Supplementary Tables and Figures

Circadian Clocks in Human Red Blood Cells

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Supplementary Table 1 "Clock" protein expression in human red blood cells. We used BLASTed full human protein sequences for Bmal1, Clock, Cry1/2, Per1-3, Dec1/2 and NPAS2 ("nuclear" clock genes) to search the Human Red Blood Cell protein database[1]. "Cytosolic" clock components such as Casein Kinase 1delta/epsilon (CSNK1D/E) and GSK3beta (GSK3B) were clearly detectable in red cells, as were "positive controls" that were checked in parallel, including beta-Actin (ACTB) and Spectrin-beta (SPTB). URL: http://proteome.biochem.mpg.de/RBC.

Supplementary Figure 1 Oppositely-phased temperature-entrainment of red blood cells. Representative immunoblots showing PRX-$SO_{2/3}$ dimer during temperature-entrainment of red cells from Subject B with oppositely-phased cycles. Quantification of the blots by densitometry is shown below each blot. Loading controls (Coomassie-stained gels showing haemoglobin monomer bands) for each blot and quantification of immunoblots by densitometry are shown below.

Supplementary Figure 2 Analysis of whole blood, white cell fractions and red cell preparations. a, Representative flow cytometry density plots (FL3-H vs. forward scatter) for whole blood, white cell fractions, and red blood cell preparations stained with Vibrant Ruby, a cell-permeant DNA stain that can be used for live imaging of cells because of its very low cytotoxicity. The plots shown are based on counting 2 x 10^4 cells from each fraction. **b**, Quantification of cell counts for three experimental subjects. Cells (2 x 10^4) from each fraction, from each subject, were counted and designated as nucleated (FL3-H > 100) or non-nucleated (FL3-H < 100) based on analysis of all of the density plots (see above). The data are shown on a linear scale and logarithmic scale below to illustrate the small absolute white cell count in whole blood and red cell preparations. The proportions attributable to nucleated cells are shown on the right. All data are means, and where errors are shown then are standard errors of the mean (SEM). *** p = 0.0002 (two-tailed t-test, whole blood vs. red cells). **c**, Whole blood or red cells were haemolysed by the addition of hypotonic PBS buffer and then re-suspended in normal PBS. Samples from red blood cells (RBC) were haemolysed at 4, 24 and 72 hours incubation and the viability of residual nucleated cells was assayed by a trypan blue based method. The percentage viability as determined by the automated counter are shown. Values are means + SEM. **d**, Immunoblots for oxidised peroxiredoxin (PRX-$SO_{2/3}$) for dilution series for each cell fraction and experimental subject. We used 300 x 10^6 cells / ml lysate for loading on gels (5 µl / lane) in all the main experiments, which equates to ~1 x 10^6 white cells / ml (0.210%) in whole blood, and ~0.1 x 10^6 white cells / ml (0.023%) in our red cell preparations obtained by gradient density centrifugation. Both these numbers of cells gave no signal on the immunoblots.

Supplementary Figure 3 Grouped and individual replicate data for transcriptional and translational inhibition experiment. Red cells were entrained under temperature cycles for two complete cycles for the

α-amanitin experiment or three cycles for the cycloheximide experiment. Cells were then put into free-running, constant conditions (at 37°C, in total darkness) and sampled as shown. Quantification of PRX-$SO_{2/3}$ immunoblots by densitometry is shown for **a**, treatment with α-amanitin (α-AMN) or **b**, treatment with cycloheximide (CHX). Vehicle-treated controls are also shown (VEH). Values were normalised to the maximum for each blot. The solid line shows the mean normalised intensity at each time-point, with boundaries of the standard error of the mean (SEM) shown by the grey lines above and below. Profiles for each experimental subject (biological replicate) are also shown on the right. Values were normalised to the maximum for each blot. For either treatment, 2-way ANOVA (drug treatment x time) did not reveal a difference (not significant, n.s.) between drug and vehicle treatment (p = 0.0606 for α-AMN vs. VEH, p = 0.3428 for CHX vs. VEH), but there was a significant effect of time across all treatment groups (p < 0.0001 for both). We noted that the period of oscillation was different in the two experiments, which is explained by the relative time that the RBCs had been in culture for – the α-amanitin experiment was performed after 2 cycles (48 hours) of temperature entrainment, whereas the cycloheximide experiment was carried out after 3 cycles (72 hours). Since there is only a finite amount of glucose present in each of the tubes housing samples, this is gradually used up over time, resulting in a prolonged period. This is in line with similar phenomena reported in transcriptional rhythms, which show that there is a clear dose-response relationship between decreasing extracellular glucose in bathing medium and lengthening period in human U2OS cell line cultures[2].

Supplementary Figure 4 Technical information about PRX immunoblots a, Immunoblots showing total 2-Cys PRX and PRX3 in white and red blood cells. Equal numbers of cells were used for loading, as shown by the total PRX blot. **b**, Analysis of red blood cells exposed to hydrogen peroxide (H_2O_2) *in vitro*. Equal numbers of red cells from three subjects (A, B, C) were incubated by with 0-1000 μM H_2O_2 for 15 mins at room temperature and then lysed and immunoblotted for PRX-SO_3 as a quantitative control for blots shown elsewhere. **c**, Whole immunoblots showing effect of reducing agent (1:10 β-mercaptoethanol) on PRX-SO_3 oligomeric form distribution (see Supplementary Figure 5).

