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mPER1-mediated nuclear export of mCRY1/2 is an important element in establishing circadian rhythm

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Receptor-mediated nucleocytoplasmic transport of clock proteins is an important, conserved element of the core mechanism for circadian rhythmicity. A systematic analysis of the nuclear export characteristics for the different murine period (mPER) and cryptochrome (mCRY) proteins using *Xenopus* oocytes as an experimental system demonstrates that all three mPER proteins, but neither mCRY1 nor mCRY2, are exported if injected individually. However, nuclear injection of heterodimeric complexes that contain combinations of mPER and mCRY proteins shows that mPER1 serves as an export adaptor for mCRY1 and mCRY2. Functional analysis of dominant-negative mPER1 variants designed either to sequester mPER3 to the cytoplasm or to inhibit nuclear export of mCRY1/2 in synchronized, stably transfected fibroblasts suggests that mPER1-mediated export of mCRY1/2 defines an important new element of the core clock machinery in vertebrates.

Keywords: circadian rhythm; nuclear export; mPER; mCRY

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INTRODUCTION

Circadian rhythms in animals measure time on a scale of 24 h. Genes that define the molecular basis for this phenomenon were first identified in *Drosophila*. A central autoregulatory feedback loop constitutes the core of the clock machinery (Allada *et al*, 2001; Reppert & Weaver, 2002; Albrecht & Eichele, 2003). The basic helix–loop–helix (bHLH) transcription factors CLOCK (CLK) and CYCLE (CYC) drive expression of the *period* (*per*) and *timeless* (*tim*) genes; in turn, Period and Timeless proteins (PER and TIM) inhibit CLK/CYC-mediated transcription of their own genes, leading to a gradual loss of PER and TIM proteins (Allada *et al*, 1998; Darlington *et al*, 1998; Lee *et al*, 1998, 1999; Rutila *et al*, 1998). At a critically reduced level of PER and TIM protein activity, CLK/CYC repression is relieved and *per*/*tim* expression returns. A corresponding mechanism operates in mice. CLK/BMAL1 (brain and muscle ARNT1-like 1) heterodimers activate transcription of period (*mPer*) and cryptochrome (*mCry*) genes, and the resulting proteins act as negative regulators on their own genes by directly interacting with and inhibiting CLK–BMAL1 (Gekakis *et al*, 1998; Bunger *et al*, 2000). However, the murine clock system is more complex, as gene duplications have generated three mPER proteins (mPER1, mPER2 and mPER3) and two mCRY proteins (mCRY1 and mCRY2), with a certain amount of functional diversity among the individual members of each of these clock protein subfamilies (see below; Albrecht *et al*, 1997; Shearman *et al*, 1997, 2000b; Sun *et al*, 1997; Tei *et al*, 1997; Zylka *et al*, 1998; Kume *et al*, 1999; van der Horst *et al*, 1999).

The subcellular distribution of clock proteins seems to be regulated on the level of active, receptor-mediated nuclear export, an aspect that has not been studied to the extent of nuclear import for the same set of proteins. Vielhaber *et al* (2001) identified a single nuclear export signal (NES) in mPER1 that is functional in both *Xenopus* oocytes and HEK293 cells. In addition, Yagita *et al* (2002) have found that mPER2 is a shuttling protein in NIH3T3 cells, with NES activity residing in three separate elements of the protein: the first is located in the amino-terminal region, the second in the carboxy-terminal region and the third in the middle portion of the protein.

In the present study, we have systematically analysed nuclear export of the murine PER and CRY proteins, either individually or in all possible heterodimeric combinations, making use of *Xenopus* oocytes as an experimental system. We have found that mPER1 is required for the nuclear export of murine CRY proteins. A dominant-negative mPER1 mutant that is designed to sequester mCRY proteins in the nucleus makes synchronized fibroblasts arrhythmic. In contrast, a different dominant-negative mPER1 variant that is designed to sequester mPER3 in the cytoplasm has no effect on circadian rhythm in the same experimental system. These observations suggest that the mPER1-mediated nuclear export of mCRY proteins has an important function in the context of the molecular machinery that drives circadian rhythm.

