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Nuclear redox imbalance affects circadian oscillation in HaCaT keratinocytes

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ABSTRACT

Circadian clock is regulated by a transcriptional/translational feedback loop (TTFL) lasting ~24 h. Circadian oscillation of peroxiredoxins (PRDX1–6) redox status has been shown in mature erythrocytes. We have recently reported that nuclear levels of PRDX2 are circadian regulated in the HaCaT keratinocytes. In this study, we addressed whether PRDX2 translocation could influence the TTFL. A reporter HaCaT cell line stably expressing the luciferase gene under control of Bmal1 promoter was lentivirally transduced either with an empty vector (EV), a vector carrying a myc-tagged wild type PRDX2 (PRDX2-Myc) or the same gene with a nuclear localization sequence (PRDX2-MycNuc). PRDX2 overexpressing cells were protected from H_2O_2-induced oxidative stress. The amplitude of the Bmal1 promoter activity was significantly dampened in PRDX2-MycNuc versus EV cells when synchronized either by dexamethasone treatment or temperature cycles. Clock synchronization was not affected in PRDX2 silenced cells. N-acetyl cysteine or melatonin treatments, significantly dampened the Bmal1 promoter activity suggesting that sustained scavenging of ROS impairs clock synchronization. Noteworthy, H_2O_2 treatment rescued proper oscillation of the clock in synchronized PRDX2-MycNuc HaCaT cells. Since the histone deacetylase Sirtuin 1 (Sirt1) modulates clock gene expression amplitude, the effect of Sirt1 activator resveratrol or Sirt1 inhibitor nicotinamide were also investigated. Interestingly, NAM enhanced the molecular clock synchronization in PRDX2-MycNuc cells. Our findings demonstrate that PRDX2 regulates the TTFL oscillation by finely tuning the cellular redox status of the nucleus likely influencing the deacetilase activity of SIRT1 enzyme.

1. Introduction

The circadian rhythms are endogenous mechanisms that synchronize (entrain), anticipate and adapt the organism physiology to cyclic environmental cues (zeigtgeber). In mammals, circadian rhythms are regulated at the systemic level by a group of ~20,000 specialized neurons located in the suprachiasmatic nucleus (SCN) of the hypothalamus. The SCN molecular clock is entrained by light/dark cycles which are transduced into neuro-hormonal signals regulating the activity of tissue specific molecular clocks present in peripheral organs including the skin (Lamia et al., 2009; Virshup et al., 2007; Vanselow et al., 2006; Geyfman and Andersen, 2009; Ndiaye et al., 2013). Despite peripheral clocks being independent and self-sustaining, their activity is tightly coordinated with the SCN master clock. Such physiological orchestration is evolutionarily conserved in both unicellular and multicellular organisms and is regulated at molecular level by an intricate transcriptional/translational feedback loop (TTFL) (Bass, 2012). In mammals, the positive arm of the loop is characterized by the heterodimerization of the transcription factors CLOCK and BMAL1. CLOCK/BMAL1 heterodimers bind the E-box elements of their target promoters and initiate the transcription of their target genes including their own repressors: Per1/2/3 and Cry1/2 genes. PER and CRY proteins

Abbreviations: TTFL, transcriptional/traslational feedback loops; PRDX1–6, peroxiredoxins 1–6; HaCaT, human adult low calcium temperature keratinocytes; NAC, N-acetyl cysteine; NAM, nicotinamide.

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are translated in the cytoplasm and once have reached a critical amount oligomerize and translocate to the nucleus where they directly repress CLOCK/BMAL1 heterodimers. The entire cycle lasts ~24 h. An additional loop involving the nuclear orphan receptors RORα and REV-ERBα directly regulates the transcription of Bmal1 stabilizing the TTFL (Geyfman and Andersen, 2009). Finally, several post-translational processes including sumoylation (Lee et al., 2008), acetylation (Asher et al., 2008; Belden and Dunlap, 2008) and phosphorylation are involved in the fine-tuning of the molecular clock and in its integration with metabolism (Vanselow et al., 2006; Eckel-Mahan and Sassone-Corsi, 2013).

It has been estimated that the molecular clock regulates the expression of around 10% of the entire genome and 20% of the soluble protein fraction (Panda et al., 2002; Reddy et al., 2006).

Indeed, several basic cellular processes such as cell division, proliferation, DNA damage repair and stress response have been found to be regulated by circadian genes (Hunt and Sassone-Corsi, 2007; Andersen et al., 2012). A very recent growing body of evidence suggests a close connection between the cellular circadian and redox systems (Tamaru et al., 2013; Wilking et al., 2013). Disruption of the circadian clock results in increased oxidative stress and premature senescence both in vitro and in vivo (Kondratov et al., 2006; Khapre et al., 2011). Indeed, several genes encoding antioxidant enzymes including the super oxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and peroxiredoxins (PRDX1–6) have been shown to be regulated by the circadian clock (Patel et al., 2013).

PRDXs are crucial ROS scavenger enzymes known to regulate intracellular signaling by local modulation and compartmentalization of H_2O_2 levels (Choi et al., 2005; Rhee et al., 2005; Mishina et al., 2011) and their deregulation may be associated to several pathologies including cancer (Ishii et al., 2012). Accordingly, it has been recently shown that PRDX2 overexpression in the nucleus or in the cytoplasm respectively induces or represses the androgen receptor transactivation in human prostate cancer cells (Shiota et al., 2011).

Interestingly, a recent and elegant study performed in human red blood cells has demonstrated that PRDX proteins display circadian rhythmicity of their redox status even in the absence of a functional TTFL (O'Neill and Reddy, 2011). Noteworthy, these redox rhythms are evolutionary conserved (Edgar et al., 2012), entrainable and temperature compensated, this allowing for the definition of a transcriptional independent metabolic clock (Bass and Takahashi, 2011). However, the reciprocal influence between the TTFL and the PRDX-based metabolic clock has not fully addressed yet.

The NAD^+-dependent histone deacetylase Sirtuin 1 (Sirt1) has been shown to deacetylate both BMAL1 and its repressor PER2 working as a circadian rheostat that permits the fine modulation of the molecular clock amplitude (Hirayama et al., 2007; Asher et al., 2008). Sirt1 is also a master regulator of several intracellular processes including the anti-oxidant intracellular stress response (Care 2014) and is inactivated by oxidative stress (Volonte et al., 2015). The existence of a crosstalk between Sirt1 and peroxiredoxins has never been addressed before.

Keratinocytes are the main cellular component of the epidermis and provides an efficient barrier against free radicals (ROS) (Ndiaye et al., 2013). Keratinocytes possess a robust peripheral molecular clock (Sandu et al., 2012; Avitabile et al., 2014; Spörl et al., 2011) as well as a relevant expression of PRDXs (Avitabile et al., 2014). We have recently shown that PRDX2 translocates in the nucleus of temperature entrained HaCaT keratinocytes in its catalytically active form and this phenomenon correlates with a more reduced nuclear redox status. Interestingly, PRDX2 nuclear levels peak in anti-phase with Bmal1 promoter activity (Avitabile et al., 2014). Since it has been proposed that the nuclear redox state is pivotal for the regulation of several transcription factors including CLOCK and NPAS2 (Rutter et al., 2001) we hypothesized that PRDX2 circadian translocation could be important for the regulation of the keratinocyte molecular clock function.

In this paper, we show that neither the overexpression nor downregulation of PRDX2 affects the synchronization of the molecular clock in HaCaT cells. However, the forced nuclear overexpression of PRDX2 results in dampened oscillations of Bmal1 promoter activity in HaCaT cells entrained either dexamethasone (Dex) treatment or temperature. This inhibitory effect on Bmal1 promoter activity correlates with the efficient scavenging of intracellular reactive oxygen species (ROS) mediated by the nuclear PRDX2. Indeed, the sustained scavenging of intracellular ROS mediated by N-acetyl cysteine (NAC) or melatonin markedly impairs the molecular clock oscillation. Importantly, we show that the normal activity of the molecular clock can be rescued in nuclear PRDX2 overexpressing cells under a specific range of H_2O_2 concentrations. This effect is lost at saturating levels of H_2O_2. Finally, althought we did not find either a direct interaction between PRDX2 and Sirt1 or a modulation of Sirt1 intracellular levels in PRDX2-Myc or PRDX2-MycNuc overexpressing cells, the inhibition of Sirt1 activity by nicotinamide was able to restore proper clock oscillation in PRDX2-Myc cells.