Supplementary Figure 5 Oligomerisation of peroxiredoxins. Oligomerisation patterns of **a**, PRX-SO_3 **a**, PRX1 and **c**, PRX2 in red cells. Following two cycles of temperature entrainment (12 h at 32°C, 12 h at 37°C), the cells were kept at constant temperature (37°C) for the rest of the experiment, and sampled 4-hourly. A loading control (Coomassie-stained gels showing haemoglobin monomer bands) is shown in Figure 4b. Molecular weights (obtained using Invitrogen Novex Sharp markers) are shown on the left, and bands representing multimer, dimer and monomer forms of the PRX protein are labelled on the right hand side.

Supplementary Figure 6 Circadian rhythms in haemoglobin oxidation and NADH/NADPH. a, Individual replicates (wells in 96-well plate) of intrinsic front-face fluorescence (FFF) measurements in red blood cells and "no cell" controls (wells filled with identical buffer, but without red cells). Representative data from experimental subject A are shown here. This technique makes use of the unique spectral properties

of Hb that are specific to the tetrameric (mostly reduced) or dimeric (mostly oxidised) forms, largely attributable to a specific tryptophan residue (Trp-37) of the Hb β-subunit. An increase in fluorescence results from formation of dimeric (oxidised) metHb subunits[3]. Fluorescence emission at 325 nm was measured after excitation at 280 nm every 15 mins. RFU = relative fluorescent units. See Fig. 5a in main text for further details. Profiles for **b**, NADH and **c**, NADPH concentrations in red blood cells are also shown. The mean value for n=3 technical replicates for each of three experimental subjects (A, B, C) is shown, as is the standard error of the mean (SEM). **d**, ATP levels in red blood cells from Subjects A, B and C, kept under free-running conditions (at 37°C, in total darkness). Data plotted are mean values for three technical replicates for each time-point with respective standard errors of the mean (SEM) shown. **e**, Bmal1 protein rhythms in mouse embryonic fibroblasts (MEFs). MEFs from wild-type or mCry1/2 double-knockout mice were entrained in temperature cycles and then put into a constant temperature of 37°C for the rest of the experiment (see Fig. 6 for details). A representative immunoblot is shown.

Supplementary Figure 7 Circadian rhythms in human U2OS cells are affected by specific *PRX* gene knockdown. Real-time bioluminescence data for *Bmal-dluc* U2OS cells treated with siRNAs directed against human **a**, *PRX1*, **b**, *PRX2*, **c**, *PRX3*, **d**, *PRX4* **e**, *PRX5*, **f**, *PRX6* are re-drawn from data retrieved from the BioGPS database (http://biogps.gnf.org/)[4]. Positive controls (treatment with siRNA directed against CRY2, resulting in a long period) and negative controls (scrambled siRNA) are shown for comparison[4].

Supplementary Table 2 Summary of the effects of siRNA directed against specific *PRX* on period or amplitude in human U2OS cells. Raw data traces are shown in Supplementary Figure 7. Each siRNA that resulted in a parameter change is listed (as a GNFid)[4].

Supplementary Figure 8 Proposed model of interconnectivity between circadian redox processes in the cytoplasm and nucleus. In the cytoplasm, oxidation (Ox) and reduction (Re) of peroxiredoxins (Prx), NADH and NADPH occur with circadian periodicity. Circadian rhythms in NADH/NADPH, which are able to move between cytoplasm and nucleus, can directly modulate the DNA-binding activity of Clock:Bmal1 and NPAS2:Bmal1, controlling rhythmic transcription at genomic loci.

Supplementary Figure 9 Proposed model of interconnectivity between circadian oscillations in the cytoplasm and nucleus. a, In a normal, nucleated cell, an interdependent non-transcriptional oscillator and transcription-translation feedback loop (TTFL) drive peroxiredoxin rhythms (in abundance and post-translational modification – e.g. sulphonylation). **b,** In red blood cells, there is no nucleus and therefore no transcription. Oscillations must therefore be driven by the non-transcriptional oscillator, which drives post-translational rhythms in peroxiredoxins (oxidation-reduction / sulphonylation). **c,** In circadian clock mutant cells, e.g. *mCry1/2* double-knockout mouse embryonic fibroblasts (MEFs), although the TTFL is incompetent, the nucleus (and thus transcription) is still intact, and can therefore still interact and

influence the non-transcriptional oscillator and peroxiredoxin rhythms, directly and indirectly. Thus, **b** and **c** are not equivalent states.

References

S1. Pasini, E.M. et al. In-depth analysis of the membrane and cytosolic proteome of red blood cells. *Blood* **108**, 791-801 (2006).

S2. Lamia, K.A. et al. AMPK regulates the circadian clock by cryptochrome phosphorylation and degradation. *Science* **326**, 437-40 (2009).

