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Regulation of Circadian Clocks by Redox Homeostasis*

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Living organisms possess biological clocks that resonate with environmental cycles in light, temperature, and food availability. Recently, circadian oscillations in the redox state of peroxiredoxin have been described as an additional non-transcriptional timekeeping mechanism. Of note, this redox cycle is conserved in both prokaryotes and eukaryotes. How the classical "transcription-translation feedback loop" model and this redox oscillation are related is still poorly understood. In this minireview, we describe the most recent evidence pointing to cross-talk between the circadian clock and the redox status of the cell.

The integration of biological clocks into cellular physiology has represented an important evolutionary advantage for multicellular and unicellular organisms, allowing them to anticipate and adapt to cyclical changes in environmental cues such as light, temperature, and food availability (1). The advantage conferred by resonating with environmental cycles has been technically challenging to demonstrate. However, pioneering experiments have shown that coordination with light/dark cycles can improve fitness in bacteria, flies, and plants (2–5).

In mammals, the timing system is composed of a series of biological clocks organized in a hierarchical manner. The main clock, also known as the "master pacemaker," resides in the paired suprachiasmatic nuclei (SCN)[2] of the hypothalamus, which receive and process light signals to achieve synchronization with the external environment. Through the release of hormones and neuropeptides, the SCN coordinate several other clocks distributed in different tissues and organs. These peripheral clocks in turn generate local self-sustained circadian rhythms (from Latin *circa diem*, about a day) of physiological processes to control tissue-specific functions (6–8).

The first insights into the molecular mechanism of cellular rhythmicity came from relatively recent studies in *Drosophila* and *Neurospora crassa*. These studies showed that rhythmic oscillations in the expression of clock-controlled genes are generated by transcription-translation feedback loops (TTFLs) and that they are necessary to coordinate behavioral rhythmicity (9, 10). Similar timekeeping logic was later described in other organisms, although with different genes involved and different levels of complexity in the transcriptional circuits (11, 12). In mammals, for example, two positive activators, CLOCK (circadian locomotor output cycles kaput) and BMAL1 (brain and muscle Arnt-like protein 1), initiate transcription of the *Period1/2* (*Per1/2*), *Cryptochrome1/2* (*Cry1/2*), *Ror*α (retinoic acid receptor-related orphan receptor α), and *Rev-erb*α/β genes. When the level of expression of PER and CRY proteins reaches a particular threshold, they translocate into the nucleus and inhibit the transcriptional activity of the CLOCK-BMAL1 heterodimer, thereby blocking their own transcription. An additional loop is created by the REV-ERBα/β and RORα proteins, which instead repress or activate transcription of the *Bmal1* gene, respectively (Fig. 1) (13). This classical model based on transcription has been slightly revisited in light of new data showing that proteasomal degradation, epigenetic modulation of gene expression, and post-translational modifications of mRNA play a key role in keeping rhythmicity (11, 14–16). For example, the turnover of PER and CRY is controlled by phosphorylation-mediated ubiquitination processes (17–21).

Although conserved in many organisms, the TTFL cannot be considered as a universal building block for circadian clocks (11). For instance, the yeast *Saccharomyces cerevisiae* and the worm *Caenorhabditis elegans* show circadian rhythms but do not express the classical "clock genes" (22–24). Also, the cyanobacterium *Synechococcus elongatus* and the filamentous fungus *N. crassa* tend to favor protein phosphorylation as their basic timing mechanism (25, 26). Very recently, biochemical oscillations of the redox state of the protein peroxiredoxin (Prx) have been described as an additional timekeeping mechanism conserved in both eukaryotes and prokaryotes (12, 27, 28). These findings have thus revealed an intriguing link between the redox status of the cell and circadian clocks. We will discuss what we know about clock-relevant redox control systems and the reciprocal regulation between the redox state of the cells and circadian clocks.