In conclusion, our study shows that PRDX2 regulates the TTFL oscillation by decreasing the nuclear redox levels this likely suggesting an enhancement of Sirt1 enzymatic activity.

2. Materials and methods

2.1. Cells and treatments

The human keratinocyte cell line HaCaT-Bluf expressing the luciferase gene under control of the Bmal1 and the Human Embryonic Kidney cell line HEK 293T were cultured in Dulbecco's Modified Eagles Medium (DMEM, Euroclone, Milan, Italy), supplemented with 10% Fetal Bovine Serum (FBS, Euroclone) plus antibiotics.

Synchronization of the molecular clock was induced in confluent cells either by temperature cycles or by Dex (Sigma–Aldrich Inc., Saint Louis, MO, USA) treatment. To measure Dex-induced synchronization, HaCaT cells were seeded in gelatin-coated 96 wells plate (Optiplate 96, Promega) and growth at confluence for two days. The third day after seeding, cells were serum starved for 16 h, washed with Phosphate Buffer Saline (PBS) and then incubated with 0.1 μM Dex for 1 h at 37 °C in serum free DMEM. After stimulation, cells were washed with PBS and kept in serum free DMEM without phenol red supplemented with 10 mM HEPES (pH 7.2), 0.38 mg/ml sodium bicarbonate and 1 mM luciferin (Promega) for consecutive 56 h (Fig. 3A) (Hirota et al., 2008). Temperature entrainment was performed by two 24 h long temperature cycles as previously described (12-h 37 °C/12-h 33 °C) (Avitabile et al., 2014).

ROS generation was induced treating cells with different concentrations of H_2O_2 (Sigma–Aldrich) (60, 125, 250, 500 μM) for 30 min at 37 °C. Saline water diluted in medium was used as negative control. ROS production was measured incubating cells with 5 μM CellROX® Deep Red Reagent (Life technologies-Invitrogen, Carlsbad, CA, USA) for 30 min at 37 °C.

To assess the effect of ROS scavenger on the molecular clock, HaCaT cells were synchronized by Dex and then treated with either N-acetyl-L-cysteine (NAC, 1, 5 mM; Sigma–Aldrich), N-acetyl-5-methoxytryptamine (melatonin, 50 or 100 μM; Sigma–Aldrich) (Fischer et al., 2006b), 3,5,4′-trihydroxy-trans-stilbene (resveratrol, 10, 25 or 50 μM; Sigma–Aldrich) or nicotinamide (NAM, 1, 2.5 or 5 mM; Sigma–Aldrich) and left in the medium during the free running period (56 h).

To rescue the proper cellular redox balance, Dex-synchronized HaCaT cells were treated with different concentrations of H_2O_2 (60, 125, 250 or 500 μM; Sigma–Aldrich).

2.2. Vector generation, lentivirus production and cells transduction

Lentiviral vectors pLenti PRDX2-Myc (PRDX2-Myc) and pLenti PRDX2-MycNuc (PRDX2-MycNuc) were generated as follow: Prdx2-myc and Prdx2-myc-nuc fragments were isolated by SalI/XbaI double digestion from pCMV-Prx2-myc and pCMV-Prx2-myc-nuc and cloned into pLenti CMV/TO PuroDEST (670-1) backbone kindly provided by Dr. Eric Campeau through Addgene (Addgene plasmid 17293) that was digested with the same enzymes (Campeau et al., 2009). The pLenti CMV/TO PuroDEST (670-1) digested with SalI/XbaI was self-ligated to obtain the empty vector (pLenti EV) used as control. The pLenti CMV V5-LUC Blast (w567-1) (CMVLuc) was kindly provided by Dr. Eric Campeau through Addgene (Addgene plasmid 21474). Lentiviral particles were produced by transient co-transfection of 70% confluent HEK293 T in 60 mm dishes with the lentiviral vectors (1 μg) and the second generation packaging plasmids pM2D (0.2 μg), pSAX2 (1.8 μg) using jetPEI® (Polyplus Transfection SA, Illkirk, France) according to manufacturer's instructions. Medium was replaced with fresh medium 24 h later. After additional 24 h, medium containing virus was collected and filtered (Ø 0.22 μm). HaCaT-Bluf cells were grown in 60 mm dishes to 30–50% of confluence and infected. Cell infection was performed as previously described (Avitabile et al., 2011).

HaCaT-Bluf cells were transduced either with Lenti-EV, Lenti-PRDX2-Myc, Lenti-PRDX2-MycNuc, or Lenti-CMVLuc using a ratio virus/medium 1:1. Virus was left on cells for two days then washed out and cultured for 7 days in medium containing 1 mg/ml blasticidin (Sigma–Aldrich) to obtain the following HaCaT-Bluf stable expressing cell lines: EV, PRDX2-Myc and PRDX2-MycNuc. PRDX2 silenced (ShPRDX2) HaCaT cells have been previously described (Avitabile et al., 2014).

2.3. Immunofluorescence

For immunofluorescence staining cells were fixed with 4% paraformaldehyde in PBS for 30 min at 25 °C followed by treatment with 0.1 M glycine for 20 min at 25 °C and with 0.1% Triton X-100 for additional 5 min at 25 °C to allow permeabilization. Cells were then incubated with the following primary antibodies: anti-myc tag monoclonal antibody (1:100 in PBS; clone 4a6, Millipore, Temecula, CA, USA), anti-PRDX2 polyclonal antibodies (1:200 in PBS; clone EPR5155, Abcam, Cambridge, UK) for 1 h at 25 °C. The goat anti-mouse IgG-Texas Red (1:200 in PBS; Jackson Immunoresearch Laboratories, West Grove, PA, USA), or goat anti-rabbit immunoglobulin IgG–FITC (1:500 in PBS; Cappel Research Products) incubated for 30 min at 25 °C were used for detection. Nuclei were stained with DAPI (1:1000 in PBS; Sigma–Aldrich) and samples were finally mounted with mowiol (Sigma–Aldrich) for observation. Fluorescence signals were analyzed with an Apo-Tome System (Zeiss, Oberkochen, Germany) connected with an Axiovert 200 inverted microscope (Zeiss); image analysis was then performed by the Axiovision software (Zeiss).

2.4. Flow cytometry analysis

Cells were washed, trypsinized, fixed with 4% paraformaldehyde for 20 min at 25 °C ROS induction was measured in H_2O_2 treated EV, PRDX2-Myc, PRDX2-MycNuc and loaded with CellROX (as described above) by flow-cytometry. At least 10,000 cells for each culture were collected and analyzed with the MACSQuant® Analyzer cytometer (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Fluorescence intensity was calculated with MACSQuantify® software (Miltenyi Biotec GmbH).

2.5. Immunoprecipitation and Western blot analysis

Cells were lysed in RIPA buffer containing 50 mM HEPES, pH 7.5, 150 mM NaCl, 1% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 5 mM EGTA, supplemented with protease inhibitors (10 mg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml leupeptin), and phosphatase inhibitors (1 mM sodium ortho-vanadate, 20 mM sodium pyrophosphate, 0.5 M NaF); 50 mg of total protein were resolved under reducing conditions by 7% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to reinforced nitrocellulose (BA-S 83, Schleider and Schuell, Keene, NH, USA). The membranes were blocked with 5% non-fat dry milk in PBS 0.1% Tween-20, and incubated with anti-Myc-tag (1:1000 in TBS-T; clone 4a6, Millipore, Temecula, CA, USA) monoclonal antibody, anti-PRDX2 (1:2000 in TBS-T; Abcam, Cambridge, UK) monoclonal antibody, anti-αTubulin (1:500 in TBS-T; clone B-7, Santa Cruz Biotechnology, Santa Cruz, CA, USA) monoclonal antibody to estimate the protein equal loading, followed by enhanced chemioluminescence detection (ECL, Amersham, Alington Heights, IL, USA).

For immunoprecipitation experiments, cells were lysed in 1% Triton X-100, 50 mM HEPES containing 150 mM NaCl 1% glycerol, 1.5 mM $MgCl_2$, 5 mM EGTA, and protease and phosphatase inhibitors as above. One milligram of total protein was immunoprecipitated with 4 mg/ml anti-Myc-Tag (Millipore) and or mouse normal IgG. Immunocomplexes, aggregated with 50 μl of γ-bind protein-A Sepharose (Pharmacia) were washed four times with 0.6 ml of buffer. The pellets were boiled in Laemli buffer for 5 min, and the protein was resolved under reducing conditions by 4–12% SDS–PAGE and transferred to nitrocellulose. The membranes were blocked with 5% non-fat dry milk in PBS 0.1% Tween-20 for 1 h and probed with anti-SIRT1 (1:1000 in TBS-T; clone H-300, Santa Cruz) and anti-Myc-Tag (Millipore) followed by enhanced chemioluminescence detection as described above.

