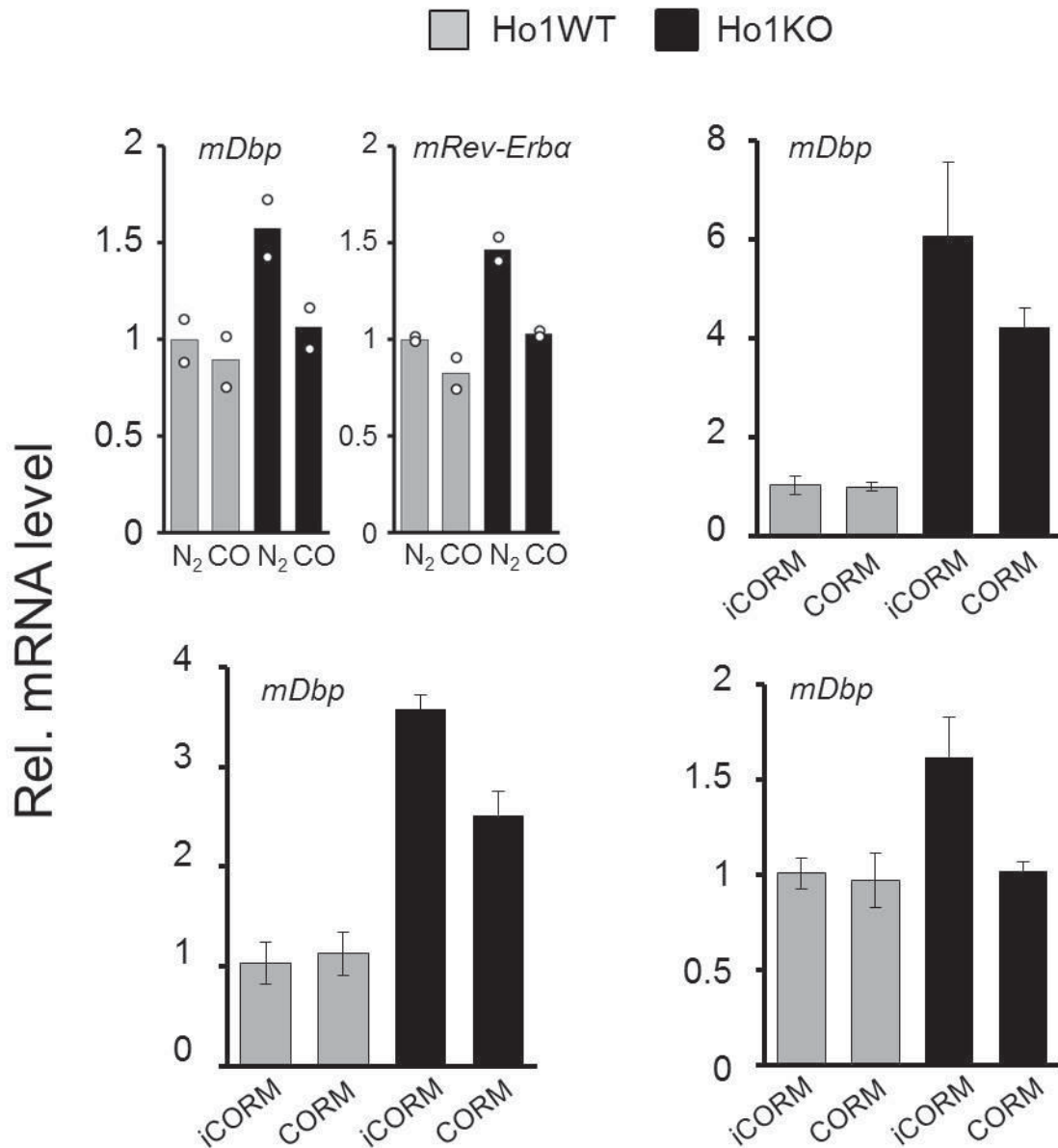## Supplementary Figure 1

Heme oxygenase 1 is regulated by the circadian clock: additional data.