Oxidative State and Redox Control Systems

The cellular redox environment is determined by the balance between the generation of oxidants and free radicals and the level of reducing agents. The most common oxidants are the reactive oxygen species (ROS), which are generated by intracellular enzymes during metabolic reactions. Some examples include superoxide anion ($O_2^{\bullet-}$), hydroxyl radical ($HO^{\bullet}$), and hydrogen peroxide (H_2O_2). To avoid oxidative damage, cells have adopted several detoxification strategies. Non-enzymatic mechanisms involve the synthesis of antioxidant molecules such as ascorbate, tocopherols (including vitamin E), and retinol (vitamin A). Enzymatic mechanisms include proteins such as superoxide dismutase, which catalyzes the dismutation of

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[2] The abbreviations used are: SCN, suprachiasmatic nuclei; TTFL, transcription-translation feedback loop; Prx, peroxiredoxin; ROS, reactive oxygen species; Trx, thioredoxin; Grx, glutaredoxin; Srx, sulfiredoxin.

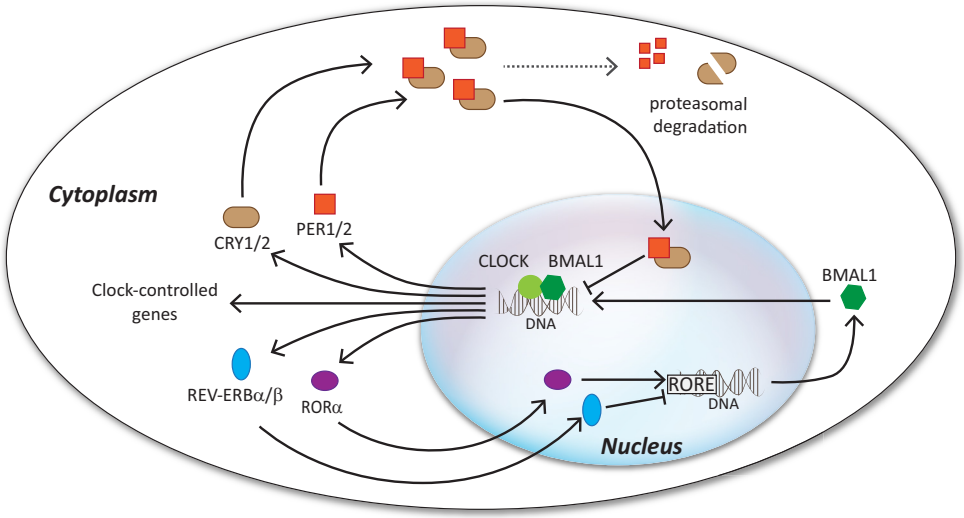

FIGURE 1. **Mammalian TTFL.** The mammalian clock is sustained by a series of feedback loops involving several genes and the proteins that they encode. The two positive activators, CLOCK and BMAL1, initiate the transcription of the clock genes *Per1/2*, *Cry1/2*, *Ror*α, and *Rev-erb*α/β. PER1/2 and CRY1/2 proteins accumulate, dimerize, and translocate into the nucleus, where they bind to the CLOCK-BMAL1 dimer, thereby inhibiting its transcriptional activity. Eventually, proteasomal degradation of PER1/2 and CRY1/2 relieves the transcriptional repression on the CLOCK-BMAL1 complex, and the cycle can restart again. An additional loop involves the nuclear receptors RORα and REV-ERBα/β, which activate and repress the transcription of *Bmal1*, respectively. *RORE*, retinoic acid receptor-related orphan receptor response element.

superoxide into oxygen and hydrogen peroxide, and catalase, which mediates the decomposition of hydrogen peroxide to water and oxygen. Additional redox buffering systems are provided by oxidize/reduced GSH and oxidized/reduced thioredoxin (Trx) (Fig. 2).