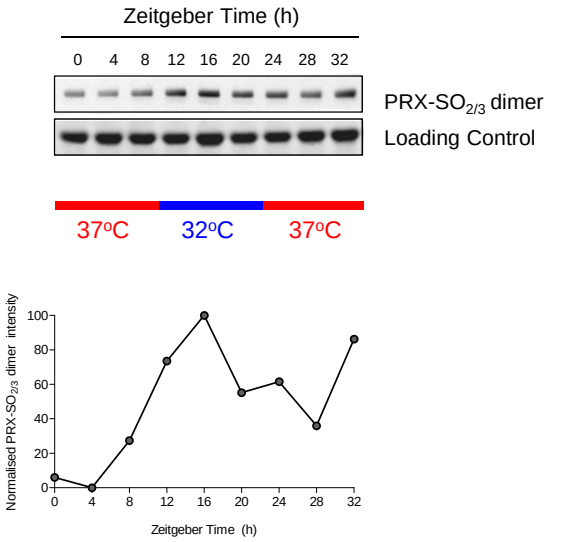
S3. Kennett, E.C. et al. Investigation of methaemoglobin reduction by extracellular NADH in mammalian erythrocytes. *Int J Biochem Cell Biol* **37**, 1438-45 (2005).

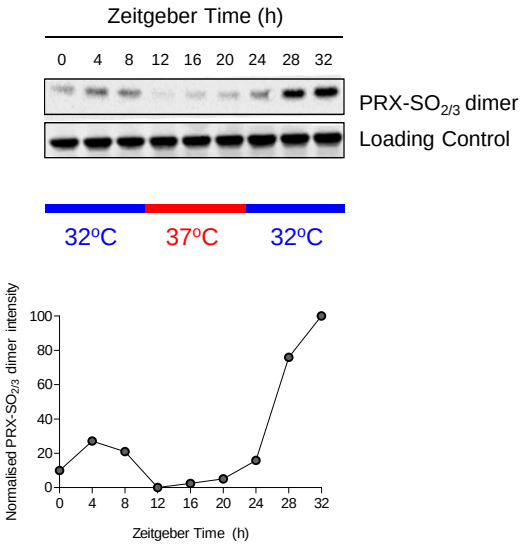
S4. Zhang, E.E. et al. A genome-wide RNAi screen for modifiers of the circadian clock in human cells. *Cell* **139**, 199-210 (2009).

Supplementary Table 1

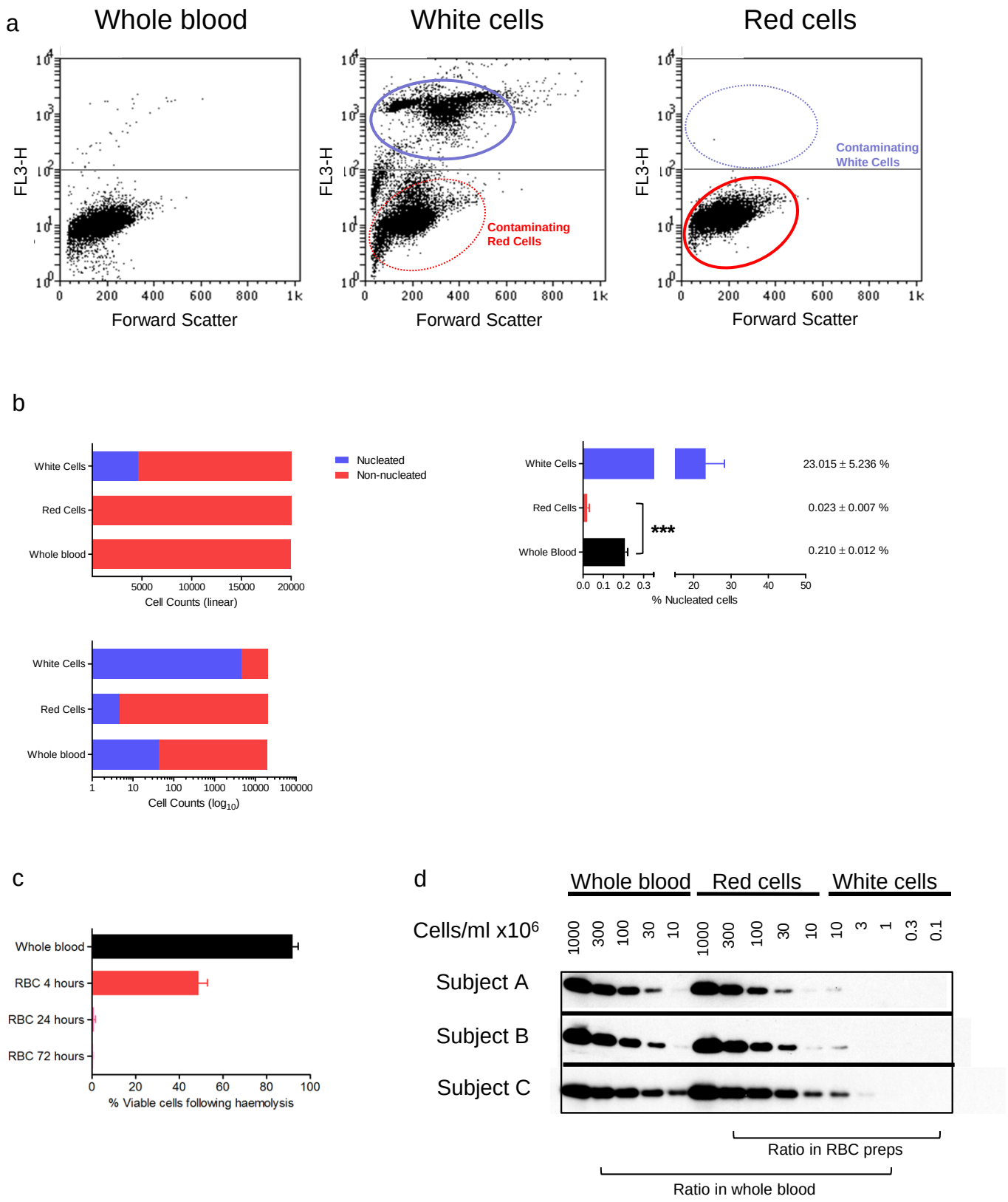
Symbol	Accession	Found in Human Red Blood Cell database ?
ARNTL (BMAL1)	NP_001025443	No
CLOCK	AAB83969	No
CRY1	NP_004066	No
CRY2	AAH35161	No
PER1	AAG01149	No
PER2	NP_073728	No
PER3	BAB63255	No
DEC1	BAA21720	No
DEC2	BAB21502	No
NPAS2	AAH28107	No
CSNK1D	AAH03558	Yes
CSNK1E	CAG30315	Yes
GSK3B	CAG38748	Yes
ACTB	AAI13037	Yes
SPTB	NP_001020029	Yes