(**a**) *Ho-1* mRNA levels oscillate in mouse peritoneal macrophages. These data are taken from our experiments published in Keller, M. *et al. Proc. Natl. Acad. Sci. USA.* **106**, 21407-21412 (2009). Cells harvested every 4 h via peritoneal lavage from four C57BL/6 mice kept in constant darkness were magnetically purified for CD11b surface expression. Three individual RNA samples of each time were pooled and subjected to global gene transcription measurement by using Affymetrix microarrays. Circadian oscillation of *Ho-1* transcript is significant ($p < 0.005$ CircWave). (**b**) *Ho-1* mRNA levels do not oscillate in primary hepatocytes from $Bmal1^{-/-}$ mice. *Ho-1* mRNA levels in hemin and CoPPIX treated (30 μM each) primary hepatocytes from $Bmal1^{-/-}$ mice. Cells were dexamethasone-synchronized and harvested after 24 hour and 40 hours, which correspond to trough and peak of *Ho-1* mRNA levels in wild-type cells (see **Fig. 2b**). Data are normalized to *Gapdh* expression and presented relative to mean expression in untreated cells. Given are means ± sd of three independent samples. (**c**) Conserved E-box (blue) in mouse, rat and human *Ho-1* promoters close the transcription start site (yellow) and start of coding region (red). (**d**) *Ho-1* mRNA levels are reduced in unsynchronized primary hepatocytes from $Bmal1^{-/-}$ mice compared to hepatocytes from wild-type littermates (wt). Data are normalized to *Gapdh* expression and presented relative to mean expression in wild-type cells. Given is mean ± sd of three independent samples.

1

Supplementary Figure 2

The heme-degradation product CO modulates circadian transcription: additional data.

Upper left: Transcript levels of *Rev-Erbα* and *Dbp* in CO or N_2 (6%) treated primary fibroblasts of *Ho-1*^{-/-} mice or wild-type littermates harvested 24 hours after dexamethasone synchronization. Other panels: Transcript levels of *Dbp* in embryonic fibroblasts from *Ho-1*^{-/-} mice (Ho1KO) or wild-type littermates (Ho1WT) 24 hours after dexamethasone synchronization, which were treated for 1 hour (or 3 x 1 hour; lower right) with 100 μM CO-releasing molecules (CORM) or inactive control molecules (iCORM) before harvesting. Data are normalized to *Gapdh* expression and presented relative to mean expression in wild-type control cells. Given is mean ± sem of three independent samples or – upper left – mean (bars) of two independent samples (small symbols).
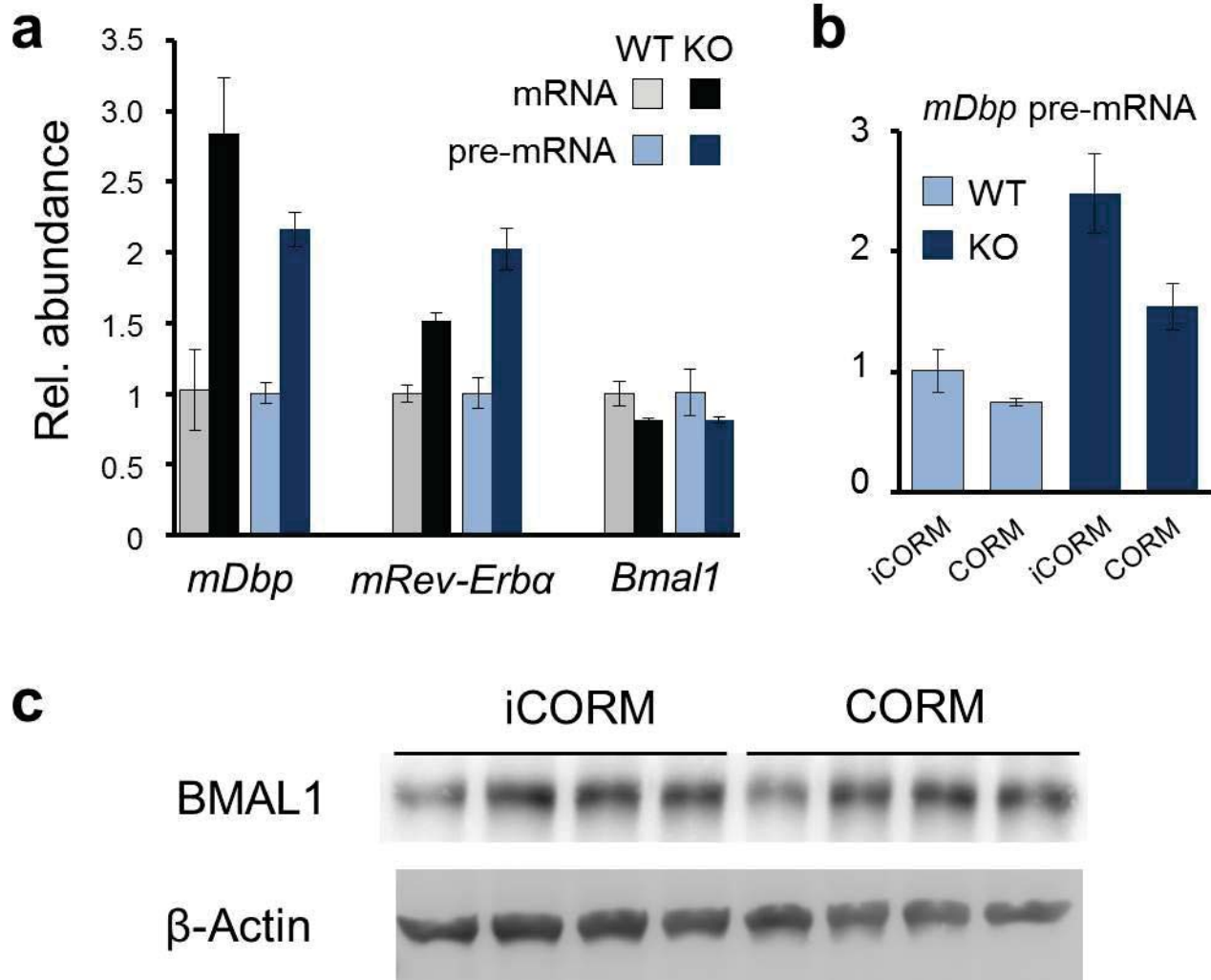
Supplementary Figure 3