GSH is a low molecular weight antioxidant involved in the reduction of disulfide bonds and in the reduction of hydroperoxides by GSH peroxidases. Oxidized GSH (the disulfide GSSG) is potentially dangerous for the cell (29, 30), but it is normally reduced to GSH by GSH reductases via an NADPH-dependent reaction. Disulfide bridges in proteins are also reduced by glutaredoxins (Grxs), which rely on GSH for their non-enzymatic regeneration. GSH can also be conjugated to Cys residues on proteins by GST in a process called glutathionylation, which protects proteins from oxidation (31, 32). Similar to Grx, Trx proteins facilitate the reduction of several proteins by cysteine thiol-disulfide exchange. Oxidized Trxs are eventually reduced by Trx reductases via NADPH-dependent reactions. Among these antioxidant systems, Prxs have recently emerged has key players in the control of circadian rhythms.

Prx Cyclical Oxidation as the Prototype for Redox-regulated Cytosolic Clocks

Prxs are a highly conserved family of antioxidant proteins classified as class 1-Cys and class 2-Cys depending on the number of Cys residues involved in catalysis. In their catalytic site, Prxs contain a "peroxidatic" Cys residue that can be oxidized to a sulfenic acid (Cys-SOH) by an incoming peroxide (Fig. 2). In class 2-Cys Prxs after oxidation, this residue reacts with a "resolving" Cys residue to form an *inter*molecular (typical and atypical class 2-Cys) or *intra*molecular (atypical class 2-Cys) disulfide bond, which is eventually reduced by Trx. Typical class 2-Cys Prxs can undergo further oxidation (termed hyperoxidation), which generates sulfinic ($Cys\text{-}SO_2H$) and sulfonic ($Cys\text{-}SO_3H$) acid forms of the catalytic cysteine (33–35). The

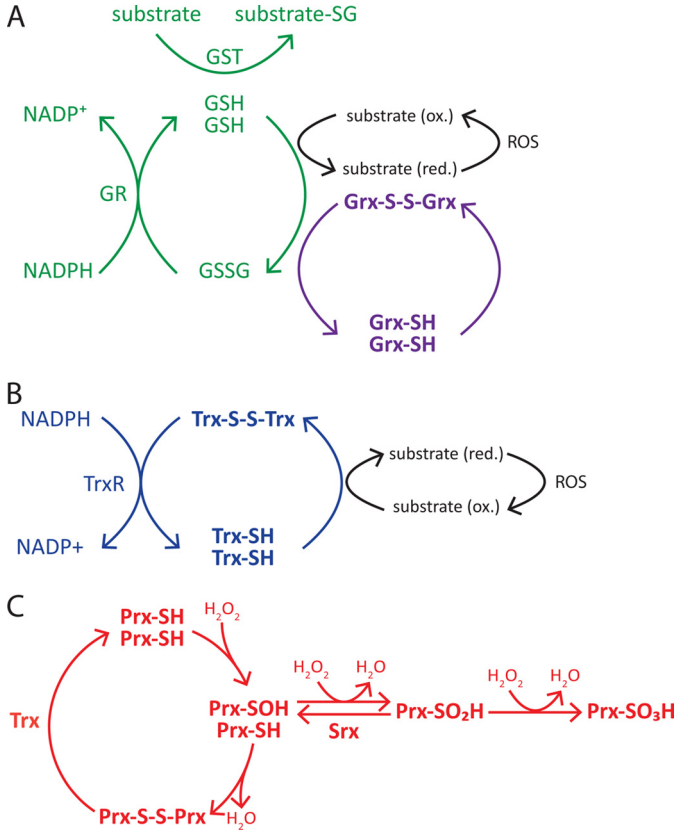

FIGURE 2. **Redox systems.** Shown is a schematic representation of the cellular redox systems and main antioxidant enzymes. *A*, GSH-Grx system. *B*, Trx system. *C*, Prx system. *substrate-SG*, glutathionylated substrate; *GR*, GSH reductase; *ox.*, oxidized; *red.*, reduced; *TrxR*, Trx reductase.

sulfinic acid form is catalytically inactive but can be reactivated by sulfiredoxin (Srx) through an ATP-dependent reduction reaction (36). In contrast, the sulfonic acid form is irreversibly oxidized, and its physiological occurrence is controversial (37, 38).

