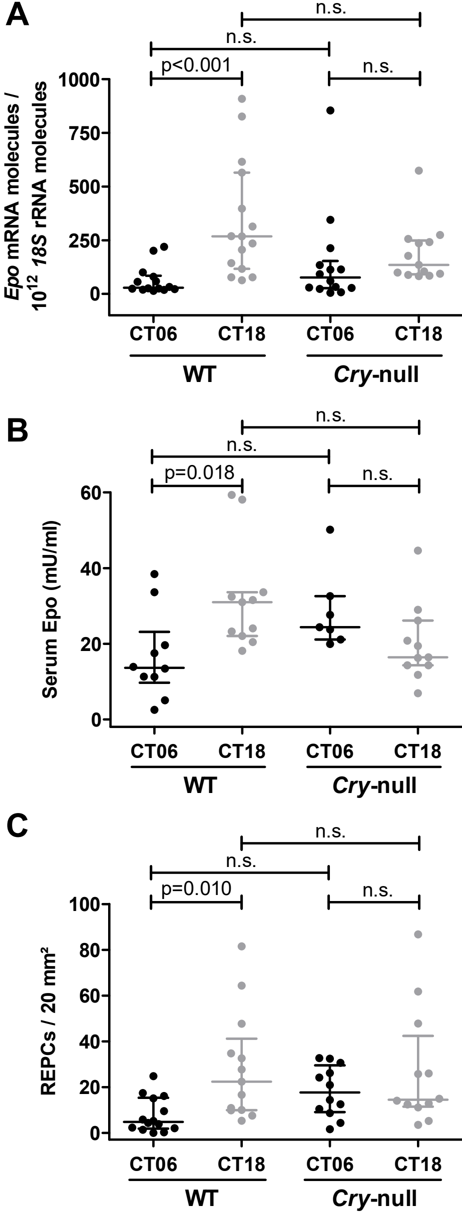

Figure 1. Circadian Epo expression. (**A**) Real-time PCR shows significantly higher Epo transcript levels at CT18 (activity phase) than at CT06 (sleeping phase) in kidneys of adult wild-type (WT) mice. This difference is abolished in *Cry*-null mice; n=13-15 per condition. (**B**) Circulating serum Epo (measured by ELISA) in WT mice exhibits a time-of-day dependent expression corresponding to *Epo* mRNA expression, while this is blunted in *Cry*-null mice; n=7-11 per condition. (**C**) Number of renal Epo-producing cells (REPC) per 20 mm^2 mid-kidney transverse sections by RNAscope® *in situ* hybridization, n=12-14 per condition. (**A-C**) Data are presented as scatter plots, with median, 25th and 75th percentile. Kruskal-Wallis test with Bonferroni as post-hoc test; n.s. not significant.

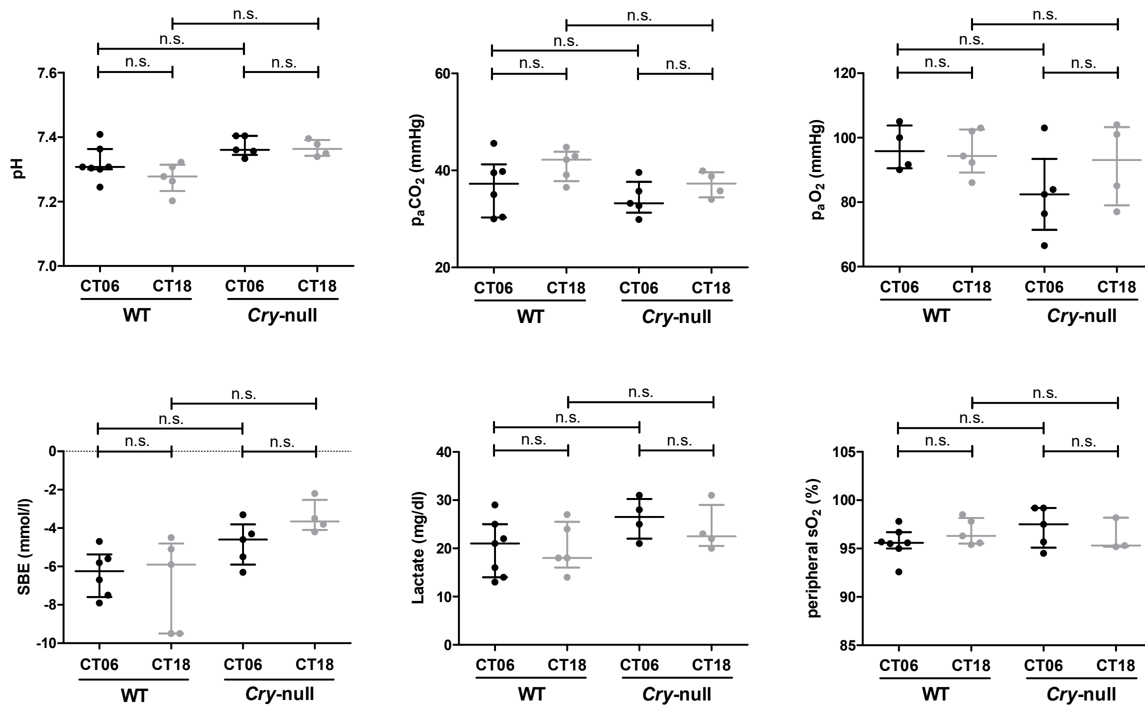

Figure 2. Analysis of arterial blood gas parameters and pulse oximetry. Specimens were obtained at CT06 (sleeping phase) or CT18 (activity phase) through rapid exsanguination via an arterial carotid catheter. Data are presented as scatter plots, with median, 25th and 75th percentile. Kruskal-Wallis test with Bonferroni as post-hoc test; n.s. not significant, n=3-7 per condition.