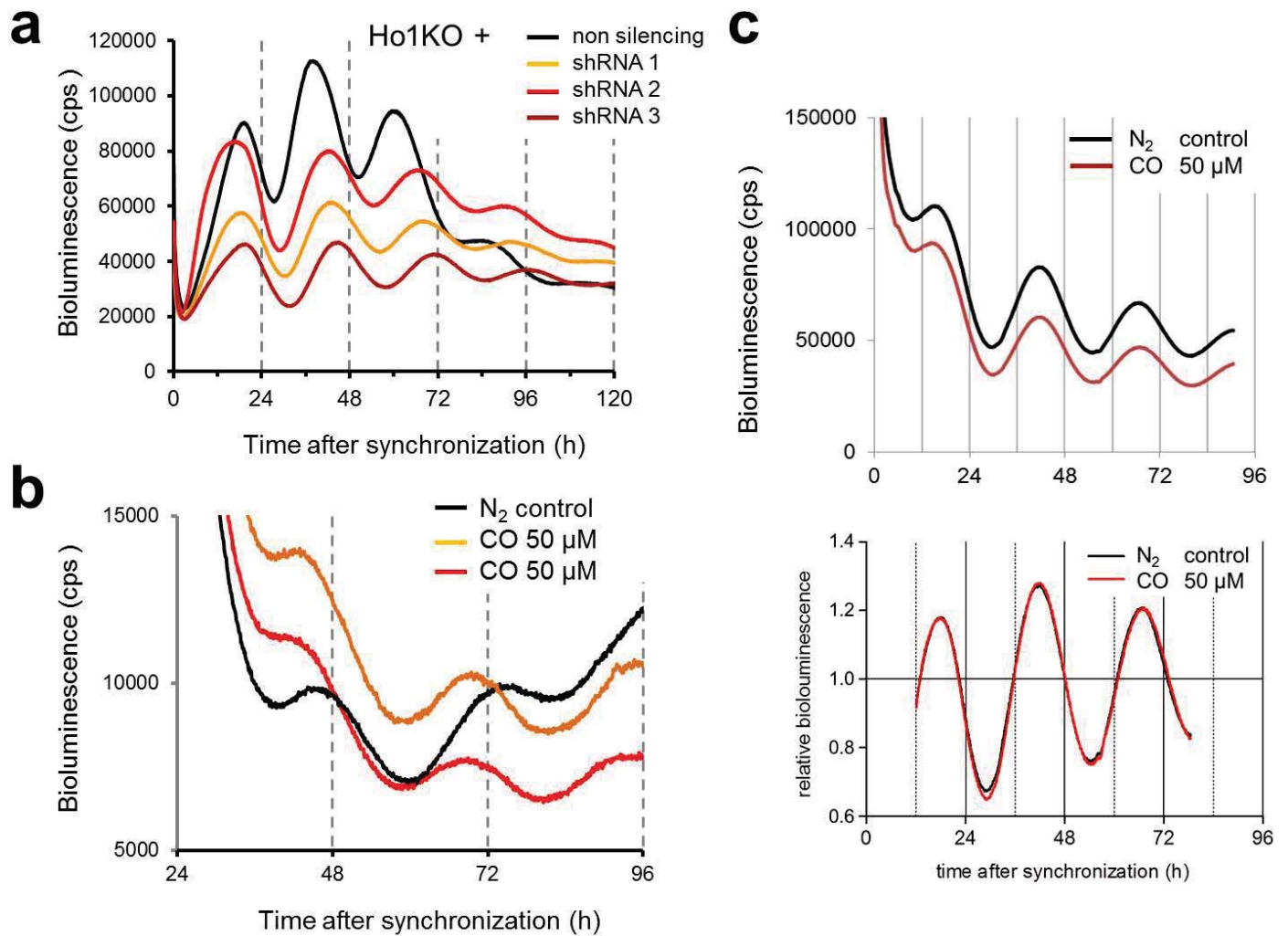CO modulates clock-gene expression at the transcriptional level.