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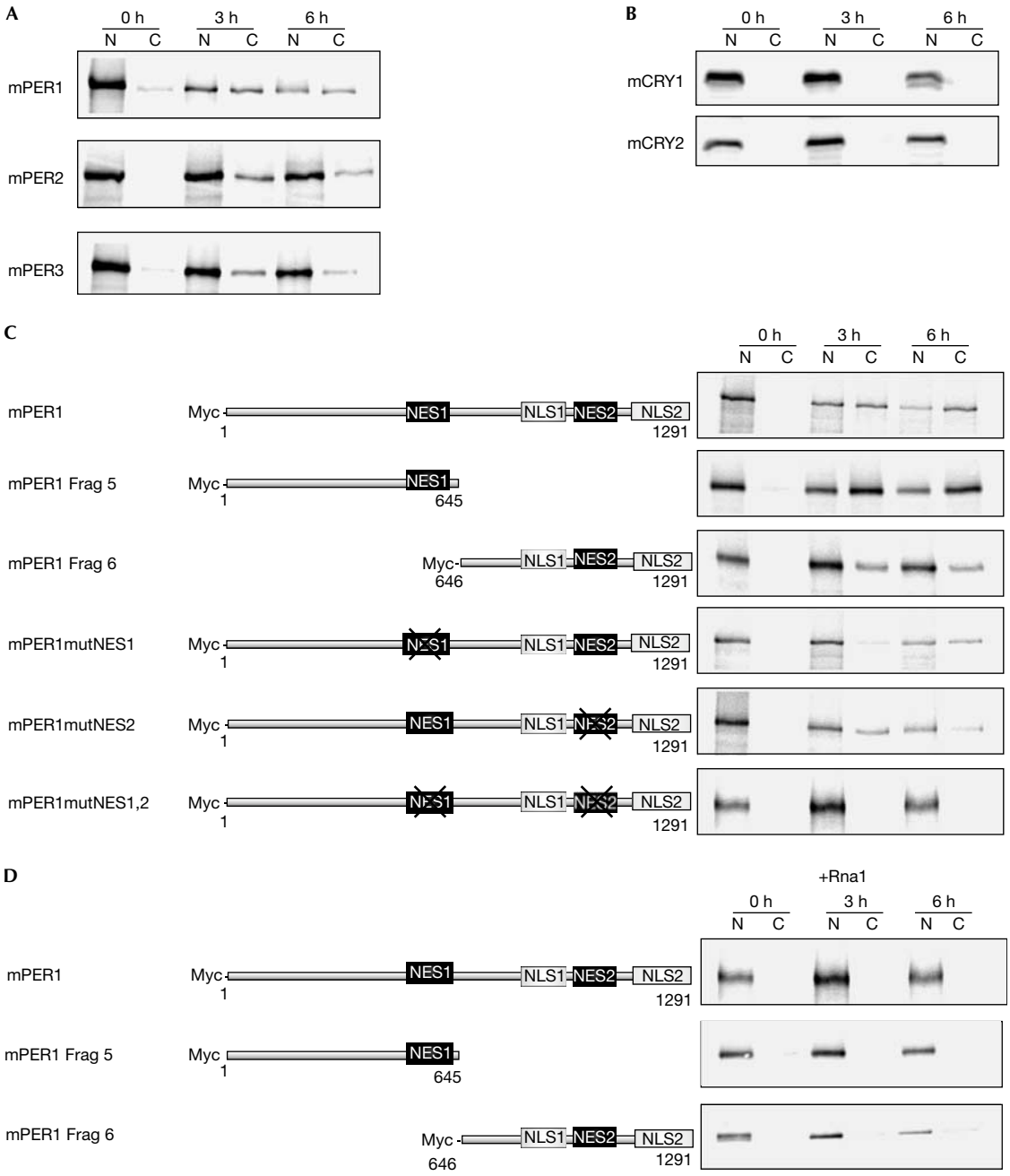