It has been recently demonstrated that Prxs follow circadian cycles of oxidation. In a recent study, in fact, the levels of dimeric Prx-$SO_{2/3}$H were shown to oscillate with a period of 24 h with peaks of hyperoxidation at 12 h (circadian time) (27). Strikingly, these oscillations were demonstrated to occur in RBCs, which do not possess DNA, showing that Prx oscillations occur even in the absence of gene transcription (27). Oscillations in Prx have also been found in the small protist *Ostreococcus tauri*, which, contrary to RBCs, possesses an endogenous clock driven by transcription and translation of recognized plant clock genes (39). Importantly, oscillations in Prx could be detected also when this organism was shifted into a dark environment, a condition under which gene transcription of *O. tauri* is known to stop (28). In addition, when the organism was brought back to light, the clock did not reset, suggesting that a mechanism must be in place to keep track of time even in the absence of gene transcription. These studies therefore show that Prx redox cycling events could be an important mechanism for timekeeping.

Of note, circadian oxidation of Prx has been found not only in eukaryotes (including algae, fungi, flies, worms, and mammals) but also in archaea and bacteria (12, 24, 27, 28), suggesting that these oscillations might have been integrated early in evolution and likely coevolved with differing TTFLs in each organism. A key unanswered question is what determines Prx oscillations. Srx, which reduces the inactive sulfinic acid form into the active sulfenic acid form, might indeed account for these oscillations. However, some organisms that display oscillations in Prx do not express Srx homologs (*i.e. C. elegans* and *N. crassa*), suggesting that other mechanisms might be in place.

Given the highly conserved redox component of circadian oscillations, it is an important goal to now understand the relationship between the classical TTFL and Prx oscillations (12). Interestingly, when the transcriptional machinery is disrupted (*e.g.* in behavioral arrhythmic *Drosophila* mutants or in *N. crassa* mutants exhibiting a lengthened period), Prx oscillations are perturbed in phase, suggesting that gene transcription is not necessary but is related to cellular metabolic cycles. Along the same lines, when the Prx clock system is abolished, as occurs in mutants of *S. elongatus* and *Arabidopsis thaliana* deficient in well annotated 2-Cys Prx genes, circadian rhythms of clock genes persist with the same period as in control organisms, but are perturbed in either phase or amplitude (12). Taken together, these studies show that TTFL and Prx cycles are intertwined but potentially autonomous components of the circadian system. These results also raise the possibility that the redox status of the cell fluctuates and that these oscillations have critical and as yet incompletely understood biological consequences.

The Reciprocal Relationship between the Redox State and Circadian System

Initial hints that redox metabolism might be linked to the circadian clock were provided by work done by Rutter *et al.* (40) in which the ratio between oxidized and reduced forms of NAD and NADP cofactors was shown to regulate the DNA-binding activity of the CLOCK/NPAS2 (neuronal PAS domain protein 2)-BMAL1 heterodimer. However, these studies were purely biochemical, based solely on the use of purified recombinant proteins, and used concentrations of reactants much higher than is seen physiologically, making their wider interpretation difficult, especially in an *in vivo* context. More recently, *in vivo* oscillations in the redox state of FAD and NADPH have been described in organotypic slices of SCN (41). This study demonstrated that the redox state of SCN oscillates in a self-sustained fashion and that these oscillations contribute to determining the excitability of SCN neurons via non-transcriptional regulation of potassium channels. However, the connection between the transcriptional clock and redox oscillations in this tissue requires further investigation. Whether redox fluctuations are an output of circadian rhythms or whether they can act as input, or indeed both, is still under intense investigation.