(**a**) Clock gene mRNA and pre-mRNA levels of primary fibroblasts of *Ho-1*^{-/-} mice (KO) or wild-type littermates (WT) harvested 24 hours after dexamethasone synchronization. Data are normalized to *Gapdh* expression and presented relative to mean expression in wild-type control cells. Given is mean ± sd of three independent samples. (**b**) Pre-mRNA levels of *Dbp* in embryonic fibroblasts from *Ho-1*^{-/-} mice (KO) or wild-type littermates (WT) 24 hours after dexamethasone synchronization, which were treated for 1 hour with 100 µM CO-releasing molecules (CORM) or inactive control molecules (iCORM) before harvesting. Data are normalized to *Gapdh* expression and presented relative to mean expression in wild-type control cells. Given is mean ± sd of three independent samples. (**c**) CO does not acutely alter BMAL1 protein level. BMAL1 protein levels in U2-OS cells 24 hours after dexamethasone synchronization, which were treated for 1 hour with 100 µM CO-releasing molecules (CORM) or inactive control molecules (iCORM) before harvesting. Shown are four independent samples.

a

b
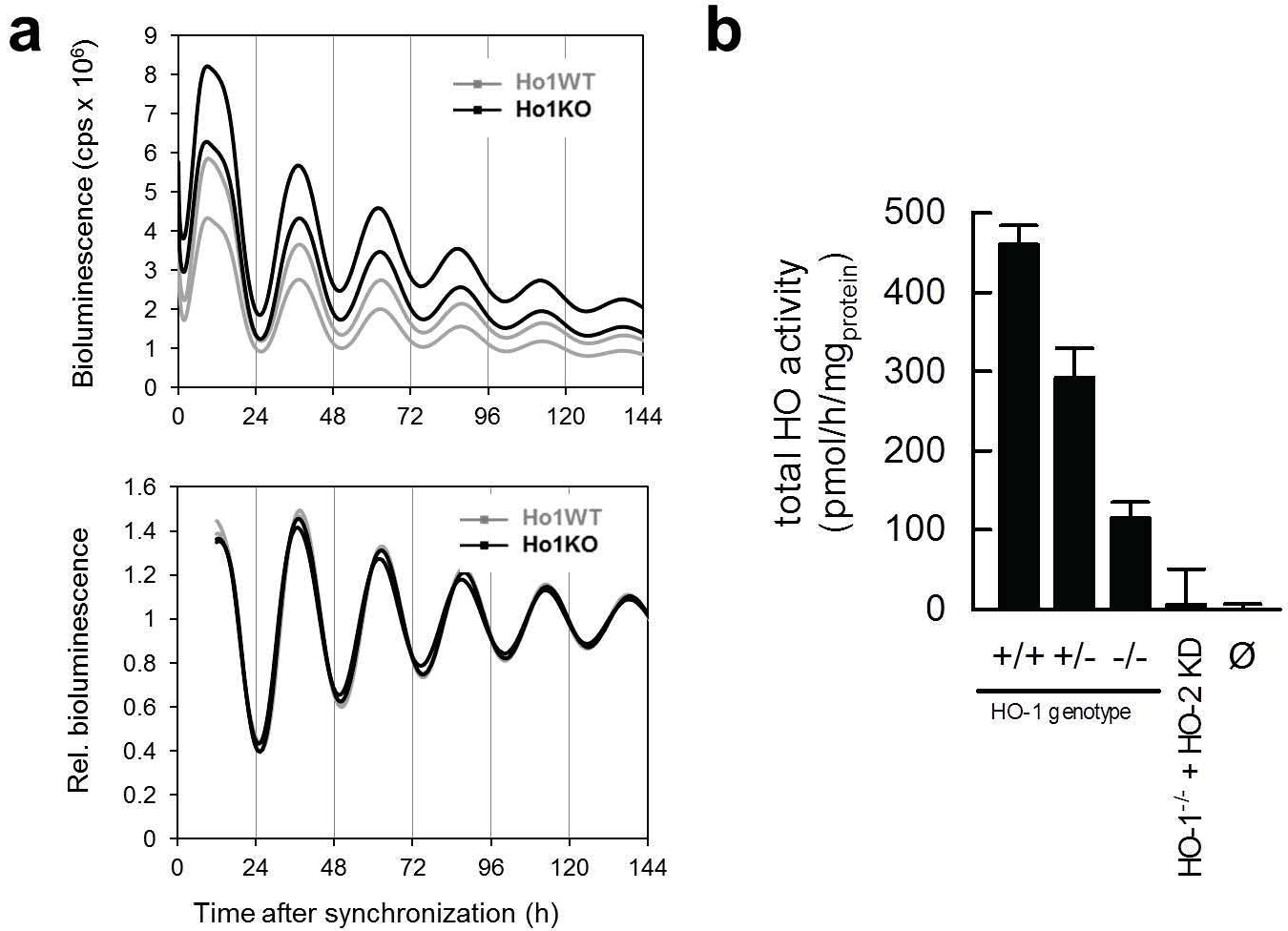
Supplementary Figure 4

Ho-1 knockout alone has no effect on circadian dynamics, probably because of residual activity from HO-2.