Fig 1 | Nuclear export of clock proteins in *Xenopus* oocytes. (**A**) All three mPER proteins are exported from the nucleus of *Xenopus* oocytes. Myc–mPER1, Myc–mPER2 and Myc–mPER3 were injected into the nucleus of *Xenopus* oocytes and the nuclear export was analysed after 3 and 6 h incubation at 18 °C by immunoprecipitation using the Myc antibody. (**B**) Myc-tagged mCRY1 and mCRY2 are not exported. The nuclear export of *in vitro*-translated mCRY proteins was assayed as above after 3 and 6 h (export efficiencies: mCRY1: 3 h 2%, 6 h 3%; mCRY2: 3 h 2%, 6 h 2%). (**C**) mPER1 contains a second leucine-rich NES in its C-terminal domain. The Myc-tagged *in vitro*-translated variants of mPER1 were injected into the nucleus of *Xenopus* oocytes and analysed as described; a schematic representation of the microinjected proteins is shown. Grey boxes indicate the location of the NLSs and the black boxes the NESs. To mutate the NES sequences, leucines were changed to alanine (NES1: IQELSEQIHRLLL; NES2: LQLNLLQL) (export efficiencies: mPER1: 3 h 46%, 6 h 70%; mPER1mutNES1: 3 h 10%, 6 h 40%; mPER1mutNES2: 3 h 37%, 6 h 35%; mPER1mutNES1,2: 3 h 2%, 6 h 4%; mPER1 Frag 5: 3 h 63%, 6 h 70%; mPER1 Frag 6: 3 h 30%, 6 h 32%). (**D**) Nuclear injection of Rna1 inhibits nuclear export of mPER1. A 1 μM portion of bacterially expressed Rna1 was co-injected with *in vitro*-translated mPER1 or mPER1 fragments (as indicated) into the nucleus of *Xenopus* oocytes and analysed for nuclear export (export efficiencies: mPER1: 3 h 5%, 6 h 3%; mPER1 Frag 5: 3 h 3%, 6 h 7%; mPER1 Frag 6: 3 h 10%, 6 h 13%). All injected PER proteins were treated with λ-protein phosphatase before gel electrophoresis.