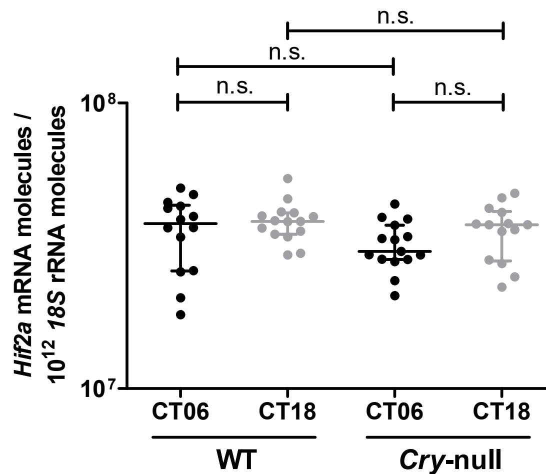

Figure 3. Expression of *Hif2a*. Real-time PCR-based quantification of the *Hif2a* mRNA expression in adult kidneys shows no difference in transcript levels at CT18 (activity phase) and CT06 (sleeping phase), neither in wild-type (WT) nor in *Cry*-null mice. Data are presented as scatter plots, with median, 25th and 75th percentile. Kruskal-Wallis test with Bonferroni as post-hoc test; n.s. not significant, n=13-15 per condition.

Figure 4. Activation of the E-box in the human minimal *EPO* promoter by the CLOCK/BMAL1 heterodimer. (**A**) Schematic representation of the minimal *EPO* promoter with E-box, hypoxia response elements (HRE1/2), and binding sites for Wilms' tumor suppressor 1 (WT1), Stimulatory Protein 1 (SP1), GATA proteins (GATA) and transcriptional start site (TSS). (**B**) The *EPO* promoter 119 [60] is significantly activated by CLOCK/BMAL1 overexpression in HEK293 cells (I, II), but not upon mutation of the E-box (III). A 6xE-box reporter served as a positive control for CLOCK/BMAL1 function (IV, V). Data are presented as scatter plots, with median, 25th and 75th percentile. Mann-Whitney-U test; n.s. not significant, n=4-6 per condition.