(**a**) Circadian oscillation dynamics of synchronized primary fibroblasts of *Ho-1*^{-/-} mice (Ho1KO) or wild-type littermates (Ho1WT) lentivirally transduced with a *Bmal1* promoter-luciferase reporter construct. Shown are two representative examples of raw data (upper panel) and detrended (lower panel) time-series for each genotype. Note, the absolute light levels slightly decrease upon *Ho-1* knockout consistent with the overall slightly lower *Bmal1* transcript levels (compare **Fig. 3a**). (**b**) Heme oxygenase activity in fibroblasts with specific depletion/knockout of heme oxygenase isoforms. Lysates of primary fibroblasts of indicated *Ho-1* genotype with or without additional RNAi-mediated depletion of *Ho-2* were analyzed for total heme oxygenase activity.

a

Bioluminescence (cps)

120000
100000
80000
60000
40000
20000
0

Ho1KO +

— non silencing
— shRNA 1
— shRNA 2
— shRNA 3

0   24   48   72   96   120

Time after synchronization (h)

b

Bioluminescence (cps)

15000

10000

5000

— N_2 control
— CO 50 µM
— CO 50 µM

24   48   72   96

Time after synchronization (h)

c

Bioluminescence (cps)

150000

100000

50000

0

— N_2 control
— CO   50 µM

0   24   48   72   96

relative bioluminescence

1.4
1.2
1.0
0.8
0.6

— N_2 control
— CO   50 µM

0   24   48   72   96

time after synchronization (h)

Supplementary Figure 5

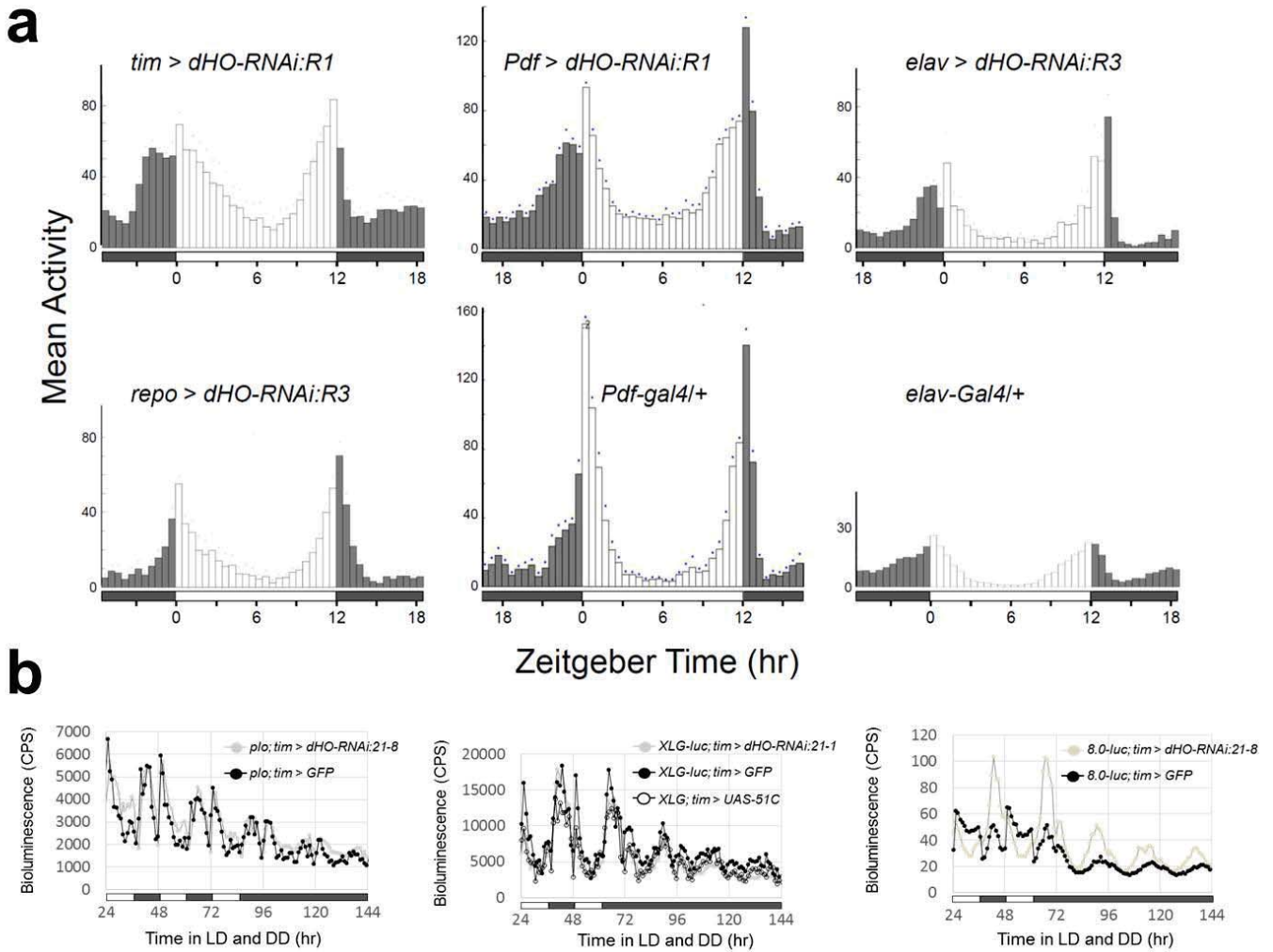Heme oxygenase–derived CO is essential for normal circadian dynamics in mammalian cells: additional data.