Supplementary Figure 1

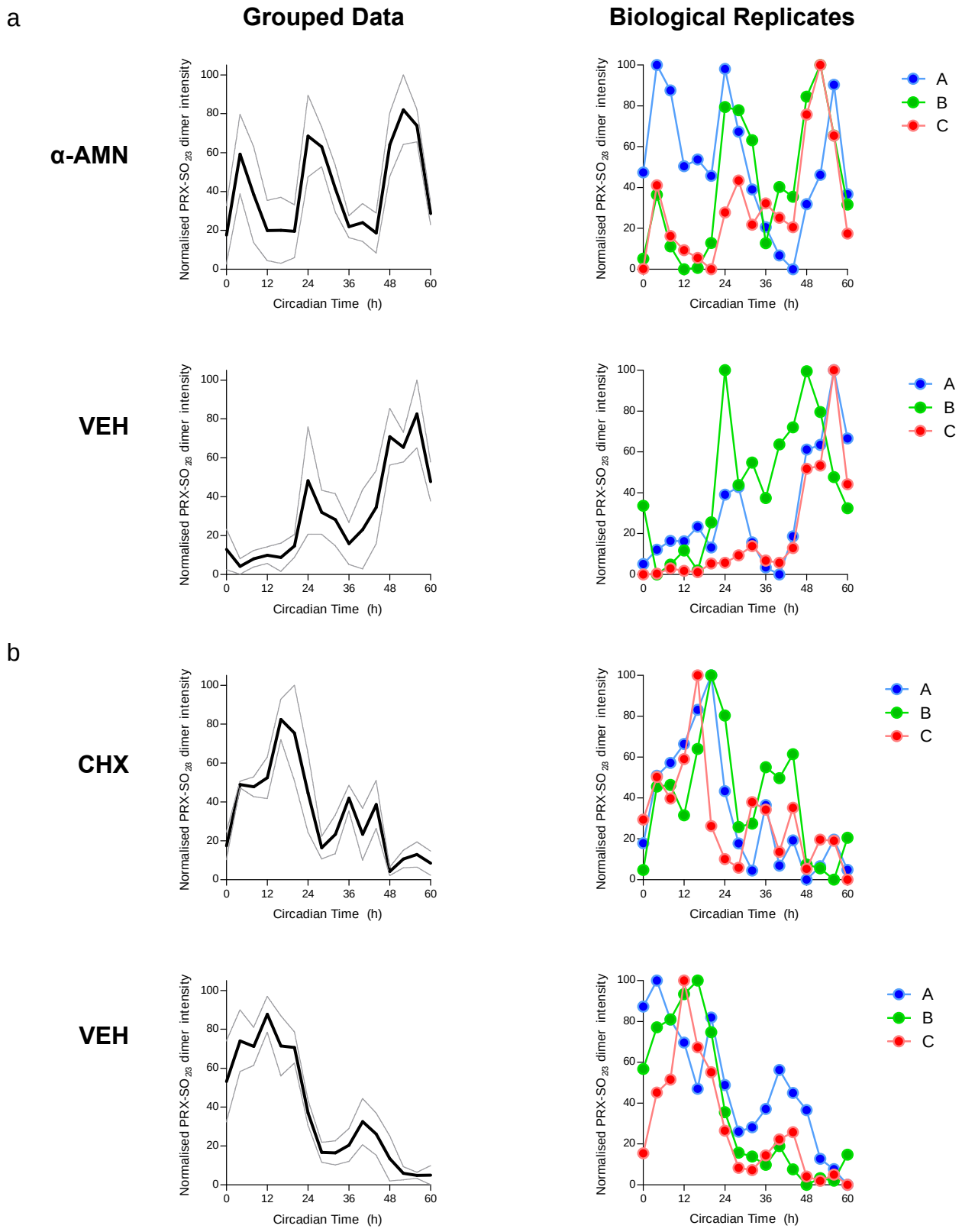




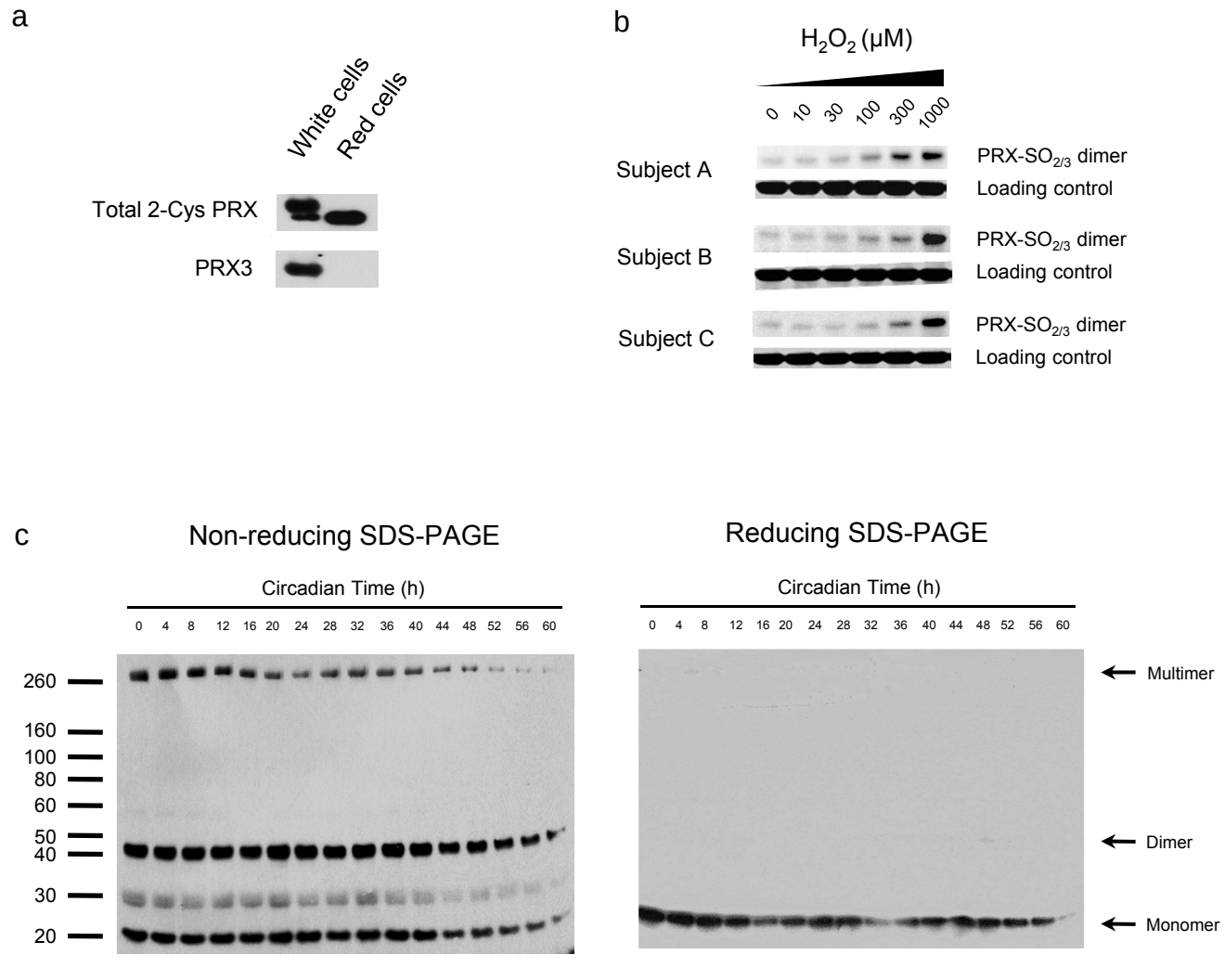
Supplementary Figure 2



Supplementary Figure 3

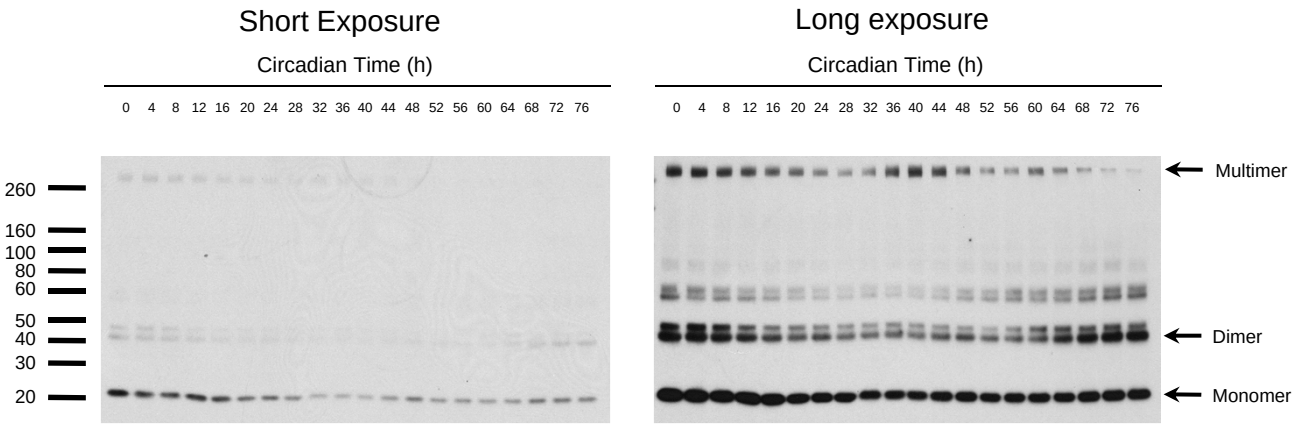