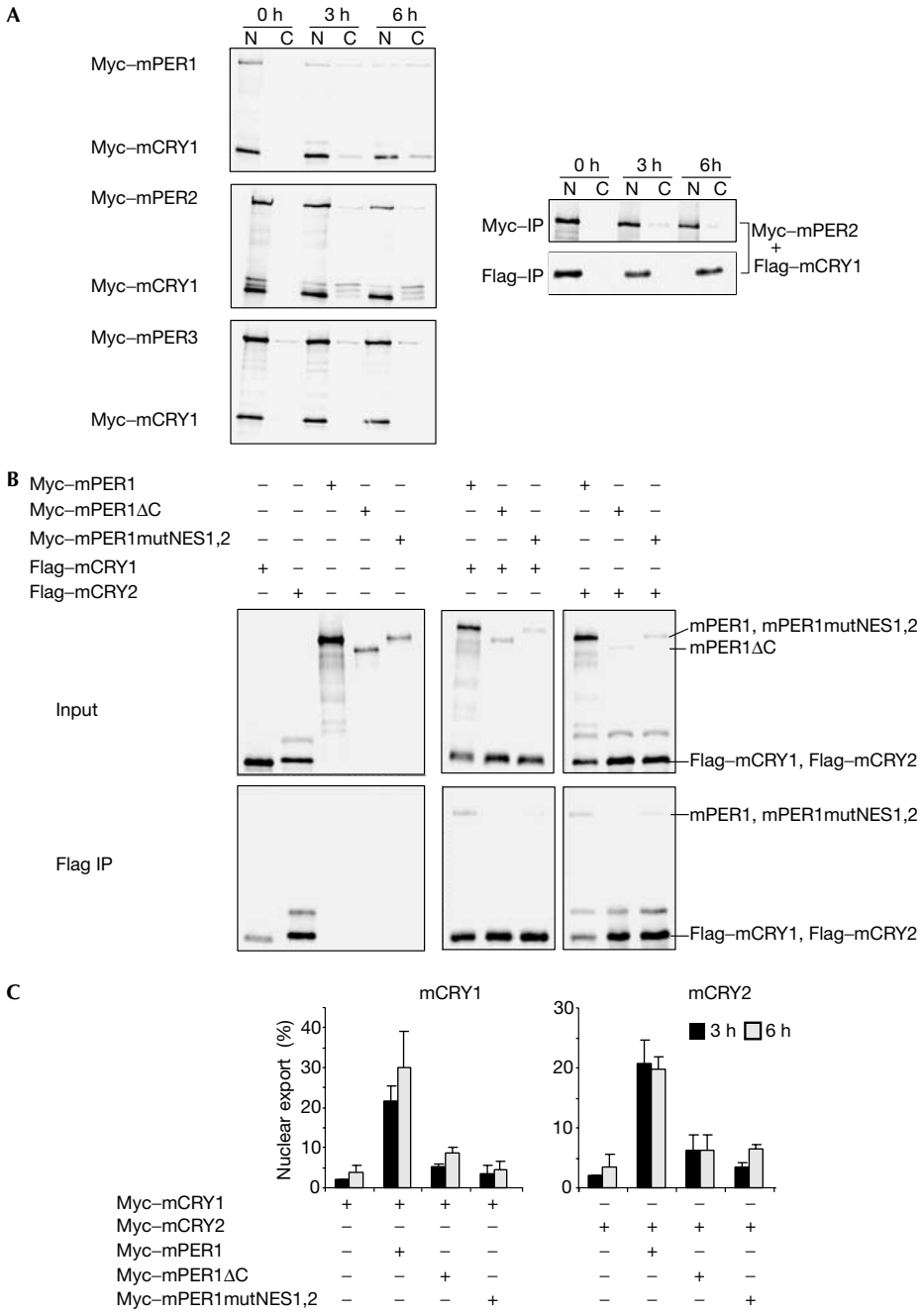


Fig 2 | mCRY1 and mCRY2 are only exported in association with mPER1. (**A**) mPER1 is required for the export of mCRY1 and mCRY2. Myc-tagged *in vitro*-translated proteins were co-injected into the nucleus of *Xenopus* oocytes and analysed as described in Fig 1 after 3 and 6 h incubation. mPER2 protein preparation from *in vitro* translation resulted in the production of three additional, smaller polypeptides of similar length, one of which was overlapping with and thus indistinguishable from mCRY1. Therefore, Myc-tagged mPER2 was co-translated with Flag-tagged mCRY1, microinjected and the resulting nuclear and cytoplasmic preparations subjected to immunoprecipitation (IP) with either Myc- or Flag-specific antibodies. (**B**) The C-terminal domain of mPER1 is required for complex formation with mCRY1 and mCRY2. Flag-tagged mCRY1 (middle panel) or mCRY2 (left panel) was co-translated *in vitro* with Myc-tagged versions of mPER1, mPER1ΔC or mPER1mutNES1,2. Heterodimerization was detected by co-immunoprecipitation using the Flag antibody (bottom panels). As a negative control, the Myc-tagged period proteins were translated without Flag-tagged mCRY1 or mCRY2 and immunoprecipitated using the Flag antibody (left-hand panel). Only 50% of the input was loaded on the SDS gel. An additional band of unknown identity was observed in all *in vitro* mCRY2 protein preparations. (**C**) Quantitative analysis of the nuclear export of mCRY1 and mCRY2 after co-injection with various mPER protein variants into the nucleus of *Xenopus* oocytes, as indicated.

Fig 3 | Circadian rhythm of synchronized fibroblasts expressing transport-deficient mPER1 mutants. (**A**) Schematic representation of constructs used and their activities. Grey boxes indicate the location of the NLSs and the black boxes that of the NESs. To mutate the NES sequences, leucines were changed to alanine (NES1: IQELSEQIHRLLL; NES2: LQLNLLQL). To mutate mPER1-NLS1, three of the basic amino acids were changed to alanine (RRHHCRSKAKRSR); mPER1-NLS2 was deleted together with the CRY-binding domain. (**B–G**) Overexpression of WT-mPER1 and nuclear sequestration of mCRY1 and mCRY2 inhibit rhythmic clock gene expression. To induce rhythmic circadian gene expression, the mouse fibroblasts, which were grown to confluence in low serum concentration, were treated with 50% horse serum for 2 h (time point 0–2 h) and then cultured in serum-free medium. Total RNA prepared at the time points indicated was analysed by real-time PCR. The messenger RNA expression level of *mPer2* and *mCry1* is shown on a logarithmic scale by measuring the absolute mRNA concentration using a standard curve. As a control for total RNA levels, *G6PDH* mRNA was analysed in the same samples. The results shown are means ± s.e.m. ($n = 3$) (Myc–Per1mutNES1,2ΔC: $n = 2$). (**H**) Rhythmicity scores for the repeated expression time series of the three genes analysed, grouped by the different constructs. Rhythmicity of the control gene *G6PDH* is always low. There is a clear trend towards weaker rhythmicity of clock gene expression in the presence of mPER1mutNES1, than following overexpression of WT-mPER1. ▶