(**a**) Circadian dynamics of synchronized primary fibroblasts from *Ho-1*^{-/-} mice lentivirally transduced with (i) shRNA constructs targeting *Ho-2* or a non-silencing (ns) control and (ii) a *Bmal1* promoter-luciferase reporter construct. Shown are representative examples of raw data time-series. For detrended time series and period quantification see **Fig. 5a**. Note, that the absolute light levels decrease upon *Ho-2* knockdown consistent with the overall increase in *Rev-Erbα* levels and thus putatively lower *Bmal1* transcript levels (compare **Fig. 5b**). (**b**) CO partly rescues the long period oscillations in heme oxygenase depleted cells. Circadian oscillation dynamics of synchronized primary fibroblasts from *Ho-1*^{-/-} mice lentivirally transduced with (i) shRNA constructs targeting *Ho-2* and (ii) a *Bmal1* promoter-luciferase reporter construct. Cells were continuously treated with 6% CO or N_2. Shown are representative examples of raw data time-series. For detrended time series see **Fig. 5c**. (**c**) Exogenous CO treatment has no effect on oscillation dynamics of wild-type cells. Circadian oscillation dynamics of synchronized primary fibroblasts from wild-type mice lentivirally transduced with a *Bmal1* promoter-luciferase reporter construct, which were continuously treated with 6% CO or N_2. Shown is a representative example of raw data (upper panel) and detrended time-series (lower panel). Note, since CO can only shorten the circadian period in *Ho*-depleted cells (**Fig. 5c**) but not in wild-type cells, period lengthening in *Ho*-depleted cells is very likely specifically due to the lack of endogenous CO rather than to other functions of heme oxygenases.
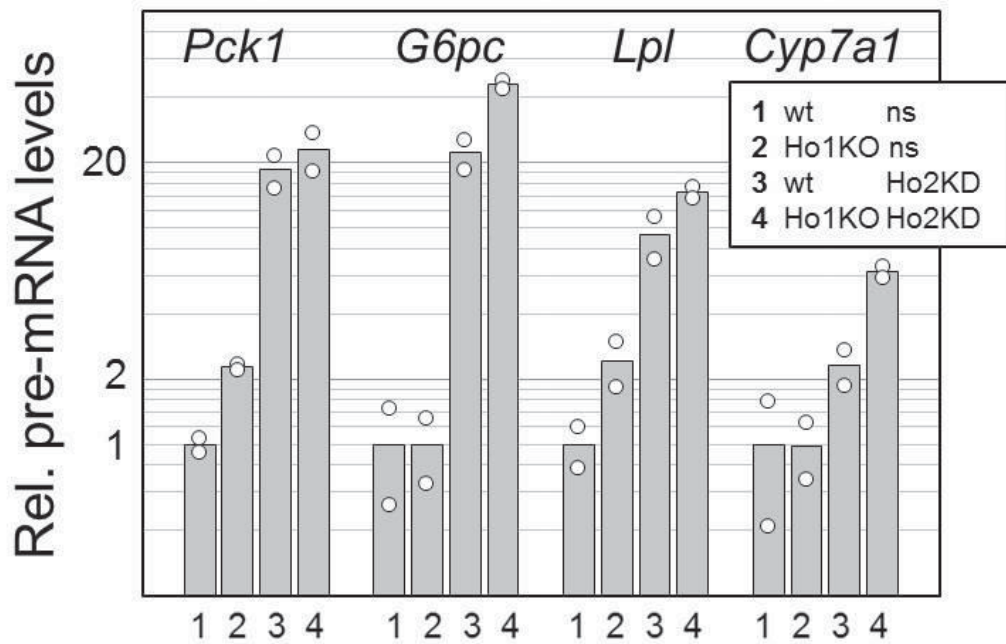
Supplementary Figure 6

Heme oxygenases modulate metabolic gene expression in primary hepatocytes at the transcriptional level.