Supplementary Figure 4

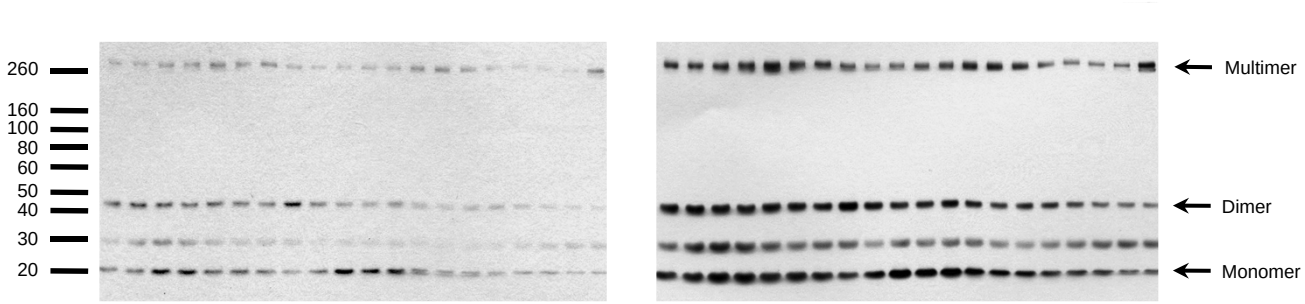


Supplementary Figure 5

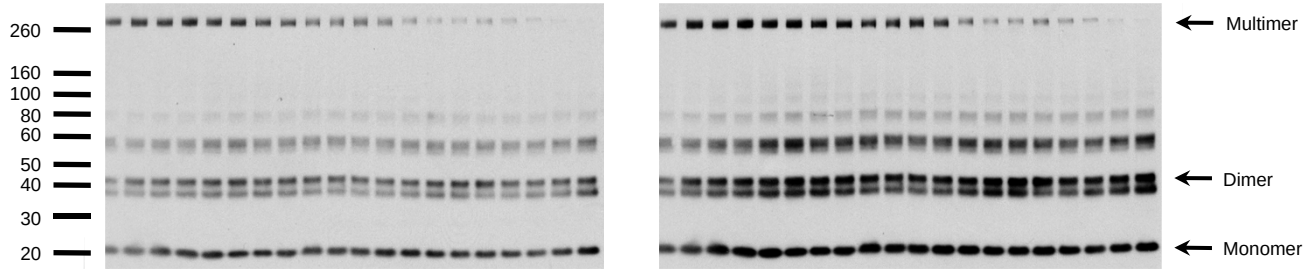
a **PRX-SO$_{2/3}$**



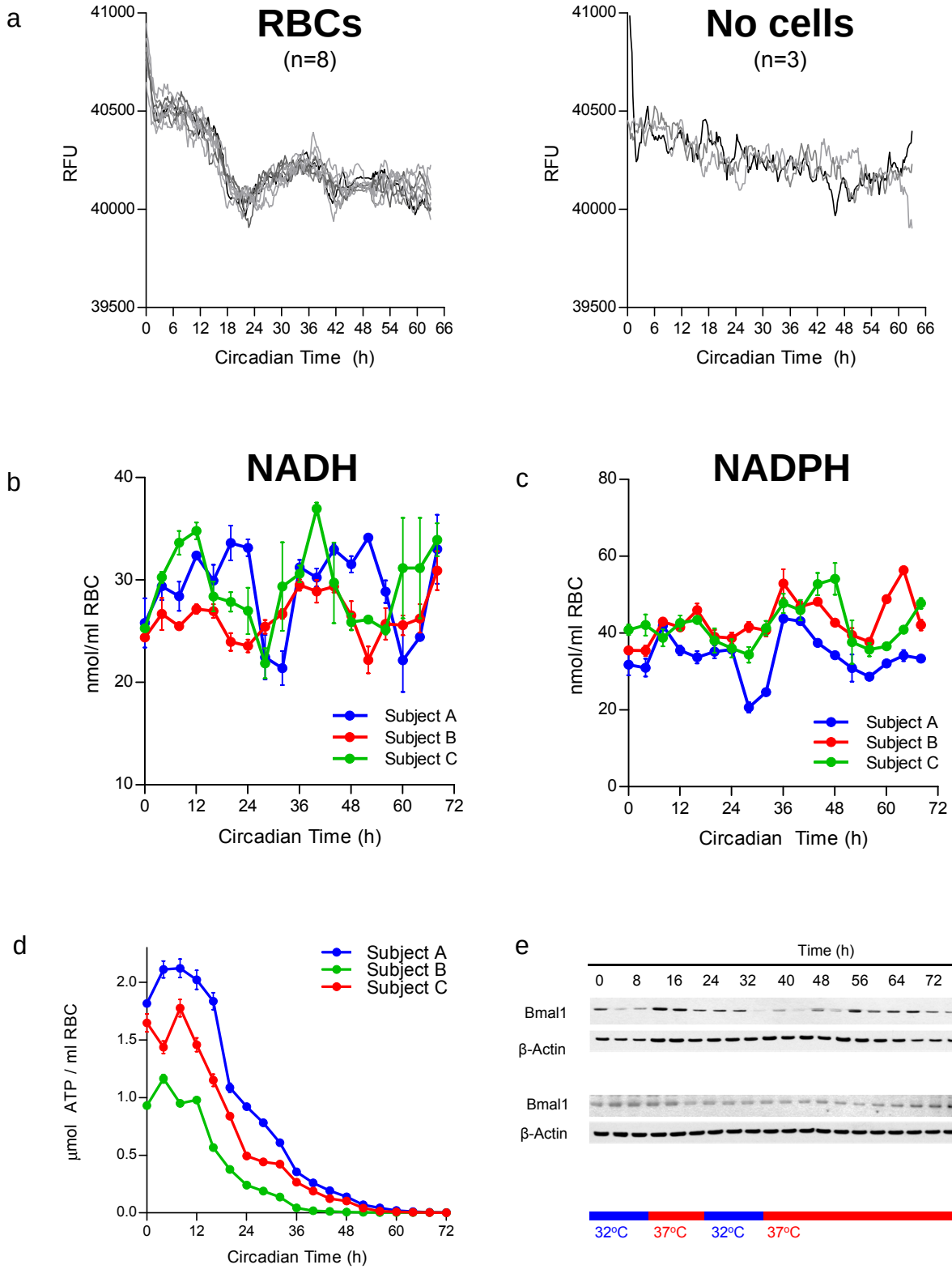