2.6. Luciferase assay

Bmal1 promoter activity was monitored by luciferase assay, using 1 mM permeable beetle luciferin (E1602, Promega, Madison, WI, USA). The plate was kept at constant temperature (37 °C) covered by an optically clear film. The luminescence was recorded every 2 h for consecutive 56 h using a Victor Wallac 3 multilabel plate reader (Perkin-Elmer, Waltham, MA, USA) set as follow: integration time 15 s, delay between readings 5760 s, number of repeats 28.

2.7. Gene expression studies

ShPrdx2 HaCaT cells (Avitabile et al., 2014) were synchronized or not (Untr) with Dex for 1 h and total RNA was collected using TRIzol (Lifetechnologies) every 6 h for consecutive 48 h. Total RNA purification was performed according to manufacturer's instructions.

Total RNA concentration was quantified spectrofotometrically using NanoDrop (Thermo Scientific) and retrotranscribed to cDNA with iScript cDNA synthesis kit (Bio-Rad) according to manufacturer's instructions. Real-time PCR was performed using the iCycler Real-Time Detection System (iQ5 Bio-Rad). The reaction was carried out in 96-well plate using iQ SYBR Green Supermix (Bio-Rad) adding forward and reverse primers for each gene and 14 ng of template cDNA to a final reaction volume of 15 μl. The thermal cycling program was performed as follows: an initial denaturation step at 95 °C for 3 min, followed by 45 cycles at 95 °C for 10 s and 60 °C for

30 s. Relative gene expression was assessed normalizing the cycle threshold (C_t) of Bmal1 gene to ribosomal 18S RNA and fold increase was calculated using the $2^{-\Delta\Delta C_t}$ method. Primer sequences have been previously reported (Avitabile et al., 2013).

2.8. Statistical analysis

All data are expressed as fold change versus the average of all the bioluminescence signals in each time series performed in EV HaCaT cells. Data are reported as mean ± SEM. The significance of circadian oscillations and amplitudes was calculated using JTK-Cycle vers. 2.1 (Miyazaki et al., 2011).

For temperature entrainment experiments, amplitude was measured averaging maxima and minima peaks of luciferase bioluminescence recorded during the free running period and significance of time series was calculated using 1-way ANOVA.

Comparison between multiple groups was performed using 1-way or 2-way ANOVA followed by Bonferroni post hoc test depending by the experimental design. Comparison between two groups by Student's *t*-test. For each statistical test, p values lower than 0.05 were considered statistically significant. Statistical analysis was performed using Graphpad Prism v 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA).

3. Results

3.1. Generation of HaCaT cell reporter lines overexpressing PRDX2 or nuclear forced PRDX2

The reporter cell line HaCaT-Bluf (Avitabile et al., 2014) stably expressing the luciferase gene under control of the Bmal1 promoter was transduced with lentiviral vectors having a selection marker for the antibiotic blasticidin and carrying either a myc-tagged wild type PRDX2 (PRDX2-Myc), a myc-tagged wild type nuclear PRDX2 (PRDX2-MycNuc) or the empty vector as control (EV) (Supplementary Fig. 1). The new stable reporter cell lines (here referred as PRDX2-Myc, PRDX2-MycNuc and EV respectively) were obtained upon 7 days of 1 mg/ml blasticidin treatment in culture medium.

The correct size and expression of ectopic PRDX2 proteins was verified by immunoblot using the anti-Myc-tag antibody. As expected, the Myc-tag specific band was absent in EV whereas PRDX2-Myc or PRDX2-MycNuc HaCaT cells displayed distinct bands respectively at 26 kDa and 30 kDa depending on the presence of the nuclear localization sequence (Fig. 1A). Densitometric analysis revealed that the endogenous PRDX2 was equally expressed in all transduced cells and detected with a single band at 23 kDa using the specific anti-PRDX2 antibody (Fig. 1A, right panel, white bars).

The intracellular localization of overexpressed PRDX2 proteins was investigated by immunofluorescence analysis using the anti-Myc-tag antibody. Ectopic PRDX2 was detected mainly in the cytoplasm and in some extent in the nucleus of PRDX2-Myc cells, whereas it was detected only in the nucleus of PRDX2-MycNuc cells (Fig. 1B). As expected, the Myc-tag signal was not detected in EV cells (Fig. 1B). The anti-PRDX2 antibody detected both the endogenous and the exogenous PRDX2 proteins. The anti-PRDX2 and the Myc-tag signals colocalized in both PRDX2-Myc and PRDX2-MycNuc transduced cells. However, in PRDX2-MycNuc the colocalization was detectable only in the nucleus. Therefore the cytoplasmic signal revealed by the anti-PRDX2 antibody correspond only to the endogenous PRDX2 (Fig. 1B).

In summary, the ectopic expression of PRDX2-Myc and PRDX2-MycNuc in HaCaT-Bluf cells does not induce any change in the expression or localization of the endogenous PRDX2. Therefore, any difference of behavior observed in different lines is only imputable to the presence and localization of the ectopic PRDX2.

3.2. Nuclear targeted PRDX2 efficiently protects against H_2O_2-induced ROS in human keratinocytes

To demonstrate that ectopic PRDX2 proteins were functional, PRDX2-Myc, PRDX2-MycNuc and EV HaCaT cells were challenged with increasing concentration of H_2O_2 (60, 125, 250 μM) for 30′ and the amount of reactive oxygen species (ROS) were evaluated by FACS analysis using the fluorescent ROS indicator CellROX. EV cells displayed a significant increase of CellROX signal starting at 125 μM H_2O_2 (Fig. 2A). The overexpression of wild type PRDX2 resulted in a significant reduction of CellROX positive cells when keratinocytes were challenged with 125 μM H_2O_2, but this protective effect was lost at H_2O_2 concentration of 250 μM (Fig. 2B). Noteworthy, the overexpression of nuclear PRDX2 resulted in sustained protection against intracellular ROS accumulation upon both 125 μM and 250 μM H_2O_2 treatment (Fig. 2B). This protective effect was lost at higher H_2O_2 concentration of 500 μM.

Our results indicate that ectopic PRDX2 proteins are functionally active as scavenger of H_2O_2-generated ROS. Interestingly, HaCaT cells overexpressing PRDX2-MycNuc were more efficiently protected against ROS accumulation than PRDX2-Myc cells.

3.3. Overexpression of nuclear PRDX2 dampens Bmal1 promoter oscillations in entrained keratinocytes

In order to analyze the effects of the ectopic expression of PRDX proteins on circadian rhythm, confluent PRDX2-Myc, PRDX2-MycNuc and EV HaCaT transduced cells were serum starved for 16 h and pulsed with 0.1 μM Dex for 1 h. Thereafter, Dex was washed out and cells were left in serum free medium for a free running period of consecutive 56 h during which luciferase activity was measured every 2 h (Fig. 3A). Dex treatment induced significant rhythmic oscillations of Bmal1 promoter activity in EV, PRDX2-myc and PRDX2-MycNuc cells as estimated by JTK-Cycle Software (Miyazaki et al., 2001) (Fig. 3B, $p < 0.05$). Interestingly, the amplitude of the oscillations was decreased in PRDX2-Myc and significantly inhibited in PRDX2-MycNuc transduced cells as compared to EV cells (Fig. 3C). Importantly, the period length was similar in all PRDX2-Myc, PRDX2-MycNuc and EV HaCaT cells.

To assess whether PRDX2 was essential for the correct entrainment of the molecular clock Dex synchronization was repeated on the HaCaT cell line stably silenced for the Prdx2 gene (ShPrdx2). The expression of Bmal1 gene was evaluated by qRTPCR on total RNA collected every 6 h for consecutive 48 h under free running conditions. Interestingly, Prdx2 silenced cells were fully responsive to Dex as demonstrated by the induction of the rhythmic expression of Bmal1 gene (Supplementary Fig. 2, $p < 0.05$, 1-way ANOVA).