In favor of a mechanistic link between redox fluctuations and the regulation of gene expression, studies in zebrafish demonstrated that changes in redox state actively control the expression of light-dependent genes. Light, which is the key entraining stimulus in this organism, generates H_2O_2, which in turn regulates the expression of the clock genes *zCry1* and *zPer2*. Interestingly, oscillations in the mRNA levels of these genes are paralleled by antiphasic oscillations in mRNA and the activity of catalase (42), suggesting that this enzyme is involved in the control of H_2O_2-mediated circadian gene expression. Recently, LdpA (light-dependent period A), a component of the cyanobacterial circadian clock, was proposed to act as a redox sensor and to be used by the clock to adjust the period length (43). LdpA contains iron-sulfur centers and can sense the redox state of the cell, which correlates with the amount of light (high light correlates with a reduced redox state, whereas low light is associated with an oxidized redox state). Interestingly, on the basis of the light conditions, LdpA modulates the levels of CikA and KaiA, the latter of which is a key component of the central oscillator (44), thereby affecting the period length. Furthermore, cyanobacteria exposed to high light conditions show short periods, whereas cyanobacteria exposed to low light conditions display long periods. Finally, the effects of altered ROS and the circadian clock have also been observed in *N. crassa* (45, 46) and in the cyanobacterium *Microcystis aeruginosa* (47), in which H_2O_2 has been shown to impact on the daily expression pattern of clock genes as well as clock-controlled genes, including those involved in coordinating photosynthesis. These results clearly show that fluctuations in the redox state of the cells have an impact on the expression of clock-related genes in multiple diverse systems.

This scenario is further complicated by the finding that clock genes can in turn regulate the expression of antioxidant enzymes, thus providing an important and novel feedback loop (Fig. 3). For instance, in *A. thaliana*, the circadian clock coordinates ROS homeostasis and ROS-responsive genes, and H_2O_2 production and scavenging exhibit diurnal rhythms (48). Importantly, mutations in the core clock regulator CCA1 (circadian clock-associated 1) or in other components of the TTFL affect this time of the day specific pattern. In addition, it was observed that ROS can feed back to affect the transcription of clock-regulated genes. The importance of this cross-talk has been underlined in *Drosophila melanogaster*, in which the *per* gene has been shown to be essential for maintaining antioxi-

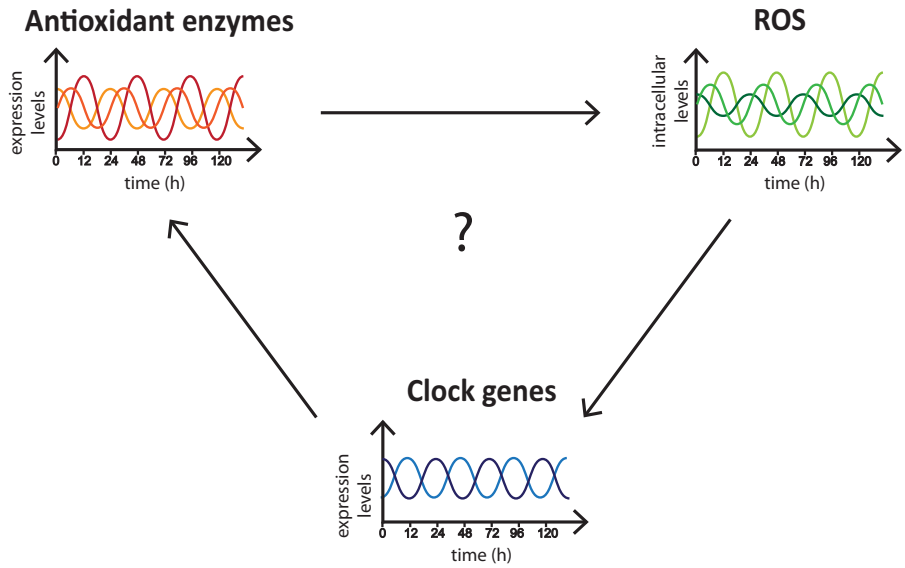

FIGURE 3. **Cross-talk between the circadian clock and redox homeostasis.** The circadian clock and the redox state of the cell are interconnected. The expression level and activity of antioxidant enzymes determine the levels of intracellular ROS, which have been shown to impinge on the expression pattern of clock genes. In addition, some antioxidant enzymes have been shown to follow a circadian pattern of expression, suggesting that the clock system can regulate redox homeostasis.