Pre-mRNA levels of *Pck1*, *G6pc*, *Lpl* and *Cyp7a1* in primary hepatocytes (24 hours after dexamethasone synchronization) of *Ho-1*^{-/-} or wild-type littermate mice with or without additional *Ho-2* depletion by RNAi. Data are normalized to *Gapdh* expression and presented relative to mean expression in wild-type cells transduced with the non-silencing control. Shown are mean levels (bars) of two independent samples (small symbols).

Supplementary Figure 7

Heme oxygenase (dHo) is essential for normal daily activity patterns in *Drosophila*: additional data.

(**a**) Mean locomotor activity of *Drosophila* males analysed in 12 hr light: 12 hr dark conditions for 5-8 days. A different *dHo-RNAi* construct inserted either on chromosome *3* (*UAS-dHo-RNAi:R1*) or chromosome *2* (*UAS-dHo-RNAi:R3*) was either driven in all clock cells: *timeless-gal4* (*tim >*); PDF-positive clock neurons: *Pdf-gal4* (*Pdf >*); all neurons: *elav-gal4* (*elav >*), or all glia cells: *repo-gal4* (*repo>*). Note the phase advance caused by *dHo* knockdown in clock cells and neurons, whereas glia-specific knockdown or 'driver-only' controls results in normal behaviour. At least 16 flies were tested for each genotype. Bars show mean activity within in 30 min; dark bars: 'lights-off', white bars: 'lights on', dots indicate sem. (**b**) Real-time luciferase recordings of flies expressing different *period-luciferase* fusion genes. Male flies were recorded in LD and DD as indicated by the bars below the plots (white and black bars indicate 'lights-on' and 'lights-off' respectively). Left: Flies expressing *luciferase* under control of the *period* promoter in all clock cells (including peripheral clocks) encoded by the *plo* transgene[29]: grey circles—*tim-gal4:27/+;plo:86-6/UAS-dHo-RNAi:21-1* (n=16); black circles—*tim-gal4:27/UAS-GFP;plo:86:6/+* (n=12). Middle: Flies expressing a PERIOD-LUCIFERASE fusion protein in all clock cells (including peripheral clocks) encoded by the *XLG-luc* transgene[30]: grey circles—*tim-gal4:27/+;XLG-luc/UAS-dHo-RNAi:21-1* (n=8); black circles—*tim-gal4:27/UAS-GFP;XLG-luc/+* (n=8); open circles—*tim-gal4:27/UAS-attP-51C;XLG-luc/+* (n=4). Note that in the left and middle panels no significant differences are observable between *dHo-RNAi* and control flies. Similar results were obtained in two independent experiments including the *dHoRNAi:21-8* line. Right: Flies expressing a PERIOD-LUCIFERASE fusion protein in dorsal clock neurons encoded by the promoter-less *8.0-luc* transgene[30]. Exact genotypes: grey circles—*8.0-luc/+;tim-gal4:67/UAS-dHo-RNAi:21-8* (n=8); black circles—*8.0-luc/UAS-GFP;tim-gal4:67/+* (n=10). Note the higher peak levels in LD and DD, and the increased amplitude of PER-LUC oscillations in *dHo-RNAi* flies.

Supplementary Note 1

It is difficult to estimate appropriate concentrations for a CO treatment experiment because of the following reasons: (i) The concentration of endogenous intracellular CO is only very roughly known and only for olfactory receptor neurons. Ingi *et al.* determined the CO production to be 1.6 nmol/mg protein per 6 hr and assumed - based on the diffusion of CO - that "the amount of CO produced for 10-30 min is an effective concentration in the cell" thus estimating 10-30 μM endogenous CO[32]. To our knowledge, there is no other report with similar measurements for other cell types. In addition, the expression levels of heme oxygenases 1 and 2 vary widely between cell and tissue types[69-71] and *Ho-1* is induced by various agents and conditions (including glucose and oxidative stress); thus, any estimation of endogenous CO concentration in a given cell type, cell compartment and under a given cellular condition should be viewed with extreme caution. (ii) When applying exogenous CO (either gaseous or as CORMs), it is similarly difficult to estimate the effective local CO concentrations that reaches a cellular compartment, since CO is a diffusible molecule with many high-affinity biological targets. In the literature, the reported CO concentrations used for such experiments vary widely (for a review see ref. 72). Based on these considerations, we decided to use 50 μM CO (in the cell culture medium) in our continuous gas treatment experiments and 100 μM CORMs (because here the CO volatilizes rapidly).