RESULTS AND DISCUSSION

Complex formation is required for nuclear export

Nuclear export of mPER and mCRY proteins was assayed by nuclear injection of *in vitro*-translated proteins. Although all three mPER proteins were readily exported into the cytoplasm (Fig 1A), mCRY1 and mCRY2 remained largely nuclear (Fig 1B). Receptor-mediated export had previously been reported for mPER1 (Vielhaber *et al*, 2001) and mPER2 (Yagita *et al*, 2002). The same studies have localized the NES function in mPER1 to one sequence element in the N-terminal portion of the protein, whereas three independent NESs were detected in mPER2. We have re-examined NES activities in mPER1 by making use of the oocyte microinjection system. Both N- and C-terminal halves of mPER1 were efficiently exported (Fig 1C); we note that export activities were weaker for the C-terminal fragment. The mPER1 N-terminal fragment contains NES as mapped previously by Vielhaber *et al* (2001), whereas the mPER1 C-terminal fragment contains a conserved sequence element corresponding to an export signal of mPER2 as mapped previously by Yagita *et al* (2002). Mutagenesis of both putative export signals, NES1 and NES2, results in a loss of export activity for mPER1, whereas mutation of either NES1 or NES2 alone maintains export activity, although at a reduced level.

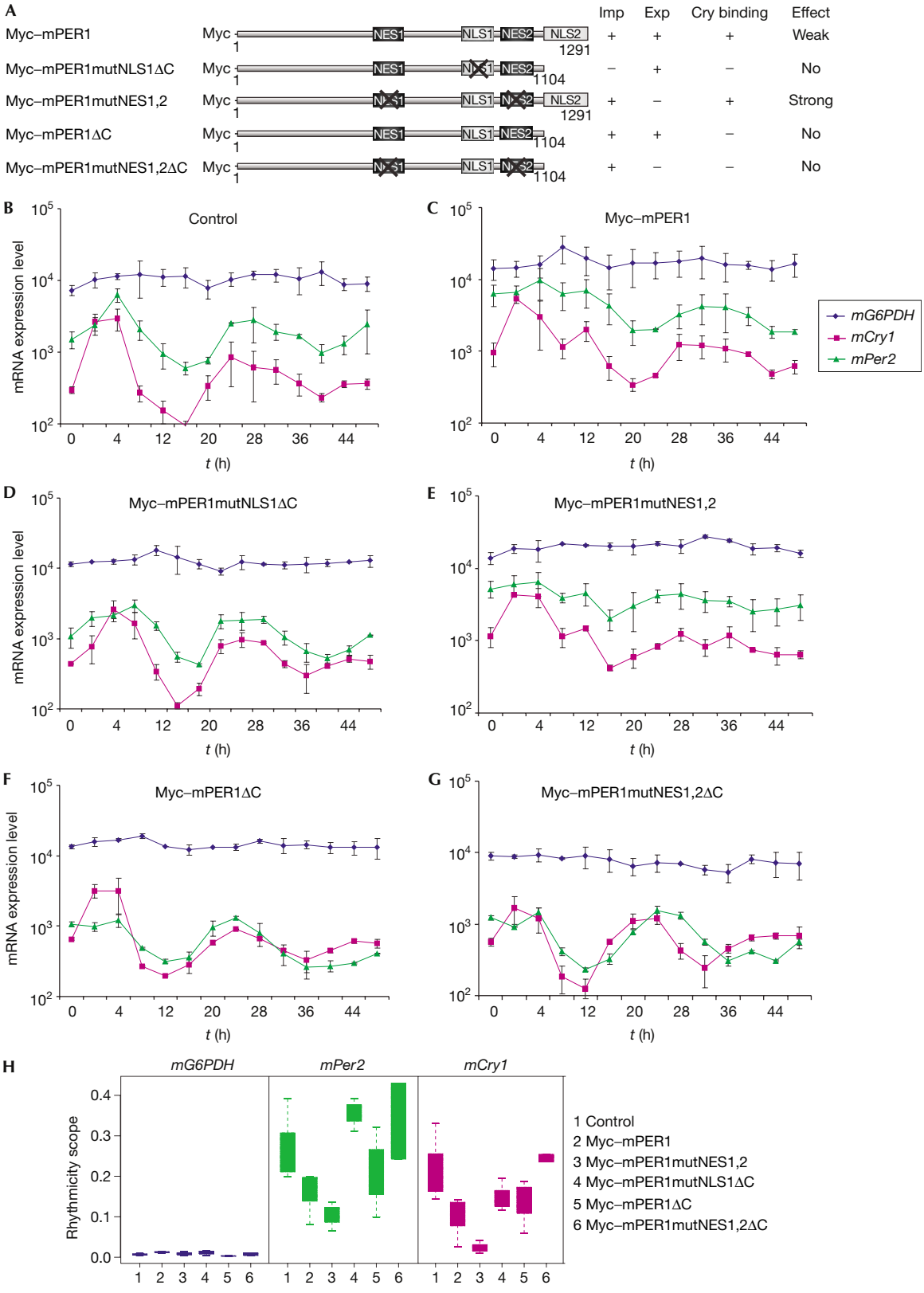
To distinguish between passive diffusion and energy-dependent, receptor-mediated nuclear export, we co-injected bacterially expressed Rna1 with mPER1, as well as with the N- and C-terminal halves of mPER1 (Fig 1D). The yeast homologue of the RanGTPase-activating protein is cytoplasmic under physiological conditions. Nuclear injection of this protein leads to a high concentration of RanGDP in this compartment and blocks nuclear export (Izaurralde *et al*, 1997). Rna1 also inhibits nuclear export of wild-type mPER1 (WT-mPER1), as well as of mPER1 Frag 5 and mPER1 Frag 6, suggesting that both NESs function in an active, receptor-mediated manner.