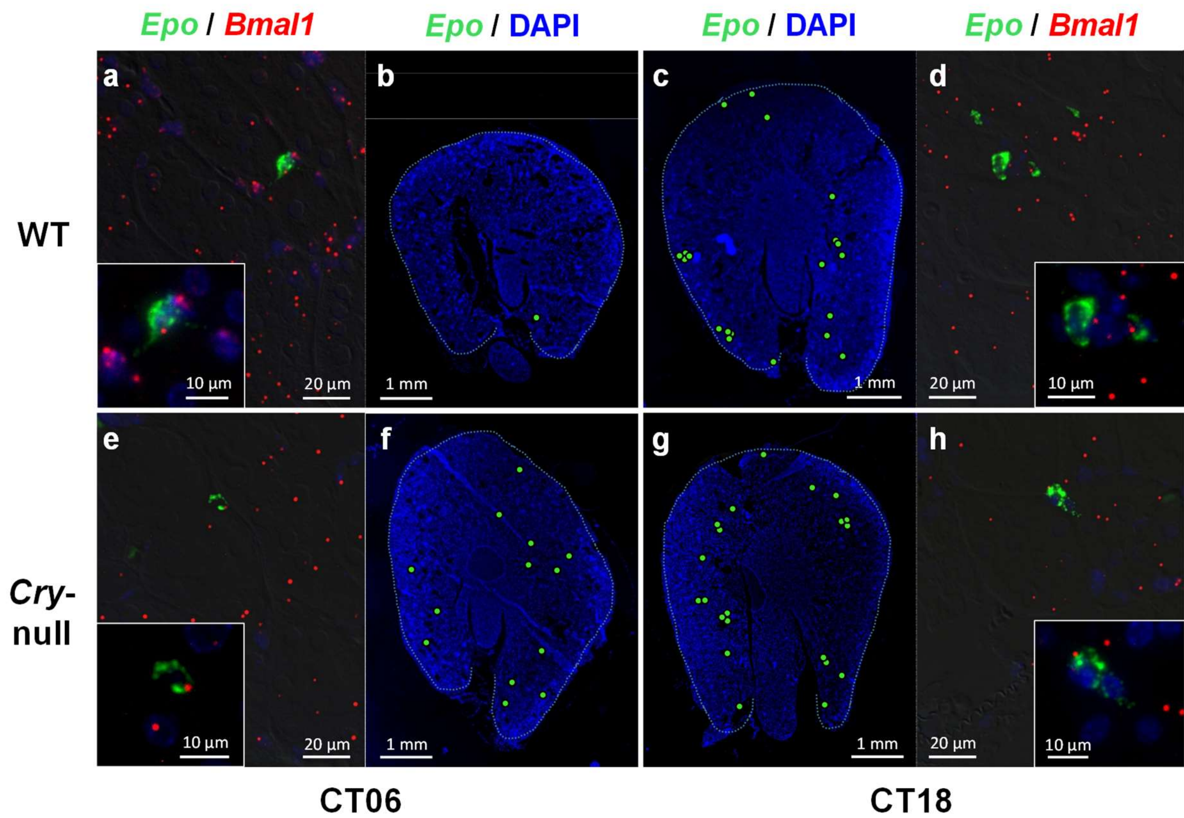

Figure 5. Co-localization of *Bmal1* and *Epo* in kidney. Double fluorescent staining of representative kidney sections from *Cry*-null and WT mice at CT06 and CT18 for *Epo* (green) and *Bmal1* (red) transcripts by RNAscope® and DAPI (blue), confirming *Bmal1* expression in REPCs (**a, d, e, h**) Lower magnification of a representative kidney with the REPC localization (green dots), showing the circadian REPC pattern in normoxic kidneys of WT (**b, c**) vs. *Cry*-null (**f, g**) mice.

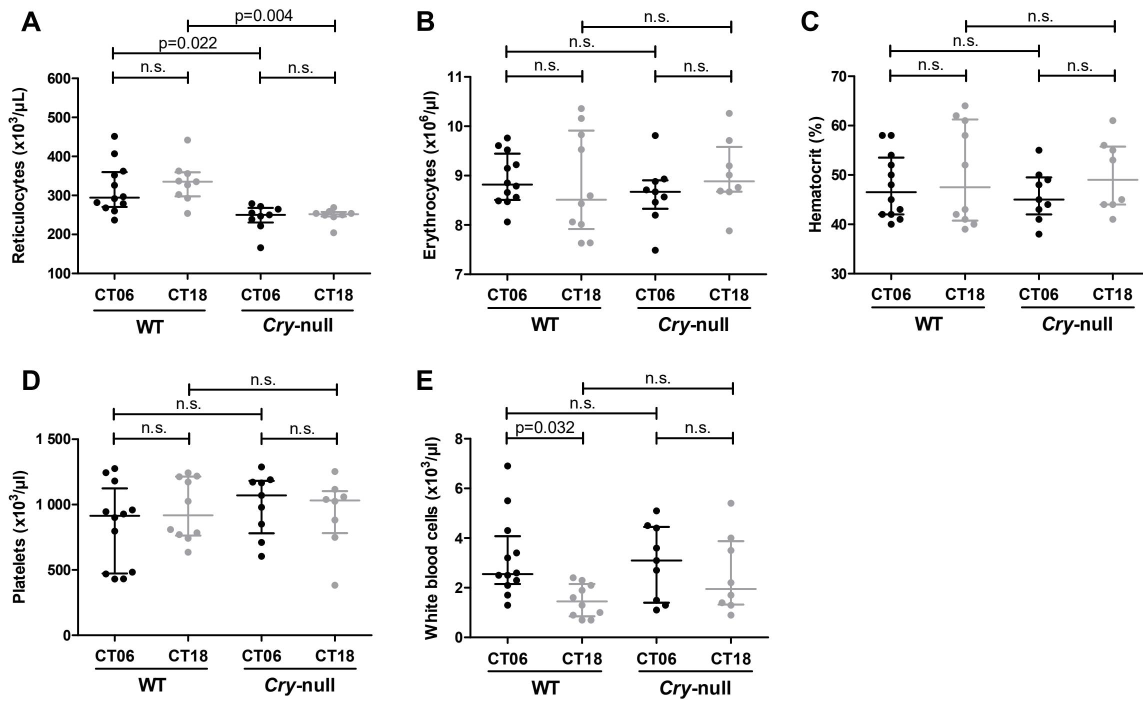

Figure 6. Analysis of hematological parameters and body weight. (**A**) Reticulocyte numbers, (**B**) erythrocyte numbers, and (**C**) hematocrit levels at CT06 vs. CT18 in WT or *Cry*-null mice. Time-of-day differences in these parameters were not significantly different. Reticulocyte numbers, however, were significantly lower in *Cry*-null than in WT mice at both time points. (**D**) Platelet numbers tended to be higher in *Cry*-null than in WT mice, both did not change significantly between CT06 vs. CT18 in both strains. (**E**) White blood cells were significantly lower at CT18 than at CT06 in WT animals; this effect was abolished in *Cry*-null mice. (**A-E**) Data are presented as scatter plots, with median, 25th and 75th percentile. Kruskal-Wallis test with Bonferroni as post-hoc test; n.s. not significant, n=8-12.