Finally, to assess whether the inhibitory effect of nuclear PRDX2 was related to a specific response to Dex, confluent PRDX2-Myc, PRDX2-MycNuc and EV cells were entrained with two 24 h-long temperature cycles (12 h 37 °C/12 h 33 °C) and then kept under free running condition at constant temperature for consecutive 48 h (Supplementary Fig. 2A). The Bmal1 promoter activity was measured every 6 h during the free running period by luciferase assay as previously described (Avitabile et al., 2014). Temperature entrainment elicited rhythmic oscillations of Bmal1 promoter activity in EV, PRDX2-myc and PRDX2-MycNuc cells (Supplementary Fig. 2B, $p < 0.05$, 1-way ANOVA). The amplitude was not significantly different between PRDX2-Myc transduced and EV cells. In contrast, PRDX2-MycNuc cells displayed significantly depressed amplitude in comparison to EV and PRDX2-Myc (Fig. 3C, $^{***}p < 0.001$, 1-way ANOVA followed by Bonferroni post hoc test).

Taken together, our results indicate that the overexpression of PRDX2 and in particular of the nuclear forced form, results in the

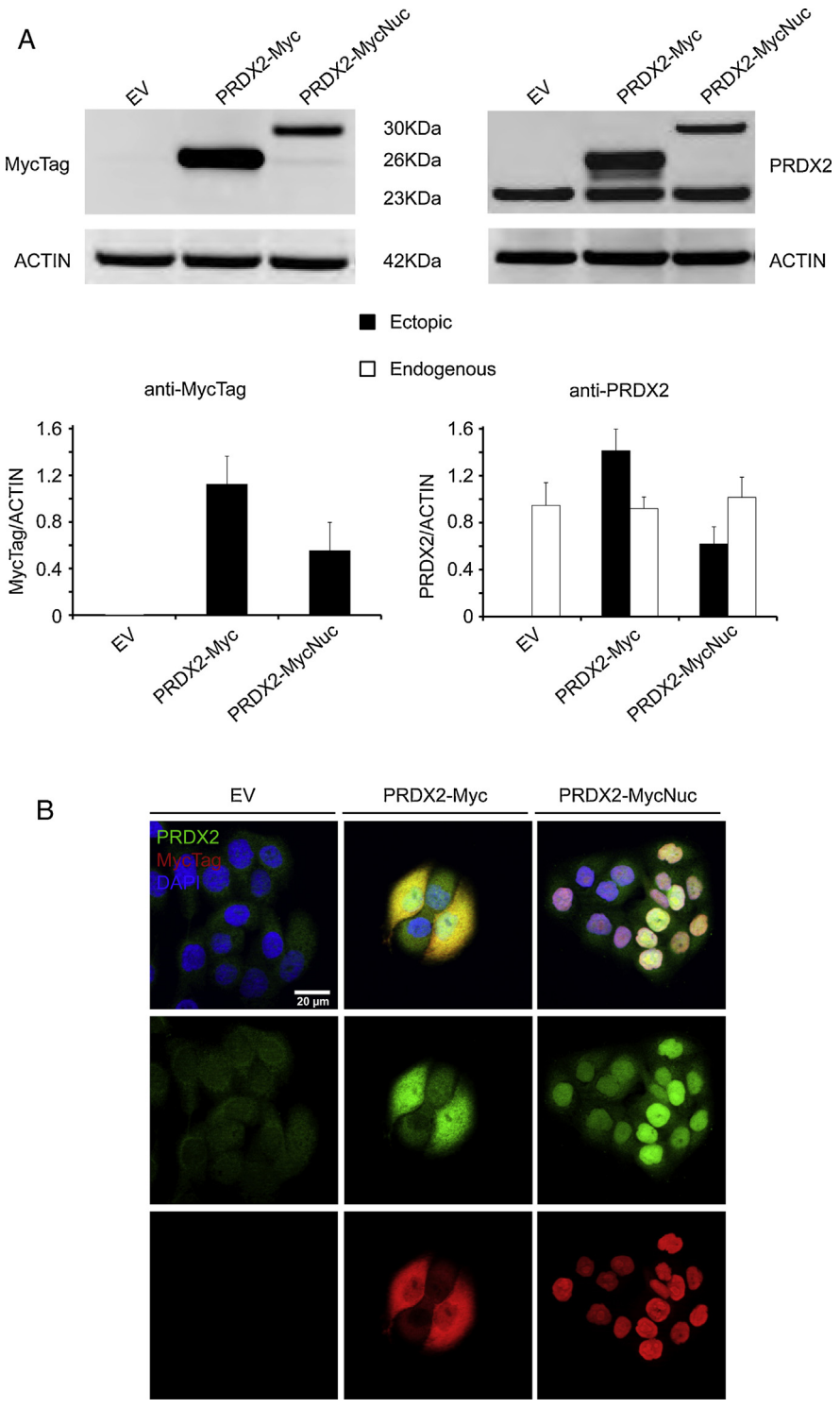

Fig. 1. Expression and localization of Myc-tag PRDX2 proteins. (A) HaCaT-BLuf reporter cells, stably expressing the luciferase gene under control of the Bmal1 promoter, were transduced with lentiviral vectors carrying either a myc-tagged wild type PRDX2 (PRDX2-Myc), a myc-tagged wild type nuclear PRDX2 (PRDX2-MycNuc) or the lentiviral empty vector as control (EV). Stable expression of the transgenes was obtained upon one week of selection with 1 mg/ml blasticidin. The new reporter HaCaT-BLuf cell lines, here referred with the name of the transgene, were analyzed by Western blot using the anti-Myc-tag antibody to specifically detect the ectopic PRDX2. Specific bands at 26 kDa or 30 kDa were evident in PRDX2-Myc or PRDX2-MycNuc respectively; as expected no band was detected in EV. Western blot analysis using the specific anti-PRDX2 antibody showed that the band at 23 kDa corresponding to the endogenous PRDX2 was present and equally expressed in all transduced cell lines. The equal loading was assessed with anti-Actin β antibody. Densitometric analysis are reported at the bottom of each Western blot and indicate the expression levels (relative to Actin β) of either ectopic (PRDX2-myc and PRDX2-MycNuc, black bars) or endogenous PRDX2 (white bars). (B) Immunofluorescence analysis of asynchronous EV, PRDX2-Myc and PRDX2-MycNuc transduced cells performed with anti-PRDX2 antibody (PRDX2, green) to detect both the endogenous and exogenous PRDX2 and anti-Myc-tag antibody (MycTag, red) to exclusively detect the ectopic PRDX2. PRDX2 signal appears both in the cytoplasm and in the nucleus of EV cells. The Myc-tag signal is absent in EV cells as expected. PRDX2 and Myc-Tag signals colocalize both in PRDX2-Myc and PRDX2-MycNuc cells. However, the colocalization is restricted to the nucleus, whereas the PRDX2 signal is clearly detectable in the cytoplasm of PRDX2-MycNuc cells. Cell nuclei were stained with DAPI (blue). Top panel, merge of PRDX2, MycTag and DAPI; middle panel, PRDX2; bottom panel, MycTag. Bar: 20 μm.