Supplementary Note 2

In *Ho*-depleted hepatocytes not only target genes of BMAL1 are significantly enriched in clusters 1 and 2 (**Fig. 6b**) but also targets of REV-ERBα/β[18], especially those which can bind all three transcription factors (not shown). Could the upregulation of cluster 1/2 gene expression also be due to CO *directly* acting on REV-ERBα/β repressor function? While this is conceivable, because REV-ERBα/β contain heme as a ligand[4,5] potentially coordinating diatomic gases such as nitric oxide (NO) or CO, it is nevertheless unlikely for the following reasons: In *Ho*-depleted hepatocytes *Rev-Erbα* transcription is upregulated (**Fig. 6a**), yet the expression of REV-ERBα/β targets is also upregulated suggesting that - if at all – *Ho*-depletion leads to a *decreased* REV-ERBα/β repressor activity. In *Drosophila*, the nuclear receptor E75 – the closest homolog to the mammalian *Rev-Erb* family – also contains heme and its repressor activity is modulated at least by NO (for CO it is still unclear[73]). However, NO *antagonizes* the receptor activity of E75 and mammalian REV-ERB proteins[5,74] – if CO would act similarly, REV-ERBα/β repressor activity should be *increased* in *Ho*-depleted (and thus CO-depleted) cells and consequently REV-ERBα/β target gene expression should be suppressed and not increased.

A second possibility is that altered (*i.e.* likely increased) heme levels in *Ho*-depleted cells positively modulate REV-ERBα/β repressor activity[4,5]. However, also this should lead to suppressed transcription of REV-ERBα/β target genes – the opposite of what we observe.

A third possibility is that increased oxidative stress or vulnerability towards oxidative stress in *Ho*-depleted cells[14,75] acts negatively on heme binding to REV-ERB proteins[76] thereby impairing their repressor activity[4,5], which may contribute to the observed upregulation of REV-ERBα/β target gene expression. Could oxidative stress in *Ho*-depleted cells also be indirectly responsible for CLOCK(NPAS2)-BMAL1 target gene expression, since oxidative stress also changes the NAD(P)H/NAD(P) ratio that is reported to influence CLOCK(NPAS2)-BMAL1 binding to the DNA[77]? Probably not, because oxidative conditions *inhibit* DNA binding of CLOCK(NPAS2)-BMAL1, while in *Ho*-depleted cells CLOCK(NPAS2)-BMAL1 target gene expression is *upregulated*.

Rather than having a *direct* effect on REV-ERB-mediated transcriptional control, it is much more likely that CO acts *indirectly* on REV-ERBα/β target gene expression *via* modulating CLOCK(NPAS2)-BMAL1 DNA binding. Given the strong overlap of the BMAL1 and REV-ERBα/β cistromes[18] it is plausible that - due to a possible competition at the cistrome level - *Ho*-depletion not only supports CLOCK(NPAS2)-BMAL1 DNA binding but thereby also alters REV-ERBα/β DNA occupancy thereby leading to a de-repression of REV-ERBα/β target gene expression.

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Genotype	Rhythmic Flies (%)	Rhythmic (n)	Arrhythmic (n)	Tested (n)	τ.Mean (h)	τ.SEM (h)	RS Mean	RS SEM
21-1/+	87	20	3	23	23.9	0.1	2.9	0.2
21-1/Pdf-Gal4	91.3	21	2	23	23.6	0.1	3.5	0.2
21-1/tim-Gal4	77.5	24	8	32	23.6	0.1	3.4	0.2
21-1/elav-Gal4	66.7	18	9	27	23.3	0.1	2.9	0.2
R3/+	87.5	14	2	16	24.1	0.2	2.6	0.2
R3/Pdf-Gal4	100	16	0	16	24.7	0.1	3.8	0.2
R3/tim-Gal4	85.7	30	5	35	24.3	0.1	3.8	0.2
R3/elav-Gal4	60.5	23	15	38	24.0	0.1	2.6	0.1
R1/+	93.4	15	1	16	24.0	0.1	3.4	0.2
R1/Pdf-Gal4	96.9	31	1	32	24.3	0.1	4.8	0.2
R1/tim-Gal4	97.8	44	1	45	24.2	0.1	4.0	0.2
R1/elav-Gal4	79.4	27	7	34	23.9	0.1	2.9	0.1
elav-Gal4/+	75	18	6	24	24.1	0.2	3.1	0.3
tim-Gal4/+	100	16	0	16	23.5	0.1	4.7	0.4
Pdf--Gal4/+	95.8	23	1	24	24.1	0.1	4.1	0.3

Supplementary Table 2: Free running behaviour of *dHo-RNAi* flies and controls. Flies were analysed for 7 days in DD and 25°C after being exposed to 12 hr: 12 hr light:dark cycles at the same temperature (**Fig. 8a, Supplementary Fig. 7b**). All genotypes show robust circadian rhythmicity with normal free-running periods. RS: Measure for rhythm strength. See Methods section for details and calculation of rhythm parameters.