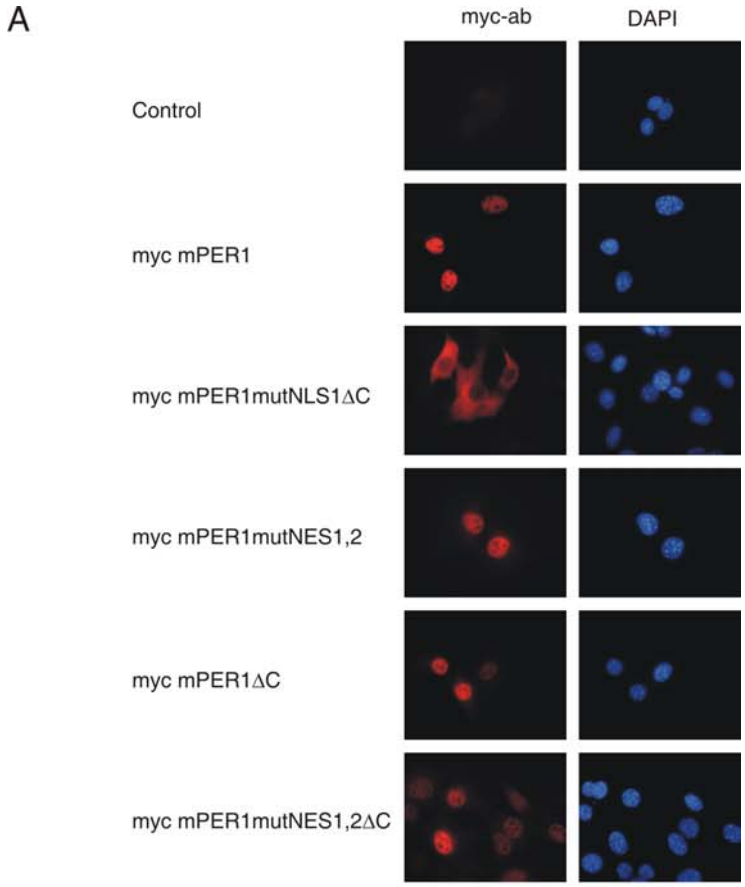
Supplementary data



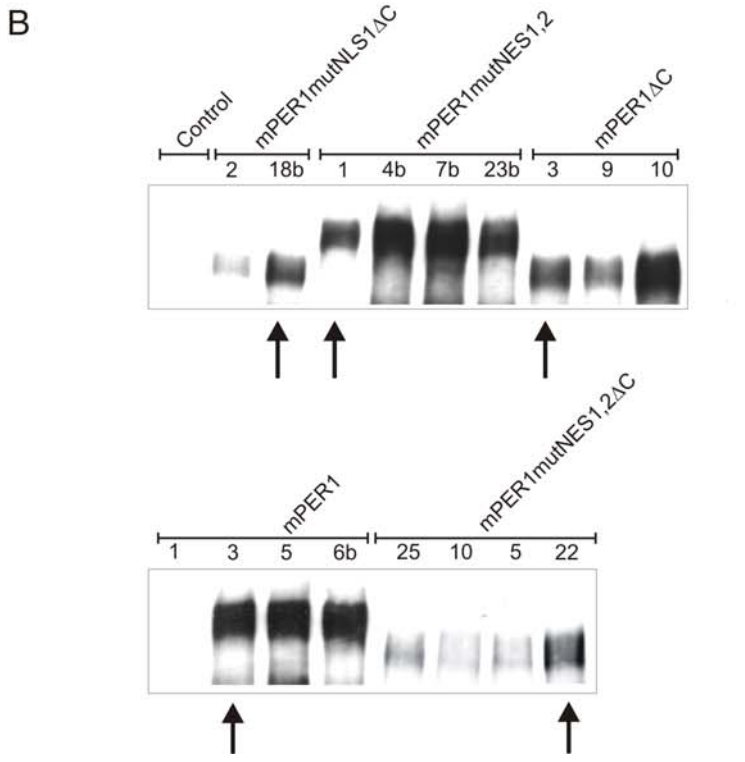


Figure 1S

Nucleocytoplasmic distribution and protein expression levels of mPER1 variants in NIH3T3 cells.

NIH3T3 cells were stably transfected with the constructs indicated.

(A) The intracellular localisation of resulting proteins was detected by immunofluorescence using myc-Cy3 antibodies (red). The nuclei were visualized by DAPI DNA staining (blue). As predicted, the NLS deficient mutant myc mPER1mutNLS1ΔC is cytoplasmic, whereas all other mPER1 variants are predominantly nuclear.

(B) 10 µg of total protein for each indicated clone was analysed by western blotting using the myc antibody. As a control the same protein amount of untransfected cells were loaded. The clones, which were indicated by the arrows, were used for real time PCR analysis.

Materials and Methods

Plasmids

To create the pCSflag vector, the myc tag in the pCSMT vector was replaced by a double-stranded oligonucleotide sequence containing a Kozak element and the flag epitope (5´-GATCGCCGCCATGGACTACAAGGACGAGGATGACAA-3´). The *mPer2* cDNA was subcloned into the NcoI restriction site of pCSMT; the resulting construct possesses five copies of the myc epitope. All *mPer1* fragments were amplified by PCR with 5´primers containing the EcoRI restriction site and 3`primers containing the StuI restriction site. The *mPer1* mutants used were constructed by using the Quick Change site directed mutagenesis kit (Stratagene) using the manufacturer´s protocol. To create stably transfected cell lines, the different *mPer1* constructs were generated by inserting the HindIII/StuI (filled in) fragment from pCSMT into pIREShyg3 (BD Biosciences), which was cut with StuI.