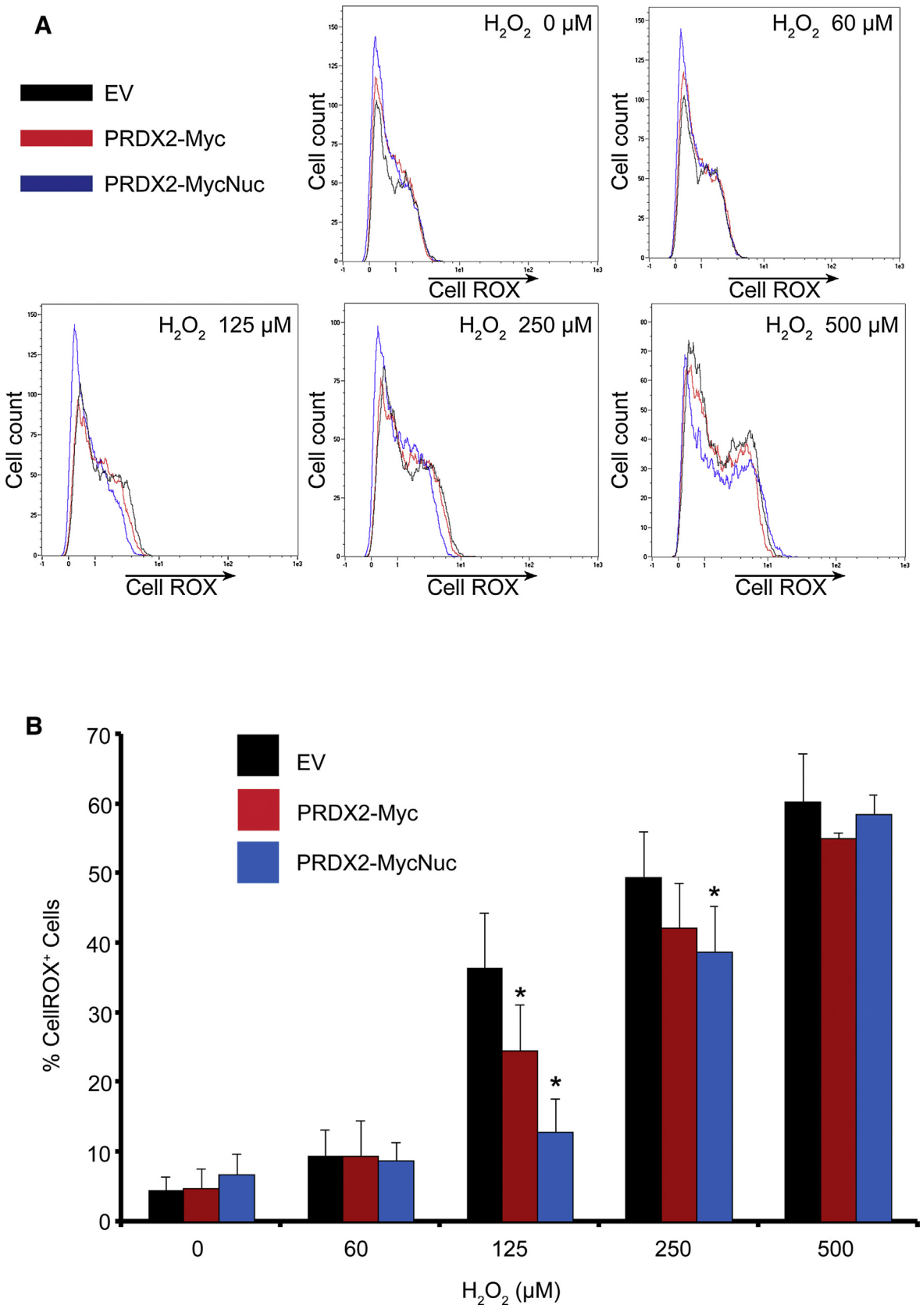

Fig. 2. Functional characterization of ectopic PRDX2 proteins. (A) To evaluate cellular ROS levels, PRDX2-Myc, PRDX2-MycNuc and EV cells treated with H_2O_2 (60, 125, 250 or 500 µM) for 30 min were incubated with CellROX and analyzed by flow-cytometry as reported in Section 2. Representative histogram plots show the amount of intracellular ROS in different cell lines (EV, black; PRDX2-Myc, red; PRDX2-MycNuc, blue). (B) Quantification analysis of FACS results. Upon treatment with 125 µM of H_2O_2, ROS levels where significantly reduced in both PRDX2-Myc and PRDX2-MycNuc cells compared to EV cells. Upon treatment with 250 µM H_2O_2 ROS protection was maintained only in PRDX2-MycNuc transduced cells (*$p < 0.05$, 2-way ANOVA followed by Bonferroni post hoc test).

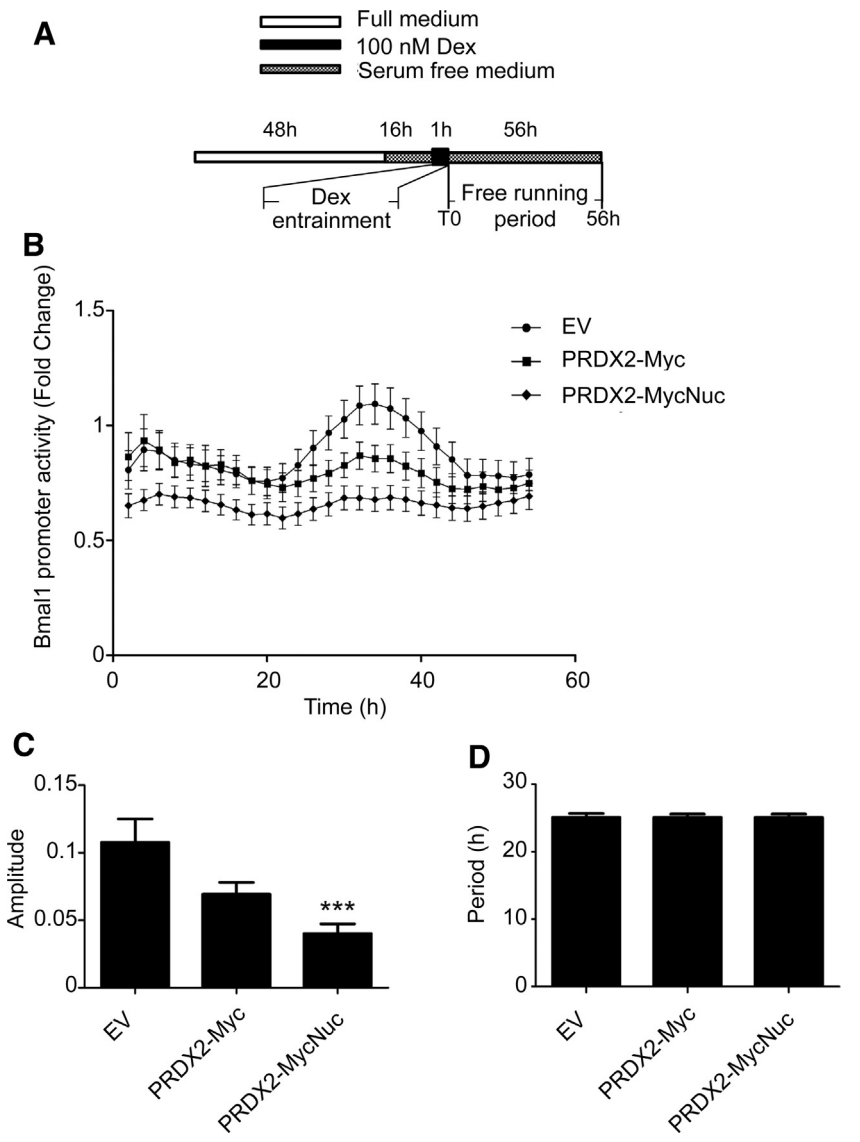

Fig. 3. Forced nuclear PRDX2 localization dampens Bmal1 promoter activity of entrained HaCaT cells. (A) Experimental scheme describing the protocol for Dex-induced synchronization. Confluent cells were serum starved for 16 h, pulsed with 0.1 μM Dex for 1 h and released in serum free medium. The luciferase activity was recorded every 2 h for consecutive 56 h as described in Section 2. (B) Luciferase assays displaying circadian oscillations of the Bmal1 promoter activity in Dex treated EV, PRDX2-myc and PRDX2-MycNuc cells. (C) The amplitude of Bmal1 promoter activity was significantly decreased in PRDX2-MycNuc as compared to EV cells. PRDX2-Myc amplitude was not significantly different to EV (***$p < 0.001$, 1-way ANOVA followed by Bonferroni post hoc test). (D) The period of circadian oscillation between the EV, PRDX2-Myc and PRDX2-MycNuc did not differ significantly.

significant repression of Bmal1 promoter activity independently of the method used to synchronize the cells.

3.4. Robust scavenging of intracellular ROS impairs Bmal1 promoter activity

To assess whether the reduction of intracellular ROS could be directly responsible for the impairment of the molecular clock function observed in PRDX2-MycNuc cells, the Bmal1 promoter activity was measured in the presence of the potent scavenger of ROS N-acetylcysteine (NAC, 1 and 5 mM). Luciferase activity was monitored for consecutive 56 h sampling every two hours during the free running period. Interestingly, both 1 and 5 mM NAC markedly inhibited the Bmal1 promoter activity that was significantly repressed in both EV and PRDX2-Myc HaCaT cells (2-way ANOVA followed by Bonferroni post hoc test; Fig. 4A and B). No additional inhibition was recorded in PRDX2-MycNuc cells (Fig. 4C). To exclude the possibility that the dampening of Bmal1 promoter oscillations was due to a generalized repression of cellular transcription, the luciferase activity was recorded in HaCaT cells stably expressing the luciferase gene under control of a constitutive CMV promoter (CMVLuc). No significant differences in luciferase activity were observed among different treatments (2-way ANOVA followed by Bonferroni post hoc test; Fig. 4D). Thus, independently from the localization of overexpressed PRDX2, sustained reduction of redox balance impaired the molecular clock function.