As mCRY proteins injected individually were not exported from the nucleus, we tested whether complex formation with the different mPER proteins would allow export of mCRY1 and mCRY2. Heteromeric complex formation was obtained by *in vitro* co-translation of mPER with mCRY proteins and verified by immunoprecipitation (Fig 2B and data not shown). mCRY1 was exported from the nucleus of microinjected oocytes if it was in a complex with mPER1, but not if it was in a complex with either mPER2 or mPER3 (Fig 2A,C). Corresponding results were obtained with mCRY2 (Fig 2C and data not shown). A mutant version of mPER1 that had both NES elements mutated and was therefore export-deficient (mPER1mutNES1,2) was still able to form a complex with mCRY1 and mCRY2; this complex was not exported, suggesting that NES activity in mPER1, and not an NES that could be masked in the isolated mCRY proteins, is required for export of the mPER1–mCRY complex (Fig 2C). Furthermore, co-injection of either mCRY1 or mCRY2 with a second mutant version of mPER1 that is unable to interact with mCRY1/2 but maintains export activity (mPER1ΔC) does not promote nuclear export of the mCRY proteins (Fig 2C). This result suggests that heteromeric complex formation is directly required for mCRY export, and it argues against mPER1 functioning indirectly to relieve inhibition of mCRY export.

To the best of our knowledge, nuclear export of murine CRY proteins has not been described previously in the literature. Earlier studies demonstrated that the nuclear entry of rPER2 is largely stimulated on complex formation with hCRY1 (Miyazaki *et al*, 2001). The function of mPER1 as an adaptor for the nuclear export of CRY proteins thus constitutes a new element in the molecular circuitry that describes the generation of the circadian rhythm.

mCRY nuclear export is required for circadian rhythm

Circadian rhythm can be induced in tissue culture by treatment with 12-*O*-tetradecanoylphorbol-13-acetate, Forskolin or a high serum concentration (as used in this study) (Balsalobre *et al*, 1998; Akashi & Nishida, 2000; Motzkus *et al*, 2000; Yagita & Okamura, 2000). We made use of this phenomenon to analyse the effects of mPER1 mutants that are predicted to inhibit either the nuclear import of mPER3 or the nuclear export of mCRY1/2 in a dominant-negative fashion. Stable cell lines ectopically expressing WT or mutant versions of mPER1 (as schematically shown in Fig 3A) under the control of a constitutively active promoter were generated and circadian gene expression was measured by real-time PCR (Fig 3B–G) and reverse transcription–PCR (data not shown). Synchronized WT fibroblasts show a circadian rhythm of endogenous *Cry1* and *Per2* transcription as described previously (Balsalobre *et al*, 1998; Yagita *et al*, 2001). Ectopic, constitutive expression of mPER1 only moderately disturbs rhythmicity (Fig 3C,H). It is also evident from an immunohistochemical analysis that ectopic WT-mPER1 is mainly nuclear (supplementary Fig 1 online). The relatively mild effect could be explained by enhanced nucleocytoplasmic shuttling of the mPER1–mCRY repressor complex and cytoplasmic passage resulting in reduced stability of both proteins.