dant defense. Indeed, flies exposed to H_2O_2 show daily mortality rhythms and are more susceptible during the late light phase. Mutation in the *per* gene abolishes this time of the day sensitivity and renders flies more susceptible to oxidative stress in general (49). *Bmal1*$^{-/-}$ mice show higher accumulation of ROS in several tissues compared with wild-type animals. This impairment in ROS homeostasis correlates with early aging and age-dependent pathologies. These data again suggest a connection between the circadian clock and redox homeostasis (50). More recently, the circadian system has been shown to also modulate the pathways involved in production and utilization of GSH (51). Wild-type Canton S flies show daily rhythms in the mRNA levels of glutamate-cysteine ligase, the rate-limiting enzyme in GSH biosynthesis, and GSTD1, which utilizes GSH in cellular detoxification. Importantly, mutants lacking the clock genes *per* and *cyc* show no rhythms in the expression of these proteins, underlying the link between GSH metabolism and the circadian system.

Compartmentalization of Oxidative State and Redox Signaling: Future Perspectives

An emerging feature of redox signaling is its spatial and temporal compartmentalization. Recent developments highlight that different ROS signaling and redox buffering systems are spatially segregated and can have unique compartmentalized functions (Fig. 4) (52–55). For example, pools of mitochondrial, cytosolic, and nuclear GSH are separated within cells, and the trafficking of GSH, from the cytosol to the mitochondrial intramembrane space, is tightly regulated by porins in their membranes (56). Importantly, the maintenance of localized redox states is critical for cell function. Mitochondria-specific depletion of GSH makes mitochondria more sensitive to oxidative damage (57), whereas overexpression of the mitochondrial glutaredoxin Grx2 protects against oxidative stress to prevent apoptosis (58). The nuclear redox state is similarly pivotal for the activation of several redox-regulated transcription factors such as CLOCK and NPAS2 (40), NF-κB (59), Nrf2 (nuclear factor (erythroid-derived 2)-like 2) (60), and Rev-erbβ (61).

Although evidence suggests that ROS are *bona fide* signaling molecules, some skepticism has been raised because of their high reactivity and low substrate specificity. However, there is evidence of tight coupling of ROS generators to the activity of antioxidant buffering systems and to specific targets, which would explain how the specificity of ROS signaling is brought about (62–64). In the adrenal gland, for example, H_2O_2 is involved in a feedback control loop to regulate corticosteroid synthesis (65). In the last phase of adrenocorticotropic hormone-induced steroidogenesis, cholesterol is imported in mitochondria, where cytochrome P450 enzymes catalyze the oxidative cleavage of its side chain. As a byproduct of their activity, cytochromes generate H_2O_2, which is eliminated by Prx3. During the catalytic cycle, Prx3 can occasionally be inactivated by hyperoxidation. Its activity is normally reverted by Srx. However, when corticosteroid synthesis increases, so does H_2O_2, and Srx activity is no longer sufficient to reduce and reactivate Prx3. This causes a further increase in H_2O_2 levels and the overflow of H_2O_2 in the cytosol. This last event triggers a signaling cascade involving p38 MAPK, which eventually inhibits corticosteroid synthesis. Of note, the levels of inactivated Prx3, activated p38 MAPK, and Srx exhibit circadian oscillations. In addition, tissue-specific ablation of Srx results in suppression of the adrenal circadian rhythms of corticosterone production, suggesting that Prx hyperoxidation, corticosteroid synthesis, and the circadian clock are interconnected.