The hormone melatonin is a neuroendocrine output of the circadian clock and a powerful natural scavenger of ROS (Kleszczynski and Fischer, 2012). Melatonin has been shown to significantly decrease the oxidative stress in several types of epithelial cells including human keratinocytes (Fischer et al., 2006; Jiang et al., 2012). Therefore, the effect of melatonin on Bmal1 promoter activity was assayed in our transduced cells. Similarly to NAC, melatonin induced dose dependent dampening of the molecular clock in EV HaCaT cells (2-way ANOVA followed by Bonferroni post hoc test; Fig. 4E), however no additional repression of clock oscillations were

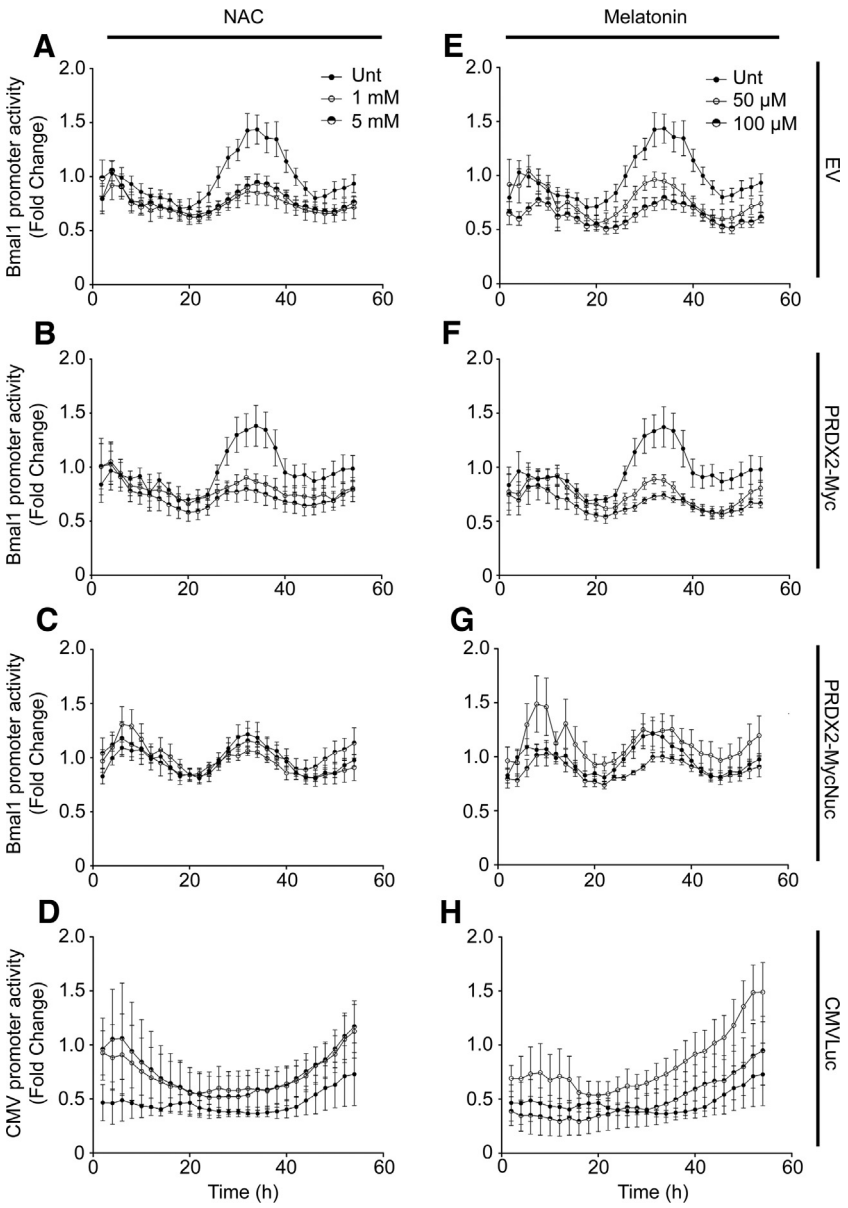

Fig. 4. N-acetylcysteine and melatonin counteract the proper induction of Bmal1 promoter activity in Dex entrained HaCaT cells. (A) EV cells were synchronized by Dex and then released in serum free medium supplemented with 1 or 5 mM NAC for consecutive 56 h. Untreated EV cells were used as control. Both NAC treatments (1 mM, 5 mM) significantly repressed the Bmal1 promoter activity as compared to untreated cells (*$p < 0.05$, 2-way ANOVA followed by Bonferroni post hoc test). (B) PRDX2-Myc cells were treated as described in (A). NAC-untreated PRDX2-Myc cells were used as control. NAC treatments significantly repressed the Bmal1 promoter activity in PRDX2-Myc cells (*$p < 0.05$, 2-way ANOVA followed by Bonferroni post hoc test). (C) PRDX2-MycNuc cells were treated as described in (A). NAC-untreated PRDX2-MycNuc cells were used as control. NAC did not induce any additional repression of Bmal1 promoter activity in PRDX2-MycNuc cells. (D) Luciferase activity was recorded in HaCaT CMVLuc cells expressing the luciferase gene under control of a constitutive CMV promoter and treated as described in (A). No significant differences in luciferase activity were observed among treatments. (E) EV cells were synchronized by Dex and then released in serum free medium supplemented with 50 or 100 μM melatonin for consecutive 56 h. Mel-untreated EV cells were used as control. Melatonin displayed a dose dependent inhibitory effect of Bmal1 promoter activity (2-way ANOVA followed by Bonferroni post hoc test). (F–G) Melatonin did not induce any additional impairment of Bmal1 promoter activity in PRDX2-Myc cells and PRDX2-MycNuc cells. (H) Melatonin treatments, had no effects on luciferase activity in CMVLuc HaCaT cells.

recorded in PRDX2-Myc or PRDX2-MycNuc HaCaT cells (Fig. 4F–G). Moreover, melatonin had no effect on the luciferase activity in CMVLuc HaCaT cells (Fig. 4H). Taken together our results overtly indicate that the sustained reduction of the redox balance has detrimental effects on the molecular clock function.

3.5. H_2O_2 restores proper levels of Bmal1 promoter activity in PRDX2-MycNuc transduced cells

We hypothesize that the excessive scavenging of nuclear ROS consequent to the nuclear overexpression of PRDX2 could be responsible for the impairment of the Bmal1 promoter activity. If this was the case, the regular activity of Bmal1 promoter could be rescued by restoring normal redox levels in PRDX2-MycNuc cells. To this aim, EV, PRDX2-Myc and PRDX2-MycNuc HaCaT cells were synchronized with Dex in the presence of increasing doses (0, 60, 125, 250 and 500 μM) of H_2O_2. The CMVLuc HaCaT cells were used as control of the treatments. The luciferase activity was recorded every 2 h for consecutive 56 h of free running period. In EV HaCaT cells, the treatment with H_2O_2 dampened the Bmal1 promoter activity dose dependently and significantly at concentration of 250 and 500 μM (2-way ANOVA followed by Bonferroni post hoc test; Fig. 5A). Interestingly, only the higher H_2O_2 concentration was effective in dampening the molecular clock oscillations