A parallel study on the nuclear import of mPER and mCRY proteins in *Xenopus* oocytes showed that mPER1 also serves as an import adaptor for mPER3 (Loop & Pieler, unpublished data), similar to what had been reported in some tissue culture systems (Kume *et al*, 1999; Lee *et al*, 2004). A mutant version of mPER1 (mPER1mutNLS1ΔC) that maintains its ability to interact with mPER3, but is not imported (supplementary Fig 1 online), should act in a dominant-negative manner on the nuclear import of endogenous mPER3 in stably transfected fibroblasts. However, we did not detect a significant effect on circadian gene expression (Fig 3D,H). This observation suggests that the nuclear import of endogenous mPER3 may not be either inhibited under these circumstances or required for circadian rhythm in this system. Similarly, mice with a targeted disruption of mPER3 showed only a rather subtle phenotype, indicating that the role of mPER3 is not crucial for a functional circadian clock (Shearman *et al*, 2000a; Bae *et al*, 2001).

Interestingly, ectopic expression of a mutant version of mPER1 that is predicted to block the nuclear export of endogenous mCRY1 and mCRY2 (mPER1mutNES1,2) has strong effects on the circadian rhythm of synchronized fibroblasts, as shown by arrhythmic expression of the circadian genes analysed (Fig 3E). Constitutive nuclear localization of the mPER1–mCRY repressor complex could result in increased stability of mPER1 and mCRY proteins and thus interfere with the timely gain of transcription activity of the clock genes. Two other variants of mPER1 were analysed for control purposes. mPER1ΔC is a shuttling protein that cannot interact with mCRY1/2, and mPER1mutNES1,2ΔC is an export-defective mutant that similarly cannot interact with mCRY1 and mCRY2. Ectopic expression of both mutants has no effect on circadian gene expression (Fig 3F–H), indicating that it is indeed mislocalization of the mPER1–mCRY complex that is responsible for the results described above.

Taken together, these results support the idea that nuclear export of mCRY1 and mCRY2 is necessary for the circadian rhythm in synchronized, cultured fibroblasts, and they support our main conclusion that the mPER1-mediated nuclear export of mCRY1/2 constitutes an essential element in the generation of circadian rhythm. Nuclear sequestration of the mPER1–mCRY protein complexes may lead to stabilization and constitutive repressor activity.

METHODS

Plasmids. For *in vitro* translation, *mPer* and *mCry* complementary DNAs were subcloned into the pCSMT vector containing six Myc epitopes (Rupp *et al*, 1994) or into the pCSflag vector. To create stably transfected cell lines, the different mPER1 constructs were inserted into pIREShyg3 (BD Biosciences, Heidelberg, Germany) (for details, see supplementary information online).

Protein expression. Radiolabelled proteins were expressed in the TNT system (Promega, Mannheim, Germany) and analysed after sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) by phosphorimaging (Amersham, Freiburg, Germany). His-tagged Rna1 was expressed as described in supplementary information online.

Co-immunoprecipitation experiments. After *in vitro* translation, 2 μl of each sample was added to protein G-Sepharose–Myc antibody pellets and incubated in NET-2 buffer at 4 °C (for details, see supplementary information online).

Microinjection into *Xenopus laevis* oocytes. Oocytes were prepared for microinjection as described previously (Claussen *et al*, 1999). About 15 nl of protein injection mix was microinjected into the cytoplasm or nucleus of oocytes (for details, see supplementary information online).

Cell culture and stable transfection. The NIH3T3 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS (Biochrom, Berlin, Germany) and stably transfected as described in supplementary information online.

Serum shock and real-time PCR. About 4×10^5 cells per well were plated in a six-well dish 4–5 days before synchronization. The NIH3T3 cells were grown in DMEM supplemented with 5% FCS. At time point 0, the medium was exchanged for 50% horse serum (Invitrogen, Karlsruhe, Germany) in DMEM, and after 2 h the medium was replaced by serum-free DMEM (Yagita & Okamura, 2000). At different time points, the cells were collected and analysed as described in supplementary information online.

Statistical analysis. Rhythmicity of a time series is scored by the difference of the strongest component of its periodogram from the average of all components. The periodogram is an estimator of the power spectrum, which contains the squared amplitudes of a decomposition of the time series into sinusoidal components. If there is a dominant periodic component in the time series, there will be a corresponding peak in the periodogram. A (gaussian) random time series produces a flat periodogram (Brockwell & Davis, 1987). Further details are described in supplementary information online.

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

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