Interestingly, oxidative signals can cause selective oxidation of specific redox couples. For example, EGF-mediated ROS signaling selectively oxidizes the cytosolic pool of Trx1 but not the mitochondrial pool of Trx2 (Fig. 4) (66, 67), suggesting that these pools are independently regulated. Furthermore, one of

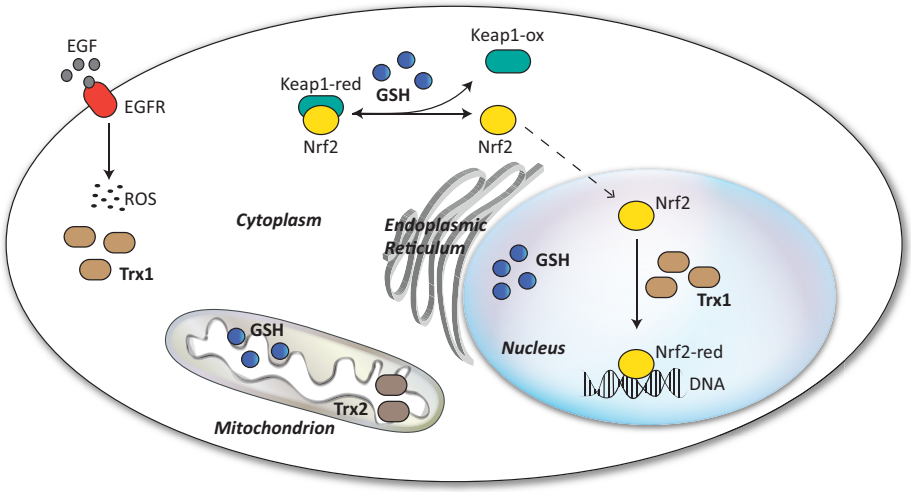

FIGURE 4. **Compartmentalization of redox systems.** Redox systems are compartmentalized, and pools of antioxidant enzymes are distributed differently in the cell. Pools of GSH and Trx have been described in the cytosolic, mitochondrial, and nuclear compartments. The cytosolic pool of Trx1 has been shown to limit ROS generated upon EGF receptor (*EGFR*) activation. The nuclear translocation of Nrf2 is regulated by a redox switch controlled by GSH and Keap1 oxidation, whereas its DNA-binding activity is regulated by a nuclear pool of Trx1. *ox*, oxidized; *red*, reduced.

the major transcription factors activated by oxidative stress, Nrf2 can be differentially activated by redox signals: its translocation is promoted by a redox switch of Keap1 (Kelch-like ECH-associated protein 1), which is controlled by GSH, whereas its nuclear activity is under the control of Trx1 (Fig. 4) (60).

It is tempting to speculate that different redox systems are strategically located within the cell not only to protect substrates from excessive oxidation but also to regulate specific signaling pathways. In addition, different redox couples might act in concert to specifically modulate the response to ROS signals in proximity of key redox-sensitive proteins. Determining how this compartmentalized nature of cellular redox systems links to the clockwork will be critical to fully understand how the cell en masse keeps daily time. We believe that this will be an exciting area of investigation in the next few years.

Conclusions

Substantial evidence highlights the capability of living organisms to resonate with environmental cycles, which confers an evolutionary advantage because perturbing the clockwork reduces fitness. However, the biological mechanisms underlying the regulation of circadian rhythms are still elusive in the light of new insights coming from redox biology. In the post-genomic era, the dominance of gene regulation at the heart of circadian rhythms needs to be reconciled with mounting evidence demonstrating the importance of redox cycles and post-transcriptional/post-translational modifications (68).

It now appears that control of ROS signaling is deeply intertwined in the circadian clock system. Disruption of circadian rhythms in humans has been linked to several diseases such as breast cancer, obesity, diabetes, sleep disorders, and neurodegenerative diseases (69). Given the role of ROS in human pathophysiology, it is tempting to speculate that some of the pathologies associated with the deregulation of clock signaling are partially caused by alteration in redox signaling and possibly their compartmentalized nature. Thus, we propose that the understanding of how localized ROS production affects the activity of oscillators within cells will have important consequences for the development of dedicated therapies aimed at restoring aberrant signaling.

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