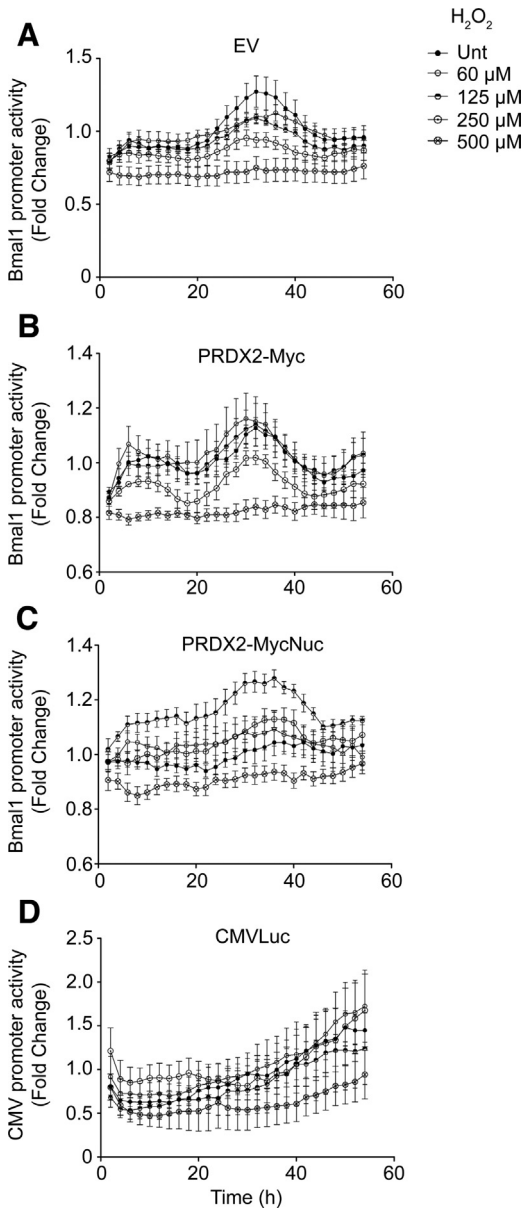

Fig. 5. Bmal1 promoter activity is restored by a specific range of H_2O_2 concentration in Dex-entrained PRDX2-MycNuc cells. (A) EV HaCaT cells were synchronized with Dex in the presence of increasing doses (0, 60, 125, 250 or 500 μM) of H_2O_2. The luciferase activity was recorded every 2 h for consecutive 56 h. In EV HaCaT cells, H_2O_2 dampened the Bmal1 promoter activity dose dependently and significantly at 250 and 500 μM (2-way ANOVA followed by Bonferroni post hoc test). (B) Similarly, H_2O_2 impaired dose dependently the luciferase activity of the Bmal1 promoter in PRDX2-Myc cells. (C) In contrast, Bmal1 promoter activity was enhanced in PRDX2-MycNuc HaCaT cells displaying a significant rescue at 125 μM H_2O_2. The positive effect on the rhythm amplitude was lost at lower (60 μM) or higher H_2O_2 concentrations (250 μM and 500 μM). (D) None of the H_2O_2 treatments showed any significant effect on the CMV promoter activity.

of PRDX2-Myc cells (2-way ANOVA followed by Bonferroni post hoc test; Fig. 5B). Conversely, the rhythmic activity of Bmal1 promoter was rescued in PRDX2-MycNuc HaCaT cells using the specific concentration of 125 μM H_2O_2 (2-way ANOVA followed by Bonferroni post hoc test; Fig. 5C). No significant effects were observed by treating the cells with either the lower (60 μM) or the higher concentrations of H_2O_2 (250 μM and 500 μM). None of the treatments had any significant effect on the CMV promoter activity (Fig. 5D).

Taken together our results clearly indicate that the impairment of the molecular clock function induced by nuclear PRDX2 overexpression can be rescued by normalizing intracellular ROS levels using a specific H_2O_2 concentration.

3.6. Sirt1 inhibition rescue Bmal1 promoter activity in Dex-synchronized PRDX2-MycNuc cells

To address whether PRDX2 could influence the Bmal1 promoter activity by modulating the Sirt1 enzymatic activity EV, PRDX2-Myc and PRDX2-MycNuc cells were entrained by Dex in the presence of different concentrations of the Sirt1 activator resveratrol (10, 25 or 50 μM) or its inhibitor NAM (1, 2.5, or 5 mM). Resveratrol treatment did not induce any significant effect either Bmal1 or CMV promoter activities at any of the concentrations tested (Fig. 6A–D). The treatment with NAM did not induce any significant change of Bmal1 promoter activity in Dex-synchronized EV cells (Fig. 6A). Strikingly, Bmal1 rhythmic promoter activity was completely rescued in Dex synchronized PRDX2-MycNuc cells when treated with different concentration of NAM (2-way ANOVA followed by Bonferroni post hoc test; Fig. 6G). A similar trend was also observed in PRDX2-Myc cells (Fig. 6F) this suggesting an inverse correlation between Sirt1 enzymatic activity and intracellular ROS levels. Neither resveratrol nor NAM had a significant effect on the activity of the constitutive CMV promoter at any of the concentrations used in the experiment (Fig. 6H).

Finally, in order to address whether PRDX2 could directly interact with Sirt1, the ectopic PRDX2 was co-immunoprecipitated using a specific antibody raised against the Myc-tag. WB analysis revealed the absence of any interaction between PRDX2 and Sirt1 in either PRDX2-Myc or PRDX2-MycNuc cells. Importantly, the basal expression levels of Sirt1 were not different in EV, PRDX2-Myc or PRDX2-MycNuc HaCaT cells (Supplementary Fig. 4).

In conclusion, our results suggest that PRDX2 indirectly favor the enzymatic activity of Sirt1 and indicate that its inhibition mediated by NAM can restore the normal oscillations of Bmal1 promoter in PRDX2-MycNuc cells.

4. Discussion

An increasing number of studies suggest the existence of an important crosstalk between the molecular clock and the redox-system. The cellular redox state is critically important for the regulation of the master clock Bmal1, Clock, Npas2 and Per2 genes transcriptional activity (Rutter et al., 2001; Nakahata et al., 2009). Several antioxidant genes are under control of the circadian clock and disruption of the circadian system is associated with increased oxidative stress both at cellular and systemic levels (Stangherlin and Reddy, 2013; Hardeland et al., 2003; Patel et al., 2013; O'Neill and Feeney, 2013). Moreover, administration of near-lethal doses of ROS inducing sustained oxidative stress has been recently demonstrated to act as an efficient resetting stimulus for the circadian clock in vitro (Tamaru et al., 2013).

The production of ROS is a direct consequence of respiration and metabolism, therefore the cellular redox balance displays rhythmic oscillations related to the circadian regulation of metabolic activities. Indeed, rhythms in ROS production have been already reported in neuronal tissue (Musiek et al., 2013). However, whether or not physiological redox fluctuations can act as input of the circadian rhythms has not fully elucidated yet.

Recently, it has been elegantly demonstrated that PRDXs display cyclic circadian oscillations of their oxidative status in enucleated adult erythrocytes, thus independently of the TTFL (O'Neill and Reddy, 2011). Such metabolic oscillations were also observed in nucleated wild type and long period or arrhythmic clock mutant cells where the oxidation rhythms of PRDX were not lost but clearly altered. On the other hand, data regarding the influence of PRDXs on