b **PRX1**



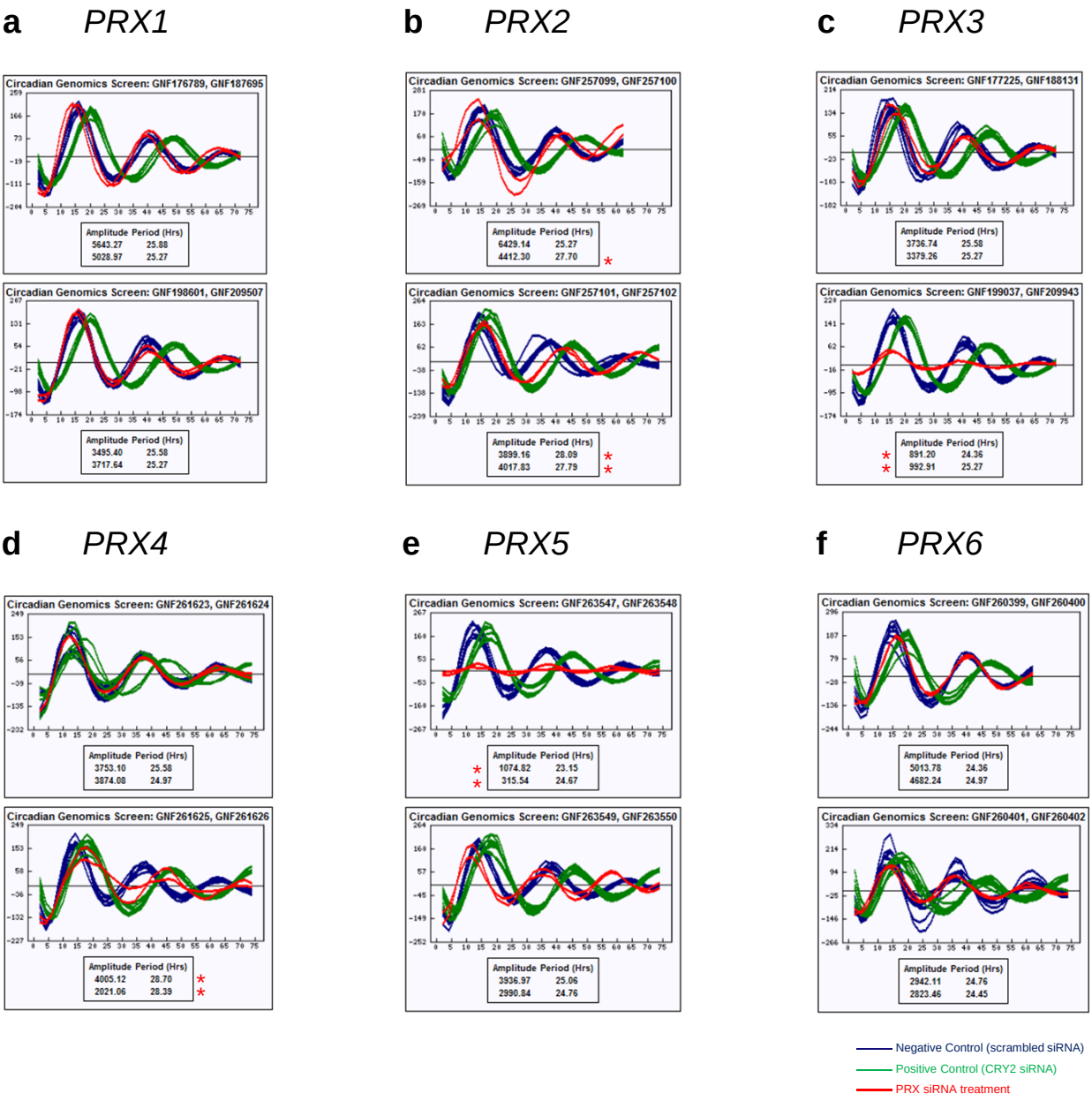
c **PRX2**



Supplementary Figure 6



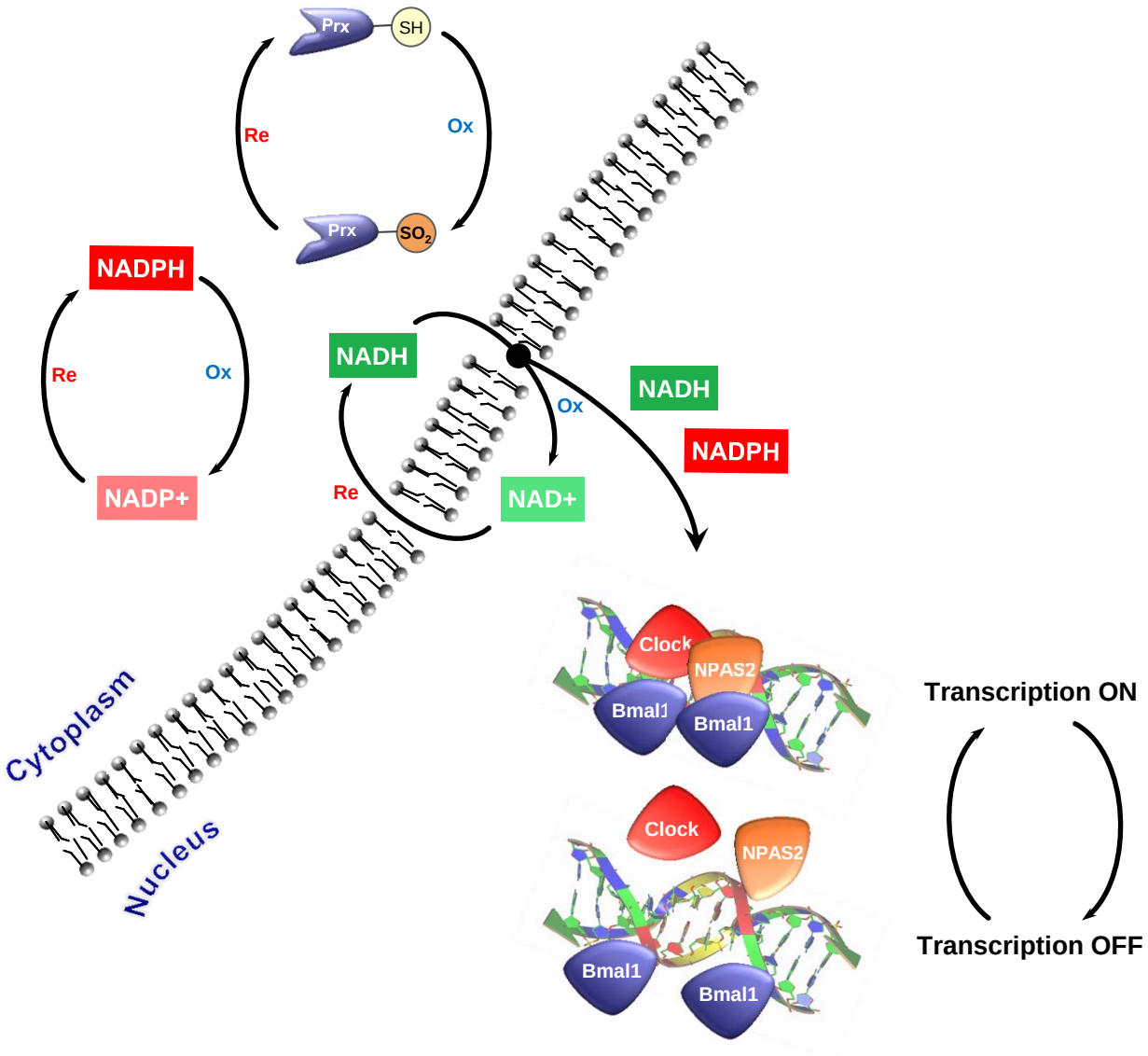
Supplementary Figure 7



Supplementary Table 2

Gene	Parameter affected	GNFid(s) of siRNAs having effects in at least one of two replicates tested
PRX1	none	
PRX2	↑ Period (prolonged to 27.9 h)	GNF257099, GNF257100, GNF257101, GNF257102
PRX3	↓ Amplitude (decreased 4-fold)	GNF199037, GNF209943
PRX4	↑ Period (prolonged to 28.6 h)	GNF261625, GNF261626
PRX5	↓ Amplitude (decreased 8-fold)	GNF263547, GNF263548
PRX6	none	

Supplementary Figure 8



Supplementary Figure 9