Protein expression

His-tagged Rna1p (pQE60-Rna1p) was expressed and purified using the protocol of Görlich et al. (1994) and stored at –70°C in Ran Storage Buffer (50 mM HEPES pH 7.6, 100 mM NaCl, 2 mM MgCl2, 0.5 mM EDTA, 1mM DTT). To enhance protein expression the bacterial strain SG13009 (pREP4) (Qiagen) was used.

Coimmunoprecipitation experiments

For coimmunoprecipitation experiments, cDNAs were mixed and then in vitro cotranslated in the coupled TNT system (Promega). 2 µl of each sample were added to protein G-Sepharose-flag-antibody pellets. The coimmuoprecipitation was performed in a final volume of 500 µl NET-2 (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.05% Nonidet P-40) for 1 h at 4°C. After washing three times with NET-2, proteins were analysed by SDS-PAGE and phosphoimaging (Molecular Dynamics).

Microinjection into Xenopus laevis oocytes

To determine the nucleocytoplasmic distribution, nucleus and cytoplasm were manually separated after different time intervals. Nuclear injection was verified via the presence of red colour from the reticulocyte lysate. Proteins fused to the myc epitope were purified from pooled nuclear and cytoplasmic fractions by immunoprecipitation as described by Rudt and Pieler 1996. The following antibodies were used: mouse anti-myc (9E10, Sigma) and mouse anti-flag M2 antibody (Sigma). After immunoprecipitation, immunopellets were resuspended in phosphatase buffer supplemented with 2 mM $MnCl_2$ and incubated with 200 U lambda protein phosphatase (NEB) for 30 min at 30°C. The reactions were stopped by the addition of SDS-PAGE sample buffer.

Cell culture and stable transfection

The NIH3T3 cells were grown in Dulbecco´s modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (Biochrom). Approximately 3 x 10^5 cells per well of a six-well-dish were plated one day before transfection. The different pIREShyg3-plasmids were linearised by Nru I; 1.5 µg of the DNA was transfected with Polyfect (Qiagen) using the manufacturer`s protocol. After 48 h incubation, the cells were 6-fold diluted and grown in complette medium containing 500 µg/ml of selective antibiotic Hygromycin B (Invitrogen) until clones were visible. These clones were picked and expanded; protein expression was verified by western blot and immunocytochemistry analysis.

Immunocytochemistry

The cells were grown on cover slips and fixed with 3% paraformaldehyde in PBS at room temperature for 15 min. After treatment with 0.5% Triton X-100 in PBS, unspecific staining was blocked with 3% BSA in PBS. The immunostaining was performed with the myc-Cy3 (Sigma). The cells were embedded with Vectashield containing DAPI (Linaris).

Serum shock and real time PCR

At different time points, the dishes were washed twice with PBS and the cells were disrupted by adding 600 µl of the RLT buffer (Qiagen), frozen in liquid nitrogen and kept at -70°C until RNA preparation by using the RNeasy mini kit (Qiagen). 500 ng of each total RNA sample was reverse transcribed using the iScript cDNA synthesis kit (Bio-Rad) and 50 ng of each cDNA was assayed by real-time PCR with iQ Sybr Green Supermix in the icycler system (Bio-Rad). As a control, *G6PDH* mRNA was amplified from the same RNA samples. The mRNA concentration was measured by using a standard curve for each analysed gene.

Statistical analysis

a) Data preprocessing

Expression data were normalized with regard to the housekeeping gene *mG6PDH* to account for variable total mRNA amounts and transformed to log scale as depicted in Fig 3 A-G.

b) Periodicity analysis

To compare the rhythmicity of the time series, we derived a score function from the smoothed periodogram (Daniell 1946). The periodogram of a time series is based on its fourier decomposition into sinusoidal components with different amplitudes and phases. The frequencies of the individual components (fourier frequencies) are determined by the length of the time series. The periodogram is the vector of squared amplitudes of the fourier components. Since the method originates from the analysis of spectra, these are interpreted as energies. The periodogram is an estimator of the power spectrum in this sense. For a periodic time series, the power spectrum is expected to have a strong peak, while a random time series (a gaussian white noise process) has a flat, however nonzero power spectrum. Therefore we use the difference of the highest energy in the periodogram from the mean energy as our scoring function to compare rhythmicity of time series. The score is required to compare the rhythmicity of time series in the presence of different constructs. Hypothesis tests for such comparisons are carried out using the nonparametric Mann-Whitney test, since the distribution of the score function is not easily derived without normality assumptions, which do not hold for our data. The comparisons of the time series for the *mCry1* and *mPer2* genes in Control and mPER1 as well as mPER1 and mPER1mutNES1,2 constructs referred to in the text are significant at the 90% (mCry1) and 80% (mPer2) levels. Given three repetitions of each time series, at most 95% significance can be reached with the Mann-Whitney test. The time series of the housekeeping gene *mG6PDH* are not rhythmic, and therefore provide typical score values for this case (approximately 0.0-0.2, see Fig. 3H).

The periodogram has been used in a similar manner for the detection of periodicities. Fisher used the flat power spectrum property of gaussian white noise processes to derive the distribution of the so-called g-statistic, which is constructed from the periodogram under the null hypothesis of no periodicity. There is a generalization of Fisher's g-test that allows to test for differences of the periodograms of two time series, but it requires much more data than is available in this study (Brockwell et al. 1987).

References in supplementary data

Brockwell, P.J., Davis, R.A. (1987) Time series: Theory and Methods. Springer.

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Görlich, D., Prehn, S., Laskey, R.A. and Hartmann, E. (1994) Isolation of a protein that is essential for the first step of nuclear protein import. *Cell*, **79**, 767-778.

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