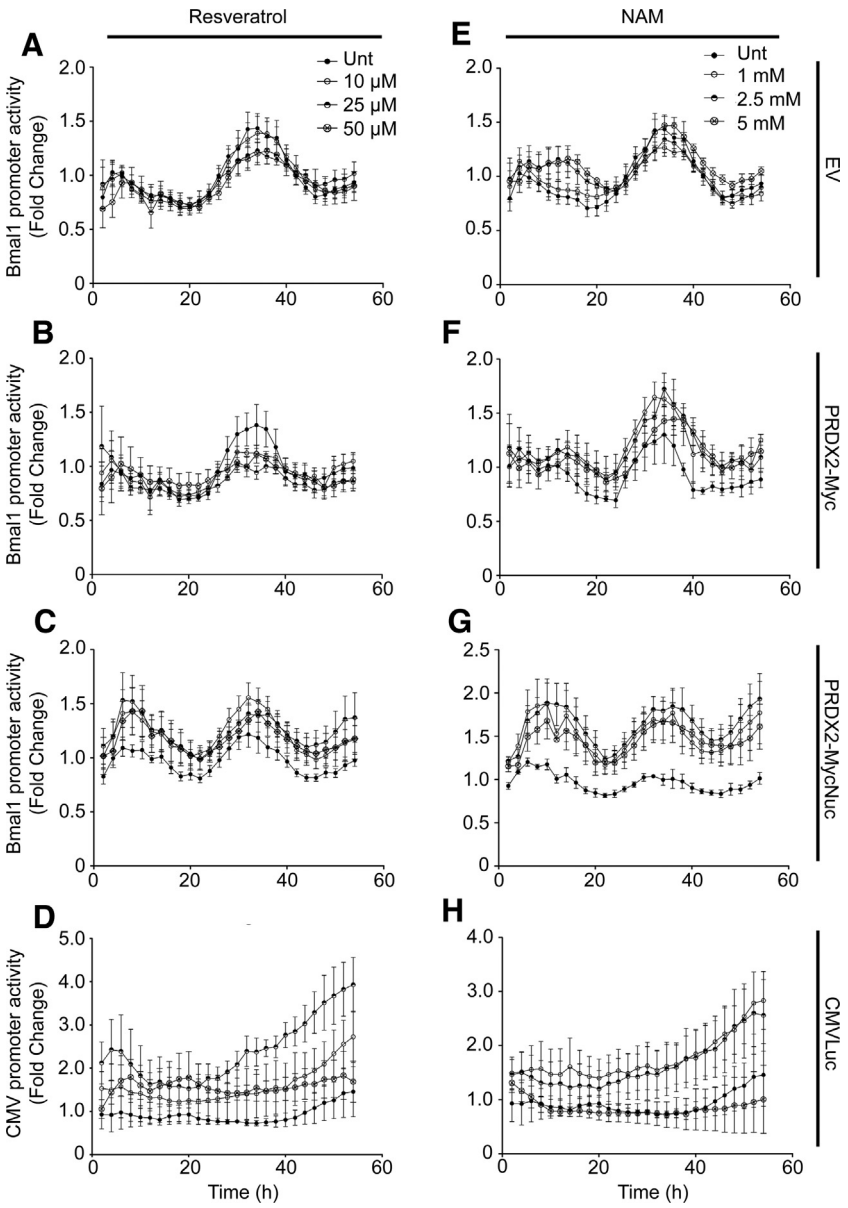

Fig. 6. Sirt1 inhibition rescues Bmal1 promoter activity in PRDX2-MycNuc cells. EV, PRDX2-Myc, PRDX2-MycNuc and CMVLuc HaCaT cells were treated with Dex in the presence of increasing doses (0, 10, 25 and 50 μM) of resveratrol. The luciferase activity was recorded every 2 h for consecutive 56 h. (A–D) Resveratrol did not induce any significant effect on Bmal1 oscillation in either EV, PRDX2-Myc, PRDX2-MycNuc cells or on the constitutive activity of CMV promoter in CMVLuc HaCaT cells. EV, PRDX2-Myc, PRDX2-MycNuc and CMVLuc HaCaT cells were treated with Dex and released in serum free medium in the presence of increasing doses (0, 1, 2.5 and 5 mM) of NAM. The luciferase activity was recorded every 2 h for consecutive 56 h. (E and F) NAM did not induce any significant effect on Bmal1 rhythmic activity of either EV or PRDX2-Myc HaCaT cells. (G) Conversely, NAM completely rescued the Bmal1 promoter activity in PRDX2-MycNuc cells (2-way ANOVA followed by Bonferroni post hoc test). (H) None of the NAM treatments showed any significant effect on the CMV promoter activity.

the TTFL are based on results obtained from an unbiased genome-wide RNAi study performed in human U2OS cells, suggesting that some PRDX proteins (including PRDX2) could influence the length or the amplitude of the circadian rhythms (Zhang et al., 2009). However, since the genuine nature of this high throughput study, many results are not validated and they could be affected by the cellular context.

We have recently demonstrated that nuclear levels of PRDX2 changes rhythmically according to the cell's circadian phase. Intriguingly, nuclear accumulation is specific for PRDX2 and correlates with the descending phase of BMAL1 promoter activity in temperature entrained keratinocytes (Avitabile et al., 2014).

PRDXs have been implicated in the regulation of H_2O_2-dependent intracellular signaling (Rhee et al., 2005) and activity of diverse proteins and transcription factors (Kang et al., 2011; Egler et al., 2005). Such regulatory activity of PRDXs depends by their ability to modulate the spatiotemporal distribution of intracellular ROS (Mishina et al., 2011) that are stringently kept under specific physiological levels (Eppler et al., 2013). In an attempt to address whether or not PRDX2 could influence the TTFL function we analyzed the rhythmic behavior of the Bmal1 promoter in human keratinocytes overexpressing PRDX2 and having normal distribution (PRDX2-Myc) or being constitutively forced in their nuclei (PRDX2-MycNuc). Our study demonstrates that the overexpression of PRDX2 in the nucleus of human keratinocytes significantly impairs the promoter activity of Bmal1 master clock gene. Since PRDX2 regulates the redox status of its milieu, our results strongly suggest that nuclear redox imbalance could be responsible for the

dampening of the Bmal1 promoter activity. This effect is independent of the synchronization stimulus since the same phenomenon has been observed both in temperature and in Dex-entrained human keratinocytes. Moreover, our results clearly demonstrate that the PRDX2-dependent ROS scavenging activity is crucial to obtain the impairment of Bmal1 promoter activity. Indeed, neither PRDX2 overexpression nor its donwregulation but only its nuclear expression had a clear effect on the amplitude of the molecular clock. Interestingly, the sustained reduction of the cellular redox balance using pharmacological concentration of NAC or melatonin scavenger molecules also resulted in the significant dampen of Bmal1 rhythms. These results together with the observation that only appropriate concentrations of H_2O_2 could rescue the normal amplitude of Bmal1 rhythmic oscillations, overtly demonstrate that correct entrainment of the circadian clock is only possible under a specific range of redox balance of the nuclear compartment.

In the attempt to elucidate the mechanism trough which PRDX2 dampens Bmal1 promoter activity we focused our attention on the NAD^+-dependent histone deacetylase Sirt1. Sirt1 acts as a rheostat for the circadian system and its activity has been correlated with either an improvement (Asher et al., 2008) or a downregulation of clock oscillations (Nakahata et al., 2008; Bellet et al., 2013). Despite PRDX2 and Sirt1 did not interact together, the inhibition of Sirt1 activity by NAM treatment resulted in a complete rescue of Bmal1 oscillatory activity in PRDX2-MycNuc cells, this suggesting that Sirt1 participates in the donwregulation of the molecular clock activity in HaCaT keratinocytes. These results are in agreement with previous studies showing that the ablation of Sirt1 results in increased amplitude of circadian gene expression (Nakahata et al., 2008) whereas its pharmacological activation impairs Bmal1 promoter activity (Bellet et al., 2013). Since Sirt1 is inactivated by oxidative stress (Volonte et al., 2015), it is possible that nuclear forced PRDX2 could favor Sirt1 enzymatic activity by keeping low the levels of ROS in the nucleus of transduced cells.

Alternatively, PRDX2 could dampen Bmal1 promoter activity through the involvement of the orphan nuclear receptors Rev-Erb α/β and the redox status of their cofactor heme. Indeed, heme, has been recently demonstrated to bind and modulate the repressor activity of nuclear orphan receptor Rev-Erb α/β (Raghuram et al., 2007) and this interaction is more efficient when heme is in its reduced state (Gupta and Ragsdale, 2011). Therefore, nuclear PRDX2 could indirectly induce the repressor activity of Rev-Erbs by lowering the nuclear redox balance and favoring the increase of the reduced heme local concentration. Indeed, PRDX2 could be directly involved in reducing heme since has been recently found to bind the cofactor in K562 cells (De Franceschi et al., 2011). Since the ability of ROS scavenging is coordinated in a circadian manner, our results have at least three important consequences: (1) in nucleated cells the molecular clock and the redox system are closely intertwined possibly through the indirect influence of PRDX2 on Sirt1 enzymatic activity; (2) the reciprocal influence of the two systems depends both by the expression levels as well as the compartmentation of the ROS scavenger functions; and (3) deregulation of antioxidant enzymes or even variation of their spatiotemporal distribution could results in the disruption of the circadian clock and consequently in the general deregulation of the metabolic pathways having a marked circadian component. Indeed, disruption of the circadian clock has been associated with chronic misregulation of redox balance and several aging-related pathological conditions including cancers (Hardeland et al., 2003).

An adequate resonance of the circadian and the redox systems might be particularly important to maintain the correct homeostasis in the skin. Skin is the first barrier against the environment and in addition to the ROS of metabolic origin has to continuously face ROS derived from external stressors such as ionizing radiations, chemical hazards and pollution. Noteworthy, many skin physiological functions are regulated by the circadian system (Geyfman and Andersen, 2009) including its ability to activate the DNA damage repair system. Indeed, skin cells of mice having genetically disrupted circadian clock are more sensitive to UV-induce DNA damage (Geyfman et al., 2012; Eppler et al., 2013).

5. Conclusion

Our study supports the idea that the redox and circadian systems are interconnected by a delicate equilibrium in peripheral organ such as the skin. The disruption of such equilibrium could induce misalignment of the peripheral with the central clock metabolism this favoring the insurgence of pathological conditions.

Competing interests

The authors declare that they have no competing interests.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biocel.2015.05.018

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